Increased cyclooxygenase-2 and nuclear factor-κB/p65 expression in mouse hippocampi after systemic administration of tetanus toxin

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Abstract. Brain inflammation has a crucial role in various diseases of the central nervous system. The hippocampus in the mammalian brain exerts an important memory function, which is sensitive to various insults, including inflammation induced by exo/endotoxin stimuli. Tetanus toxin (TeT) is an exotoxin with the capacity for neuronal binding and internalization. The present study investigated changes in inflammatory mediators in the mouse hippocampus proper (CA1-3 regions) and dentate gyrus (DG) after TeT treatment. The experimental mice were intraperitoneally injected with TeT at a low dosage (100 ng/kg), while the control mice were injected with the same volume of saline. At 6, 12 and 24 h after TeT treatment, changes in the hippocampal levels of inflammatory mediators cyclooxygenase-2 (COX-2) and nuclear factor kappa-B (NF-κB/p65) were assessed using immunohistochemical and western blot analysis. In the control group, moderate COX-2 immunoreactivity was observed in the stratum pyramidal (SP) of the CA2-3 region, while almost no expression was identified in the CA1 region and the DG. COX-2 immunoreactivity was increased by TeT in the SP and granule cell layer (GCL) of the DG in a time-dependent manner. At 24 h post-treatment, COX-2 immunoreactivity in the SP of the CA1 region and in the GCL of the DG was high, and COX-2 immunoreactivity in the SP of the CA2/3 region was highest. Furthermore, the present study observed that NF-κB/p65 immunoreactivity was obviously increased in the SP and GCL at 6, 12 and 24 h after TeT treatment. In conclusion, the present study demonstrated that systemic treatment with TeT significantly increased the expression of COX-2 and NF-κB/p65 in the mouse hippocampus, suggesting that increased COX-2 and NF-κB/65 expression may be associated with inflammation in the brain induced by exotoxins.

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Introduction

In the immune system, excessive innate immunity in defense against bacterial or viral infections is a response to a variety of pathological conditions, such as chronic inflammation (1,2). During the inflammatory process, numerous pro-inflammatory mediators are generated and the major mediators of inflammatory events are members of the cyclooxygenase (COX) family (3). Two major COX isoforms, COX-1 and COX-2, catalyze the first step of the synthesis of prostaglandin E_2 (PGE_2), which is the transformation of arachidonic acid (4). In particular, COX-2 expression is enhanced by stimuli from

inflammatory mediators, including lipopolysaccharide (LPS) and several pro-inflammatory cytokines (5-8). In addition, COX-2 is located in the perinuclear membrane and exerts pathological effects though the biosynthesis of prostaglandins several hours after the stimuli (9,10).

Nuclear transcription factor kappa-B (NF-κB), one of the most important transcription factors, has critical roles in inflammation and immunity as well as cell proliferation, differentiation and survival (11). The activation of NF-κB involves the phosphorylation of inhibitor of NF-κB (IκB). Once IκB is phosphorylated, the resulting free NF-κB then translocates to the nucleus, where it binds to κB binding sites in the promoter regions of target genes and induces the transcription of pro-inflammatory mediators, including inducible nitric oxide synthase (iNOS), COX-2 and tumor necrosis factor (TNF)-α (2,10,12).

It is well known that brain inflammation has a crucial role in various diseases of the central nervous system (CNS), including Alzheimer's disease and epilepsy (13,14). The hippocampus in the mammalian brain, which is important in memory function (15), is a vulnerable to certain types of brain damage (16-20). In particular, it is highly sensitive to various insults, including inflammation induced by exo/endotoxin stimuli (5,21-24). Tetanus toxin (TeT), an exotoxin, has a capacity for neuronal binding and internalization (25-27). When systematically administered to animals, TeT reaches the CNS via retrograde transportation through nerve axons (28). A previous study by our group reported that systemic administration of TeT caused responses in the mouse hippocampus, including the secretion of certain inflammatory cytokines and glial activation, while neuronal death was not observed (27,29). However, in studies regarding TeT, few have focussed upon the effect of TeT treatment on alterations in inflammatory mediators in the hippocampus. Therefore, to further investigate changes of inflammatory mediators induced by TeT, the present study observed changes in the immunoreactivities and protein levels of COX-2 and NF-κB/p65 in the mouse hippocampus after the systemic administration of TeT.

