Celecoxib Attenuates Kainic Acid-induced Neuronal Cell Death Through Suppression of Microglial c-Jun N-terminal Kinase Phosphorylation

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ABSTRACT

In the present study, neuroprotective property of celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, and its underlying mechanism were examined in the animal model of kainic acid (KA)-induced excitotoxicity. KA, administered intracerebroventricularly (i.c.v.), induced marked neuronal cell death with concurrent microglial activation and subsequent induction of inducible nitric oxide synthase (iNOS) in the hippocampus. Histopathological analysis demonstrated that celecoxib (100 mg/kg), pre-treated 1 hr before or post-treated 2 hr after KA i.c.v. injection, significantly attenuated KA-induced death of pyramidal neurons in CA3 region. Celecoxib obviously suppressed KA-induced microglial activation and subsequent iNOS expression. KA-induced phosphorylation of c-Jun N-terminal kinases (JNK) was attenuated with celecoxib treatments. The results of the present study demonstrate that suppression of JNK phosphorylation by celecoxib contributes to its neuroprotective action against KA-induced excitotoxicity suggesting that celecoxib may be a potentially valuable in the treatment of acute brain pathologies associated with excitotoxic neuronal damage such as epilepsy, stroke, and traumatic brain injury.

Key words: kainic acid, celecoxib, cyclooxygenase-2 (COX-2), iNOS, c-Jun N-terminal kinases (JNK), microglia, neuronal death

INTRODUCTION

Excessive release of excitatory amino acids may play an important role in the pathogenesis of neuronal injury (Choi and Rothman, 1990). Kainic acid (KA), a potent excitotoxin, binds to specific kainite-type non-N-methyl D-aspartate (NMDA) receptors and causes depolarization of neurons resulting in status epilepticus (SE), neurodegeneration, and memory loss (Izquierdo et al., 2000; Zagulaska-Szymczak et al., 2001). The systemic or intracerebroventricular (i.c.v.) injection of KA produces pyramidal cell death...
in hippocampus and causes reactive gliosis (Sperk et al., 1983; Giusti et al., 1996).

c-Jun N-terminal kinases (JNK) are strongly activated in cellular responses to various stresses such as UV radiation, heat shock, DNA-damaging agents, and metabolic inhibitors as well as proinflammatory cytokines (Minden and Karin, 1997; Ip and Davis, 1998). Activation of JNK has been also reported to play a causal role in excitotoxin-induced apoptotic neuronal cell death (Yang et al., 1997; Tournier et al., 2000). In accordance with previous reports that excitotoxins such as glutamate and KA activate the JNK pathway, disruption of JNK pathway has been reported to result in the resistance to excitotoxic neuronal death in mice (Schwarzschild et al., 1997; DeCoster et al., 1998).

Cyclooxygenase-2 (COX-2) is constitutively expressed in neurons within the brain, particularly in pyramidal neurons of the hippocampus and cortex (Breder et al., 1995; Yamagata et al., 2001). COX-2 expression is markedly and transiently up-regulated in neurons in response to KA injection (Chen et al., 1995; Yamagata et al., 2001) and after cerebral ischemia (Collaco-Moraes et al., 1996; Sanz et al., 1997). It has been reported that COX-2 inhibitors attenuated neuronal cell death in ischemic brain damage (Nogawa et al., 1997). Further, KA treatment of transgenic mice over-expressing COX-2 leads to lethal epileptic seizures, indicating a cause-effect relationship between neuronal expression of COX-2 and excitotoxicity (Kelley et al., 1999). However, the mechanism by which COX-2 inhibition exhibits protection against excitotoxin-induced neuronal cell death was not clearly understood. In the present study, we investigated whether celecoxib, a selective COX-2 inhibitor, exerts neuroprotective effect on KA-induced cell death of pyramidal neurons and the underlying mechanism by which celecoxib attenuates KA-induced neuronal cell death in CA3 region of hippocampus.

MATERIALS AND METHODS

Animals and reagents

Male ICR mice weighing 23 ~ 25 g were obtained from Myung-Jin Ltd (Seoul, Korea). All animal experiments were conducted in accordance with the animal care guidelines of the National Institute of Health (NIH) and Korean Academy of Medical Sciences (KAMS). Mice were housed 5 per cage in a room maintained at 22±2°C with an alternating 12/12 h light/dark cycle. Food and water were available ad libitum. KA was obtained from Sigma Chemical Co. (St. Louis, MO, USA). KA was prepared as stock solutions at a concentration of 5 mg/ml in sterile 0.1 M phosphate-buffered saline (PBS, pH 7.4) and aliquots were stored at −20°C until use. Celecoxib (a generous gift from Pfizer) was administered intraperitoneally 1 hr before or 2 hr after KA injection.

