# Changes in Cyclooxygenase-2 Immunoreactivity in the Hippocampus in a Model of Streptozotocin-Induced Type 1 Diabetic Rats

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ABSTRACT. In this study, we investigated diabetic stage dependent cyclooxygenase-2 (COX-2) immunoreactivity in the dentate gyrus in streptozotocin (STZ)-induced type 1 diabetic rats. The animals were sacrificed at 2, 3 and 4 weeks after STZ treatment. Blood glucose levels were increased after STZ treatment. COX-2 immunoreactivity in dentate gyrus was significantly increased in these regions 3 weeks after STZ treatment and restored to its basal level to 4 weeks after STZ treatment. In contrast, COX-2 immunoreactivity was not changed in CA3 region in all groups. These results suggest that STZ-induced type 1 diabetes transiently, but not permanently, decreased synaptic transmission and plasticity 3 weeks after STZ treatment in the dentate gyrus.
KEY WORDS: cyclooxygenase-2, hippocampus, streptozotocin, type 1 diabetes.

Commonly, there are two types of diabetes mellitus (DM); type 1 DM shows massive loss of pancreatic β cells causing absolute insulin deficiency, while type 2 DM demonstrates relative insulin deficiency in addition to insulin resistance triggered by hyperglycemia which is the key to diabetic clinical symptoms [15]. In brain, neuronal insulin signaling plays an important role in synaptic formation and regeneration of neurons in brain [4] and decreased levels or sensitivity is related with type 1 or 2 DM, respectively. Diabetes impairs the neuronal adaptive ability to oxidative and metabolic stress [16], and alters hippocampal neurogenesis and neuropathy in dorsal root ganglion [12, 27]. Duration-dependent differences like hippocampal neuronal apoptosis are reported [21].

For an animal model of type 1 DM, we selected the streptozotocin (STZ) treated rat. STZ interacts with β-cell selectivity via the glucose transporter 2 [20]. Its toxicity is induced by DNA alkylating activity of its methylnitrosourea moiety, poly-ADP-ribose polymerase overstimulation, and cellular energy depletion [20]. In many studies including ours, STZ-induced animal models are used for studying the effects of type 1 diabetes mellitus [5, 18, 32].

Cyclooxygenase (COX), a membrane-bound protein, is involved in inflammation, angiogenesis, colon cancer and Alzheimer's disease [8, 11], and classified into two types, COX-1 and COX-2. Both isoenzymes convert arachidonic acid into multifunctional prostanoids. COX-2 enzyme is reported as a key player [11] in neuro-inflammation which is involved in most neurodegenerative disorders by eliciting neuronal death and influencing formation of new neurons [9]. In several tissues, mRNA level of COX-2 was studied and level in the brain was lowest [24], but its expression in brain was not the lowest [35]. In brain, COX-2 is involved in normal neuronal function and expressed constitutively at high levels in neurons of the hippocampus, cortex and amygdala, and lower levels in the caudate putamen, thalamus and hypothalamus [34]. Also, the function of COX-2 includes memory consolidation, acquisition and retrieval [26, 29, 31].

In our previous studies, we observed that the expression of COX-2 and hippocampal neurogenesis is closely associated in a type 2 DM model, the Zucker diabetic fatty rat [13]. However, there are no comparative studies on the expression of COX-2 in the type 1 diabetic animals. In the present study, therefore, we investigated changes in COX-2 immunoreactivity in the hippocampus of STZ-induced type 1 diabetic animals.

## MATERIALS AND METHODS

*Experimental animals*: Seven-week-old male Wistar rats were purchased from Orient Bio, Inc. (Seongnam, South Korea). The animals were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12-hr light/12-hr dark cycle, and were given *ad libitum* access to water and food. The procedures for handling and caring of animals follow the Guide for the Care and Use of Laboratory Animals issued by Institute of Laboratory Animal Resources, U.S.A., 1996, and the experimental protocol was approved by the Institutional Animal Care

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and Use Committee (IACUC) of Seoul National University (approval no. SNU-080128-6). 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.

*Induction of type 1 DM and experimental design*: STZ (Sigma-Aldrich, St. Louis, MO, U.S.A.) was dissolved in a 0.1 M sodium citrate buffer (pH 4.3). Following one week acclimation to the *vivarium*, diabetes was induced by a single intraperitoneal injection of 75 mg/kg of STZ. For the control group, sodium citrate buffer was treated intraperitoneally as the same volume in STZ. After 72 hr, fasting blood glucose levels were monitored and rats with blood glucose levels of >8.0 mmol/*l* were utilized for the study and divided into 4 groups.

*Check for blood glucose levels*: Blood was sampled by a "tail nick" at 9–11 a.m. using a 27 G needle before sacrifice and glucose in blood from a non-fasting state was analyzed by using a blood glucose monitor (SureStep$^{TM}$ blood glucose meter, Lifescan, CA, U.S.A.).