Materials and methods

Experimental animals. A total of 56 male ICR mice (BW; weight, 25-30 g; age, eight weeks) were purchased from the Jackson Laboratory (Maine, ME, USA). The animals were housed under standard conditions with a 12-h light/dark cycle at 23±3˚C, 55±5% relative humidity and free access to food and water. All animal care and experimental procedures were performed according to the National Institutes of Health (NIH) guidelines (NIH Guide for the Care and Use of Laboratory Animals; NIH publication no. 85-23, 1985) and were approved by the Institutional Animal Care and Use Committee (IACUC) at Kangwon National University (approval no. KIACUC-140409-1; Chuncheon, Republic of Korea). All efforts were made to minimize animal suffering, as well as the number of animals used.

Treatment with TeT. The mice were intraperitoneally injected with a low dose of TeT (100 ng/kg; Dawinbio, Seoul, Republic of Korea) and the control animals were injected with the same volume of saline (pH 7.4). The mice (n=14 at each time-point) were sacrificed at 6, 12 and 24 h following treatment with TeT.

Preparation of tissue samples for histology. Animals were anesthetized with sodium pentobarbital (40 mg/kg; JW Pharmaceutical Co., Ltd., Seoul, Republic of Korea) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS; pH 7.4; Sigma-Aldrich, St. Louis, MO, USA) followed by 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M PBS. The brains were removed and post-fixed in 4% paraformaldehyde for 6 h. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight (Sigma-Aldrich). Subsequently, tissues were frozen and serially sectioned using a cryostat (Leica Microsystems GmbH, Wetzlar, Germany) to obtain 30-μm coronal sections, which were then collected in six-well plates containing PBS.

Immunohistochemical analysis. The expression of neuronal nuclei (NeuN), COX-2 and NF-κB/p65 was determined using immunohistochemistry. Coronal sections from control- and TeT-treated animals (n=7 for each time-point) were incubated with with 0.3% hydrogen peroxide (Sigma-Aldrich) in PBS for 30 min at room temperature. Subsequent to washing three times with PBS (each for 10 min), the sections were incubated with 10% normal goat serum (Vector Laboratories, Inc., Burlingame, CA, USA) in 0.05 M PBS for 30 min at room temperature. The samples were subsequently incubated with polyclonal rabbit anti-NeuN (ABN78; 1:1,000; Chemicon; EMD Millipore, Billerica, MA, USA), polyclonal rabbit anti-COX-2 (160126; 1:500; Chemicon) or polyclonal rabbit anti-NF-κB/p65 (sc-372; 1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibody overnight at 4˚C. Subsequent to washing three times with PBS (each for 10 min), the sampels were exposed to biotinylated goat anti-rabbit immunoglobulin (Ig)G (BA-1000) and streptavidin-biotinylated horseradish peroxidase complex (SA-5004) (1:200; Vector Laboratories, Inc.) for 2 h at room temperature. Antibodies were then visualized using 3,3'-diaminobenzidine tetrachloride (Sigma-Aldrich) in 0.1 M Tris-HCl buffer (pH 7.2) and samples were mounted on gelatin-coated slides. Following dehydration by immersion in serial dilutions of ethanol, the sections were mounted in Canada balsam (Kanto Chemical, Tokyo, Japan). In order to test the specificity of the immunostaining, a negative control sample was prepared using pre-immune serum instead of primary antibody (data not show).

Eight sections per animal were selected to quantitatively assess immunoreactivity for COX-2 and NF-κB/p65. Digital images of the hippocampus proper and dentate gyrus were captured using an AxioM1 light microscope (Carl Zeiss, Oberkochen, Germany) equipped with a digital camera (Axiocam MRc 5; Carl Zeiss) connected to a PC monitor. According to the method used in previous studies by our group (27,30), immunostaining intensities were semi-quantified using digital image analysis software (MetaMorph 4.01; Universal Imaging Corp., Bedford Hills, NY, USA). The level of the immunoreactivity was scored as (-), (±), (+), (++) or (+++) representing no staining (gray scale value ≥200), weakly positive (gray scale value, 150-199), moderate (gray scale value, 100-149), high (gray scale value, 50-99) or very high (gray scale value ≤49), respectively.