Intracerebroventricular (i.c.v.) injection of KA

The administration of KA (0.1 μg/5 μl) was performed according to the procedure established by Laursen and Belknap (Laursen and Belknap, 1986). Briefly, KA was injected at bregma with a 50 μl Hamilton microsyringe fitted with a 26-gauge needle that was inserted to a depth of 2.4 mm.

Immunohistochemistry

All mice were sacrificed 24 hr after KA injection. Mice were transcardially perfused and post-fixed for 4 hours in 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose, sectioned coronally (45 μm) on a freezing microtome and collected in cryoprotectant for storage at −20°C. Free floating immunohistochemistry of brain sections was processed as described previously (Baker and Farbman, 1993). Sections were performed cresyl violet stainings and sections collected from cryoprotectant were placed in PBS for washes and then pre-incubated for 30 min in 0.1 M PBS with 1% bovine serum albumin and 0.2% Triton X-100 and incubated for 2 days at 4°C with the following primary antibodies; iNOS (1:1,000, BD Pharmingen, San Jose, CA, USA), p-JNK (1:1,000, BD Pharmingen, USA) or OX-42 (1:1,000, BD Pharmingen, USA). After 2 days incubation with primary antibody, antigens were finally detected with 3,3-diaminobenzidine tetrahydrochloride using Vector Elite ABC kit (CA, USA). Sections were mounted, air-dried and dehydrated through graded ethanol, cleared in histoclear, and coverslipped using Permount (Fisher, USA).

In situ labeling of DNA fragmentation

Analysis of cells exhibiting DNA fragmentation was performed according to the manufacturer’s instructions using terminal deoxynucleotidyl transferase using
Celecoxib Inhibits KA-induced Microglial JNK Activation

Fig. 1. Neuroprotective action of celecoxib against KA-induced neuronal cell death in CA3 region of mouse hippocampus. Celecoxib was intraperitoneally administered 1 hr before (Cel + KA) or 2 hr after (KA + Cel) KA i.c.v. injection. Mice (n=6 per each group) were killed 24 hr after KA injection. Neuronal cell death was examined with cresyl violet staining (A) and Terminal deoxytransferase-mediated dUTP-nick end labeling (TUNEL) (B, C), respectively. KA injection showed marked loss of CA3 pyramidal neurons (KA) and pre- and post-treatment of celecoxib significantly attenuated KA-induced neuronal cell death in CA3 region. However, post-treatment of celecoxib showed increased protection compared to pre-treatment. Neuronal death in vehicle-treated mice was negligible (data not shown). Quantitative data represent three independent experiments and were expressed mean ± SEM. *p < 0.05 and **p < 0.01 indicate statistically significant difference from the KA-only treated group. #p < 0.05 indicates significant difference between pre-treatment and post-treatment of celecoxib. Arrows indicate CA3 region. Scale bar: 100 μm.

RESULTS

Pre- and post-treatment of celecoxib attenuated KA-induced neuronal death

Intracerebroventricular (i.c.v.) injection of KA resulted in an extensive death of hippocampal neurons in CA3 region (Fig. 1). To elucidate whether celecoxib has a protective effect against KA-induced neuronal death, celecoxib was administered 1 hr before or 2 hr after KA i.c.v. injection and neuronal death was assessed by analysis of digitized images from 5 or more microscopic fields of TUNEL-stained cells from TIFF files (Adobe Photoshop).

Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis for multiple group comparison was done using one-way analysis of variance followed by the Tukey post doc test. p values lower than 0.05 were considered statistically significant.

peroxide-12-UTP nick end labeling (TUNEL) (Roche Molecular Biochemicals, Indianapolis, IN, USA) to label double stranded DNA breaks suggestive of apoptosis as described previously (Henshall et al., 2001). The percentage of TUNEL-positive cells was assessed by analysis of digitized images from 5 or more microscopic fields of TUNEL-stained cells from TIFF files (Adobe Photoshop).
determined with cresyl violet staining and confirmed with TUNEL staining. Celecoxib significantly attenuated KA-induced neuronal death in CA3 region (Fig. 1). Especially, post-treatment of celecoxib showed significantly increased protection against KA insult compared to pre-treatment of celecoxib. Celecoxib itself showed negligible changes in the viability of pyramidal neurons compared to vehicle-treated control (data not shown). The representative and quantitative analysis of neuronal death, determined with TUNEL staining, was in accordance with the data from cresyl violet staining. The number of TUNEL-positive neurons was significantly decreased with celecoxib treatment. However, administration of celecoxib did not completely block KA-induced neuronal death.

**Celecoxib suppressed microglial activation and subsequent iNOS expression**

Administration of KA resulted in extensive microglial activation and subsequent induction of iNOS in the region that neuronal death occurred (Fig. 2). Microglial activation was determined with immunoreactivity of a microglial marker OX-42 and the increased expression of iNOS was localized to activated microglia. Administration of celecoxib significantly attenuated microglial activation and subsequent induction of iNOS (Fig. 2) while celecoxib itself exhibited neither microglial activation nor induction of iNOS (data not shown). The magnitude of microglial activation and iNOS expression was in accordance with the degree of neuronal death. As expected, post-treatment of celecoxib was more effective compared to pre-treatment of celecoxib.