*Tissue processing*: For immunohistochemical analysis, vehicle- and STZ-treated rats at 2, 3 and 4 weeks after STZ treatment (n=5 per group) were anesthetized with 30 mg/kg Zoletil 50 (Virbac, Carros, France) and perfused 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 6 hr. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. The 30-$\mu$m-thick brain sections in coronal plane were serially cut using a cryostat (Leica, Wetzlar, Germany). The sections were collected into six-well plates containing PBS for further processing.

*Immunohistochemistry for COX-2*: To obtain accurate data for immunohistochemistry, the free-floating sections were carefully processed under the same conditions. The tissue sections were selected between −3.00~−4.08 mm to the bregma in reference to a rat atlas [25] for each animal. The sections were sequentially treated with 0.3% hydrogen peroxide ($H_2O_2$) in PBS for 30 min and 10% normal goat serum in 0.05 M PBS for 30 min. They were then incubated with diluted rabbit anti-COX-2 (1:200, Cayman, Ann Arbor, MI, U.S.A.) overnight at room temperature and subsequently exposed to biotinylated goat anti-rabbit IgG and strep-tavidin peroxidase complex (1:200, Vector, Burlingame, CA, U.S.A.). They were then visualized by reaction with 3,3'-diaminobenzidine tetrachloride (Sigma, St. Louis, MO, U.S.A.) in 0.1 M Tris-HCl buffer (pH 7.2) and mounted on gelatin-coated slides. The sections were mounted in Canada Balsam (Kanto, Tokyo, Japan) following dehydration.

Analysis of a region of interest in the hippocampus was performed using an image analysis system. Images were calibrated into an array of 512 × 512 pixels corresponding to a tissue area of 140 × 140 $\mu$m (40× primary magnification). Each pixel resolution was 256 gray levels. The intensity of COX-2 immunoreactivity was evaluated by means of a relative optical density (ROD), which was obtained after the transformation of the mean gray level using the formula: ROD=log (256/mean gray level). ROD of background was determined in unlabeled portions and the value subtracted
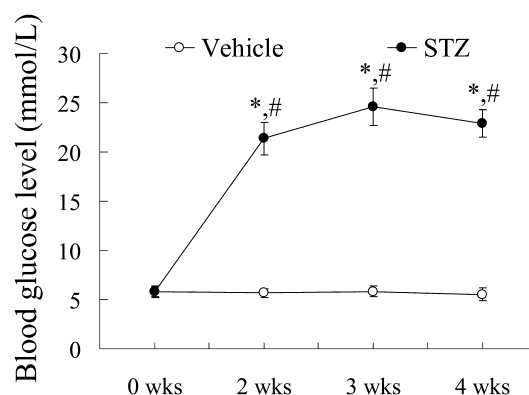


Fig. 1.　Changes in blood glucose levels at 0, 2, 3 and 4 weeks after vehicle or STZ treatment. Differences among the means were analyzed statistically by two-way analysis of variance followed by Duncan's new multiple range method (n=5 per group; *$P$<0.05, significantly different from vehicle-treated group, #$P$<0.05, significantly different from the 0 week post-treatment group). The bars indicate SE.

for correction, yielding high ROD values in the presence of preserved structures and low values after structural loss using NIH Image 1.59 software. A ratio of the ROD was calibrated as %.

*Statistical analysis*: The data shown here represent the means of experiments performed for each experimental area. Differences among the means were statistically analyzed by two-way analysis of variance followed by Duncan's post-hoc analysis in order to elucidate differences.

RESULTS

*Changes in blood glucose levels*: In the pre-diabetic stages, the blood glucose levels were similar between vehicle- and STZ-treated groups (5.81 and 5.83 mmol/*l*, respectively). In the vehicle-treated group, the blood glucose level did not change significantly 4 weeks after vehicle treatment. However, in the STZ-treated group, blood glucose level significantly increased at 2 weeks after STZ treatment compared to the vehicle-treated counterpart and higher blood glucose levels were maintained 4 weeks after STZ treatment. At 3 weeks after STZ treatment, the blood glucose peaked and slightly decreased 4 weeks after STZ treatment (Fig. 1).