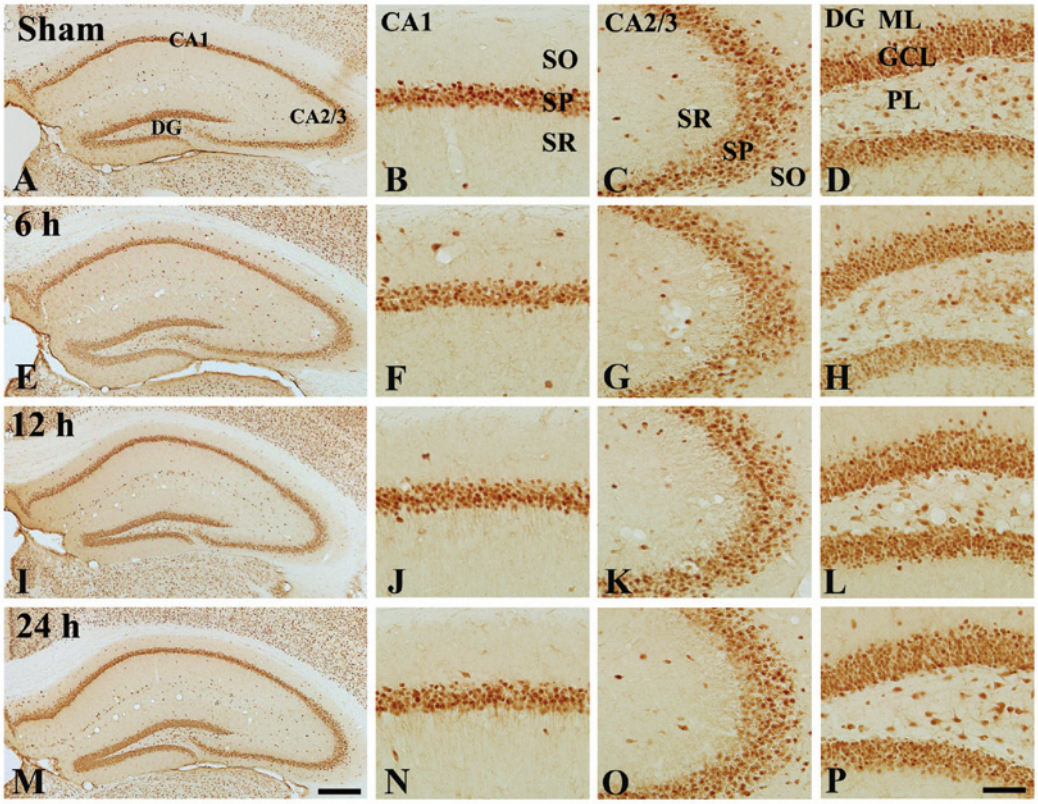

Figure 1. Immunohistochemical analysis of NeuN in the hippocampi of (A-D) the control mice and (E-P) 100 ng/kg TeT-treated mice. Numbers of NeuN-immunoreactive cells in the TeT-treated groups were similar to those in the control-group. CA, cornu ammonis; DG, dentate gyrus; GCL, cell layer; ML, molecular later; PL, polymorphic layer; SO, stratum oriens; SP, stratum pyramidal; SR, stratum radiatum. Scale bar, 400 μm (A, E, I and M) or 50 μm (B-D, F, G, H, J-L, N-P). TeT, tetanus toxin; NeuN, neuronal nuclei.