**Celecoxib inhibited KA-induced phosphorylation of C-Jun N-terminal kinases (JNK)**

To understand the underlying mechanism by which celecoxib suppresses the KA-induced neuronal cell

![Fig. 2](image_url) Suppression of hippocampal microglial activation and subsequent iNOS expression through inhibition of JNK phosphorylation by celecoxib. KA resulted in increased microglial activation, determined with immunoreactivity of microglial marker OX-42, and increased iNOS expression. KA injection showed marked phosphorylation of JNK in the region where neuronal cell death occurred. Treatment of celecoxib, with greater intensity in post-treatment, extensively suppressed KA-induced microglial activation and subsequent iNOS expression. In accordance, treatment of celecoxib considerably suppressed KA-induced microglial JNK phosphorylation. Significant JNK phosphorylation was not observed in vehicle-treated mice and celecoxib per se showed negligible effect on JNK activation (data not shown). The red box in the map from "The Mouse Brain (Academic Press, Inc)" indicates the area of images taken in this experiment. Insets in the upper left corner show enlarged images of single microglia. Scale bar: 20 μm.
death, phosphorylation level of JNK, which is known to play a role in apoptotic neuronal cell death to excitotoxic damage, was examined. KA treatment exhibited an increased level of phospho-JNK, which is localized to activated microglia in CA3 region of hippocampus (Fig. 2). Treatment of celecoxib suppressed the level of JNK phosphorylation (Fig. 2). Especially, post-treatment of celecoxib showed increased suppression of JNK phosphorylation compared to pretreatment of celecoxib.

**DISCUSSION**

In the present study, we demonstrated that celecoxib, a selective COX-2 inhibitor, significantly attenuated KA-induced neuronal cell death and that the underlying protective mechanism of celecoxib was attributed to the suppression of microglial JNK phosphorylation in mouse hippocampus. However, celecoxib did not completely abolish KA-induced neuronal cell death in this study where celecoxib was intraperitoneally administered once with a dose of 100 mg/kg 1 hr prior to or 2 hr after KA injection.

The present data demonstrated that celecoxib was effective against KA-induced excitotoxicity in both pre- and post-administration to KA i.c.v. injection. Especially, post-treatment of celecoxib was significantly more protective than pre-treatment although both regimens were beneficial. The discrepancy on the magnitude of protection between the pre- and post-treatment of celecoxib may suggest that low level of endogenous prostaglandins (PGs) is beneficial against excitotoxic stimuli at the beginning of excitotoxicity whereas the excessive amount of PGs from pathologically induced microglial iNOS were detrimental. It has been previously demonstrated that COX-2 expression is induced in response to ischemic damage and KA in mouse hippocampus (Chen et al., 1995; Nogawa et al., 1997).

The present data that exhibit celecoxib was protective irrespective of administration points were not in accordance with previous reports that pretreatment of COX-2 inhibitors such as rofecoxib and celecoxib aggravated or showed no effect on KA-induced epileptic activity and neurotoxicity (Baik et al., 1999; Kunz and Oliw, 2001). This discrepancy might be attributed to the different experimental conditions such as frequency and doses of the drugs.

KA-induced excitotoxicity has been reported to result in the pathologically activated microglia and subsequent release of a variety of cytotoxic mediators such as nitric oxide (NO) (Minami et al., 1991; Milatovic et al., 2002). It has been reported that pathologic amount of NO from iNOS contributes to detrimental neuronal damages such as neurological disorder (Koprowski et al., 1993; Sugimoto and Iadecola, 2002) and apoptotic cell death (Kim et al., 1999). It has been also reported that inhibition of iNOS activity decreases glutamate release and improves stroke outcome after experimental ischemia (Perez-Asensio et al., 2005). In accordance with previous reports, the present study demonstrated that celecoxib attenuated KA-induced iNOS expression, which was proportional to the degree of neuronal cell death in CA3 region.

It has been reported that JNK signaling plays a key role in the cyclic AMP-mediated suppression of IL-1β plus IFNγ-induced iNOS expression in hepatocytes (Zhang et al., 2004). Further, it has been, recently, reported that celecoxib unlike other selective COX-2 inhibitors such as rofecoxib and NS-398 decreased the expression of endothelial tissue factor through inhibition of JNK phosphorylation (Steffel et al., 2005).

In conclusion, the present data demonstrate that selective COX-2 inhibition with celecoxib significantly attenuates KA-induced excitotoxicity through the suppression of JNK phosphorylation in pathologically activated microglia suggesting that celecoxib might be valuable in the treatment of brain injury associated with excitotoxic insults such as epilepsy, ischemia, and traumatic brain injury.

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