Western blot analysis. The protein expression of COX-2 and NF-κB/p65 in the hippocampus of the control- and TeT-treated animals (n=7 at each time point) was determined by western blot analysis. Subsequent to sacrifice by cervical dislocation, mice were decapitated and the brains were removed. The brains were then serially and transversely cut into 400-μm section using a vibratome (Leica Microsystems GmbH), and the hippocampal region was dissected using a surgical blade. The tissues were homogenized in 50 mM PBS (pH 7.4) containing 0.1 mM ethylene glycol tetraacetic acid (pH 8.0), 0.2% Nonidet P-40, 10 mM ethylendiamine tetraacetic acid (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol (DTT) (All from Sigma-Aldrich). Subsequent to centrifugation at 16,000 x g for 20 min, the protein concentration of the supernatants was determined using a Micro Bicinchoninic Acid protein assay kit with bovine serum albumin as a standard (Pierce Biotechnology, Inc., Rockford, IL, USA). Aliquots containing 50 μg total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS (Sigma-Aldrich), 0.3% bromophenol blue (Sigma-Aldrich) and 30% glycerol (Junsei Chemical Co., Ltd., Tokyo, Japan). Aliquots were then subjected to 5% SDS-PAGE and electrotransferred to nitrocellulose membranes (Pall Corp, Port Washington, NY, USA). To reduce background staining, the membranes were incubated with 5% non-fat dry milk (Sigma-Aldrich) in PBS containing 0.1% Tween 20 (Sigma-Aldrich) for 45 min and subsequently incubated with rabbit polyclonal anti-COX-2 (160126; 1:1,000; Chemicon), rabbit polyclonal NF-κB/p65 (sc-372; 1:1,000; Santa Cruz Biotechnology, Inc.) and rabbit polyclonal anti-β-actin (ab8227; 1:2,000; Abcam, Cambridge, UK) overnight at 4˚C. Subsequently, the membranes were washed three times with PBS/0.1% Tween 20 (each for 10 min), followed by incubation with the peroxidase-conjugated goat anti-rabbit IgG (A6154; 1:10,000; Sigma-Aldrich) secondary antibody for 1 h at room temperature. Antibodies were visualized using an enhanced chemiluminescence kit (Pierce Biotechnology, Inc.). The blots were exposed to X-ray film (X-max; Kodak, Rochester, NY, USA) and scanned using a Hewlett Packard ScanJet 3200C at 300dpi (HP, Inc., Palo Alto, CA, USA). Subsequently, densitometric analysis was conducted using Scion Image software (Scion Corp., Frederick, MD, USA) in order to quantify the bands, with normalization to β-actin.

Statistical analysis. Values are expressed as the mean ± standard error of the mean. SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Differences between groups were assessed using one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference between values.

Results

TeT does not cause neuronal damage in hippocampi of mice. In the present study, the neuronal damage/death in the hippocampi

Table I. Semi-quantitative analysis of cyclooxygenase-2 immunoreactivity in hippocampal regions after treatment of mice with 100 ng/kg TeT.

Region	Layer	Time after TeT treatment			
		Control	6 h	12 h	24 h
CA1	SO	-	±	±	+
	SP	±	+	+	++
	SR	-	±	±	+
CA2-3	SO	-	±	±	+
	SP	+	++	++	+++
	SR	-	±	±	+
DG	ML	-	±	±	±
	GCL	±	+	+	++
	PL	-	±	±	+

Levels of immunoreactivity were defined as five grades: (-), negative; (±), weakly positive; (+), moderate; (++), high; and (+++) very high. CA, cornu ammonis; DG, dentate gyrus; GCL, granule cell layer; ML, molecular layer; PL, polymorphic layer; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; TeT, tetanus toxin.

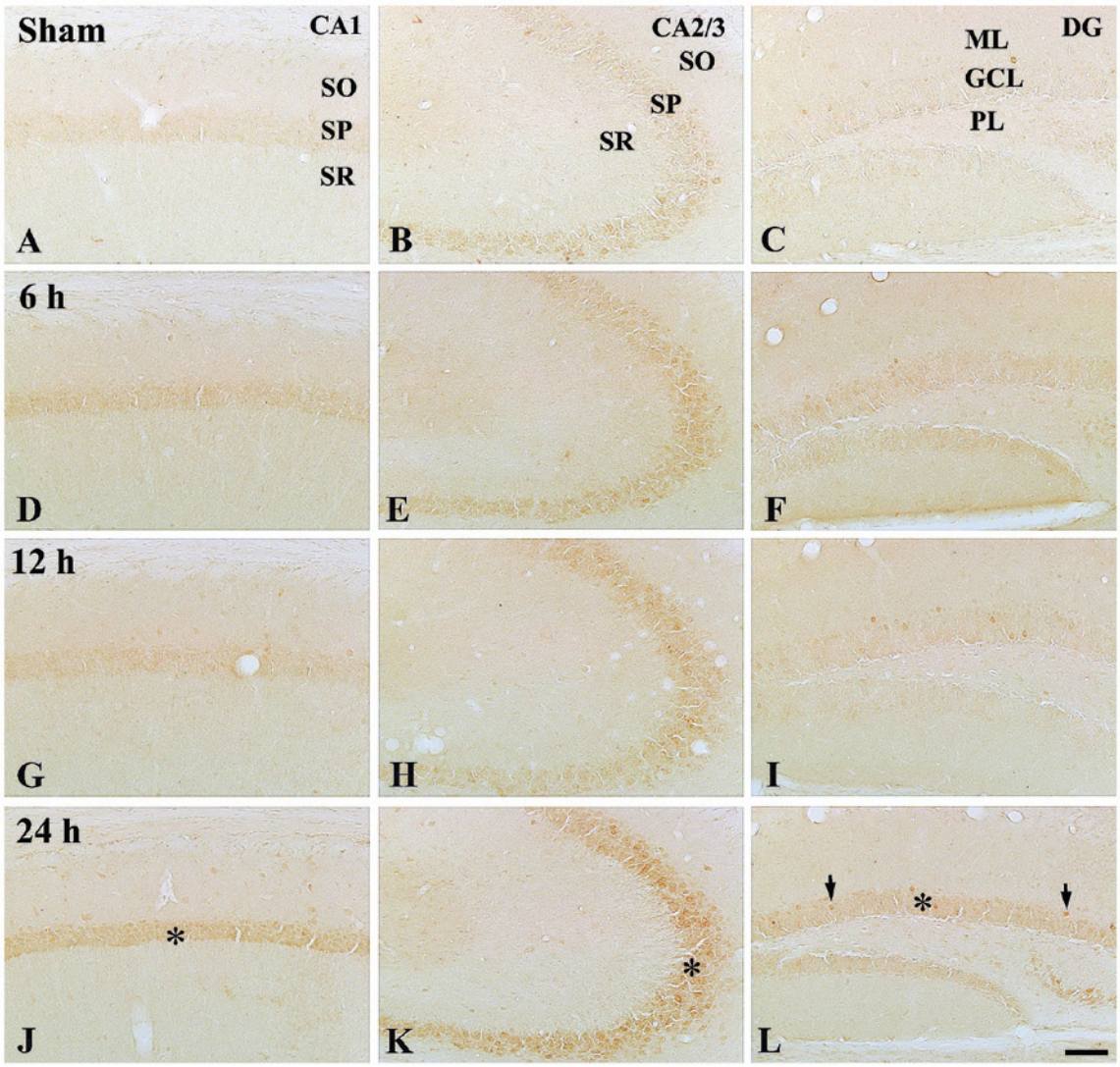

Figure 2. Immunohistochemical detection of COX-2 in the hippocampi of (A-C) the control mice and (D-L) 100 ng/kg TeT-treated mice. At 24 h post-treatment, COX-2 immunoreactivity was distinctively increased in the SP (asterisks) and GCL (asterisk and arrows). TeT, tetanus toxin; COX, cyclooxygenase; SP, striatum pyramidal; GCL, granule cell layer; SO, stratum oriens; SR, stratum radiatum; ML, molecular later; PL, polymorphic layer. Scale bar, 50 μm.

Table II. Semi-quantitative analysis of nuclear factor-κB/p65 immunoreactivity in hippocampal regions after treatment with 100 ng/kg TeT.

		Time after TeT treatment (h)			
Area	Layer	Control	6	12	24
CA1	SO	±	+	+	+
	SP	+	++	++	++
	SR	±	+	+	++
CA2-3	SO	±	+	+	+
	SP	+	++	++	++
	SR	±	+	+	+
DG	ML	-	+	+	+
	GCL	±	++	++	++
	PL	±	+	+	+

Levels of immunoreactivity were defined as four grades: (-), negative; (±), weakly positive; (+), moderate; and (++), high. CA, cornu ammonis; DG, dentate gyrus; GCL, granule cell layer; ML, molecular layer; PL, polymorphic layer; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; TeT, tetanus toxin.

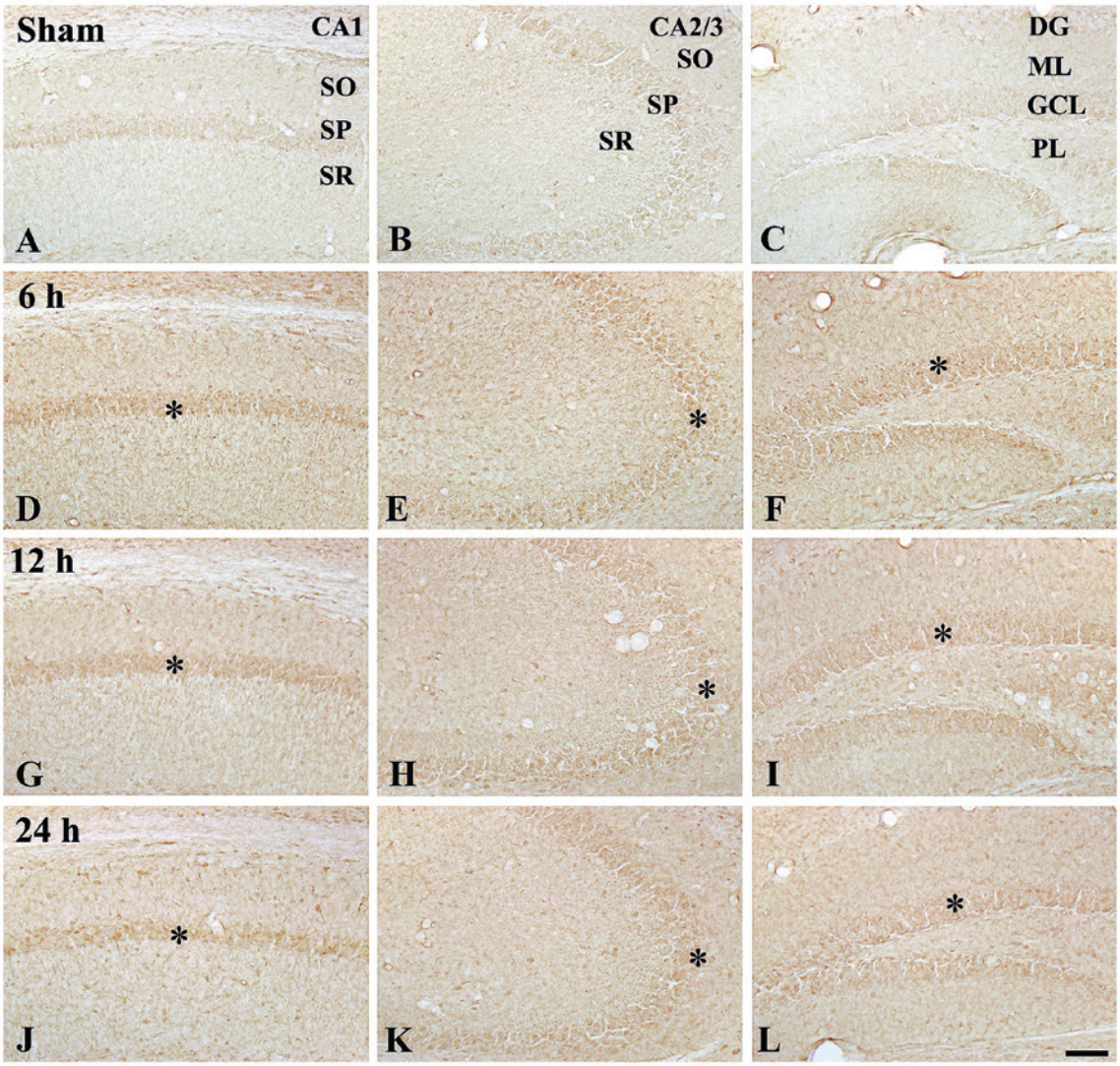

Figure 3. Immunohistochemical detection of NF-κB/p65 in the hippocampi of (A-C) control mice and (D-L) 100 ng/kg TeT-treated mice. NF-κB/p65 immunoreactivity was apparently increased in the SP (asterisks) and GCL (asterisks) after TeT treatment. TeT, tetanus toxin; NF, nuclear factor; SP, striatum pyramidal; GCL, granule cell layer SO, stratum oriens; SR, stratum radiatum; ML, molecular later; PL, polymorphic layer. Scale bar, 50 μm.

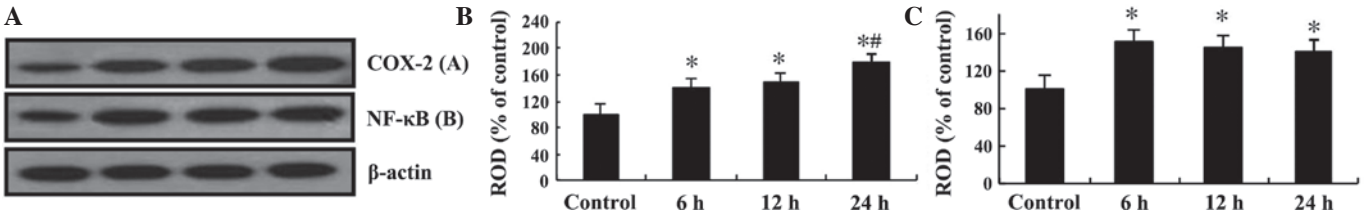

Figure 4. Western blot analysis of COX-2 and NF-κB/p65 in the hippocampi of control- and 100 ng/kg TeT-treated mice (n=7 in each group). (A) Representative western blot. ROD of (B) COX-2 and (C) NF-κB/p65 normalized to β-actin. Values are expressed as the mean ± standard error of the mean. *P<0.05 vs. control group; #P<0.05 vs. preceding time-point. NF, nuclear factor; COX, cyclooxygenase; TeT, tetanus toxin; ROD, relative optical density.

of mice treated with TeT was observed by immunostaining for NeuN (Fig. 1). NeuN-immunoreactive cells were observed in the hippocampus proper (CA1-3 regions) and dentate gyrus of the control group (Fig. 1A-D). In the TeT-treated groups, the distribution pattern of NeuN-immunoreactive cells was not different from that in the control group at any time-point after TeT treatment (Fig. 1E-P).

TeT increases COX-2 expression in mouse hippocampi. In the control group, COX-2 immunoreactivity observed in the stratum pyramidal of the CA1 region was low and that in the stratum pyramidal of the CA2/3 region was moderate; furthermore, low COX-2 immunoreactivity was observed in the granule cell layer of the dentate gyrus (Table I; Fig. 2A-C).

At 6 h post-treatment, COX-2 immunoreactivity was slightly increased in the stratum pyramidal and granule cell layer in all the hippocampal sub-regions compared with that in the control group (Table I; Fig. 2D-F). COX-2 immunoreactivity at 12 h post-treatment was similar to that at 6 h post-treatment (Table I, Fig. 2G-I). Of note, at 24 h post-treatment, COX-2 immunoreactivity in the stratum pyramidal and granule cell layer was significantly increased compared with that at 12 h post-treatment; in particular, the immunoreactivity in the stratum pyramidal of the CA2/3 region was high (Table I; Fig. 2J-L).

TeT increases NF-κB/p65 expression in mouse hippocampi. In the control group, moderate NF-κB/p65 immunoreactivity was detected in the stratum pyramidal of the CA1-3 regions, while low NF-κB/p65 immunoreactivity was identified in the granule cell layer of the dentate gyrus (Table II; Fig. 3A-C).

At 6 h post-treatment, NF-κB/p65 immunoreactivity was markedly increased in all layers of all hippocampal sub-regions compared with those in the control group (Table II; Fig. 3D-F). At 12 h post-treatment, the pattern of NF-κB/p65 immunoreactivity was similar to that at 6 h post-treatment (Table II; Fig. 3G-I). At 24 h post-treatment, NF-κB/p65 immunoreactivity in all layers was not significantly changed compared with that at 12 h post-treatment; however, the immunoreactivity was higher than that in the control group (Table II; Fig. 3J-L).

Effects of TeT on COX-2 and NF-κB p65 protein levels. Western blot analysis was performed to confirm the changes in the protein levels of COX-2 and NF-κB/p65 in the mouse hippocampi after TeT treatment (Fig. 4). COX-2 protein levels steadily increased in a time-dependent manner until 24 h post-treatment, while NF-κB/p65 levels were significantly increased at 6 h post-treatment and then remained constant until 24 h post-treatment (Fig. 4).

Discussion

TeT has been commonly used in experimental studies on neurological disorders or animal models of diseases (31,32). It was reported that intrahippocampal TeT injection induced neuronal damage or death in certain brain regions, particularly in the hippocampus (21,33). However, results of previous studies regarding the induction of neuronal damage or death were inconsistent due to differences in animals, dosages of TeT, routs of administration and time of sacrifice (27,29,34). In the present study, following intraperitoneal injection of TeT, no neuronal damage or loss in any of the sub-regions of the hippocampus was identified using immunohistochemical analysis of NeuN expression.

In the present study, COX-2 immunoreactivity and expression levels were significantly increased compared with those in the control group at 24 h after injection of 100 ng/kg TeT; in particular, enhanced COX-2 immunoreactivity was identified in the stratum pyramidal of the hippocampus proper (CA1-3 regions) and in the granule cell layer of the dentate gyrus. In analogy with this finding, a previous study showed that systemic administration of LPS increased COX-2 immunoreactivity in the mouse hippocampus (5). The brain and the immune system are extensively interconnected and regulate each other (35,36). The communication within the hippocampus contributes to region-specific vulnerability to certain insults (35). In the hippocampus, certain types of stimulation, such as bacterial toxin stimuli, evoke a rapid immune response accompanied with specific cellular and molecular changes (5,37). For example, intraperitoneal treatment with LPS caused a reduction in the mRNA levels of interleukin-1β and 6 in the cerebral cortex and hippocampus of mice (38). In addition, upon activation with LPS, microglia in the olfactory bulb were shown to secrete a variety of cytokines in a rat model of neuroinflammation (39). A previous study also showed that microglia in rat hippocampi were activated following injection of TeT into the ventral hippocampus (40). Furthermore, recent studies by our group revealed marked changes in inflammatory cytokines accompanied with glial activation in all hippocampal sub-regions after intraperitoneal administration of 100 ng/kg TeT (27,29).

NF-κB, a heterodimer of its p65 and p50 sub-units, is located in the cytoplasm as an inactive complex bound to its inhibitor, which is phosphorylated and subsequently degraded and dissociated to produce activated NF-κB (10,12). The present

study observed changes of NF-κB/p65 immunoreactivity and expression levels in mouse hippocampi after systemic administration of TeT. NF-κB/p65 immunoreactivity was increased in the cytoplasm of pyramidal neurons and granule cells after TeT treatment. It was reported that NF-κB was activated by numerous different types of stimuli and NF-κB regulated the expression of COX-2 in the CNS (41). Therefore, these results lead to the hypothesis that the increased COX-2 expression may be closely associated with the increase of NF-κB/p65 immunoreactivity in neurons after TeT treatment.

In conclusion, the present study suggested that the systemic administration of 100 ng/kg TeT did not cause neuronal damage; however, it markedly increased the expression of COX-2 and NF-κB/p65 in mouse hippocampi after TeT treatment.

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