*Changes in COX-2 immunoreactivity*: COX-2 immunoreactivity was mainly detected in the granule cells and some interneurons in polymorphic layer of dentate gyrus in all groups (Fig. 2). However, there were significant differences in COX-2 immunoreactivity in dentate gyrus between groups. In the vehicle-treated groups, there were no significant changes with time after treatment (data not shown). At 4 weeks after vehicle treatment, COX-2 immunoreactivity was detected mainly in the granule cell layer of the dentate gyrus (Fig. 2A). At 2 weeks after STZ treatment, COX-2 immunoreactivity was slightly increased in the dentate gyrus
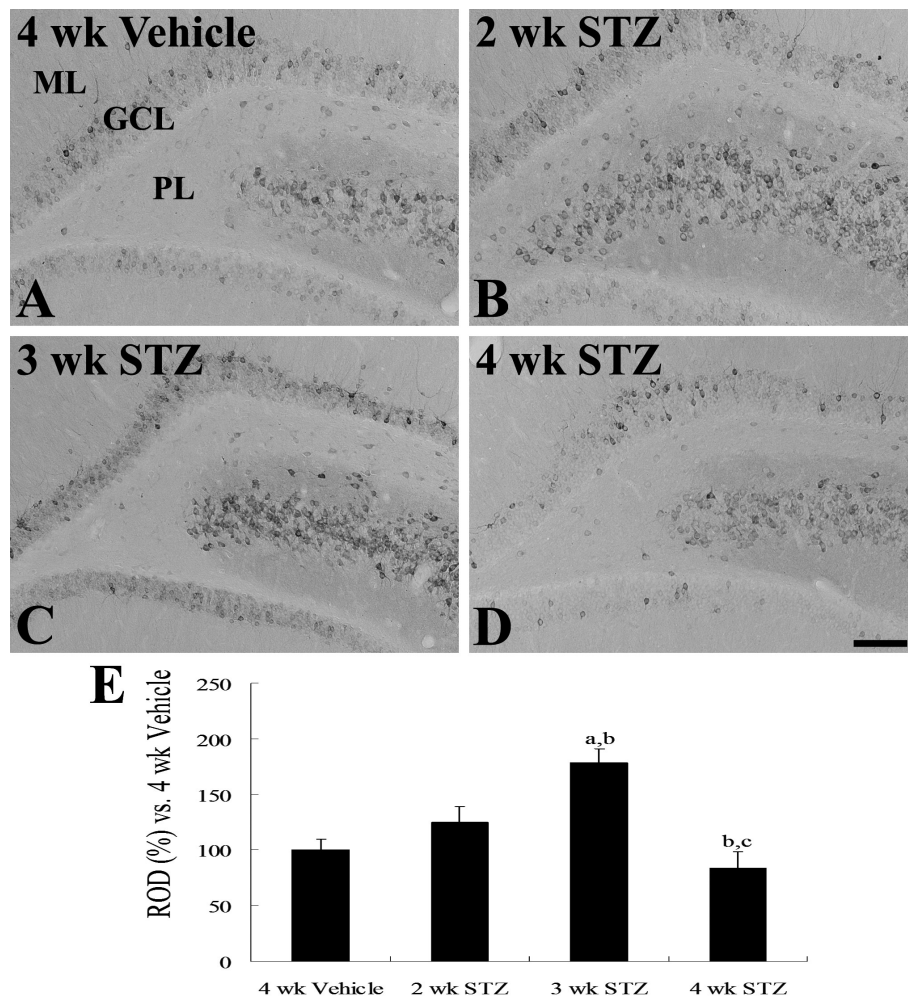
Fig. 2. Cyclooxygenase-2 (COX-2) immunoreactivity in the dentate gyrus of groups at 4 weeks after vehicle treatment (A), and 2 (B), 3 (C) or 4 weeks (D) after STZ treatment. COX-2 immunoreactivity is found in the granule cell layer (GCL) of the dentate gyrus. PL, polymorphic layer; ML, molecular layer. Scale bar=100 $\mu$m. E: Relative optical densities (ROD) as percentage values of COX-2 in the dentate gyrus of rats groups at 4 weeks after vehicle treatment, and 2, 3 or 4 weeks after STZ treatment. Differences among the means were analyzed statistically by one-way analysis of variance followed by Newman-Keuls method (n=5 per group; a $P<0.05$, significantly different from vehicle-treated group, b $P<0.05$, significantly different from the 2 weeks post-treatment group; c $P<0.05$, significantly different from the 3 weeks post-treatment group). The bars indicate the SEM.

compared to that in the control group (Fig. 2B and 2E). At 3 weeks after STZ treatment, COX-2 immunoreactivity was significantly increased in the dentate gyrus compared to the control or 2 weeks post-STZ group (Fig. 2C and 2E). At 4 weeks after STZ treatment, COX-2 immunoreactivity was significantly decreased in the dentate gyrus compared to that in the 2 or 3 weeks post-STZ group and slightly low compared to that in the control group (Fig. 2D and 2E).

COX-2 immunoreactivity was mainly detected in the stratum pyramidale of CA3 region (Fig. 3). However, there were no significant differences in COX-2 immunoreactivity in CA3 region between groups (Fig. 3).

DISCUSSION

Diabetic brain shows cognitive deficits and increased risk of developing dementia, especially the incidence of Alzheimer's disease [18]. In a previous study, the MRI differences in brain lesions between the two type diabetes were compared and more prominent cognitive disturbances were found in type 2 DM [3]. In particular, hippocampus-based memory impairments and cognitive dysfunction are reported in both type of DM [1, 10]. In non-obese, type 1 DM mice, reduction of hippocampal neurogenesis is reported [2] and in our previous report with STZ-induced type 1 DM rats, that pattern was also observed [5].
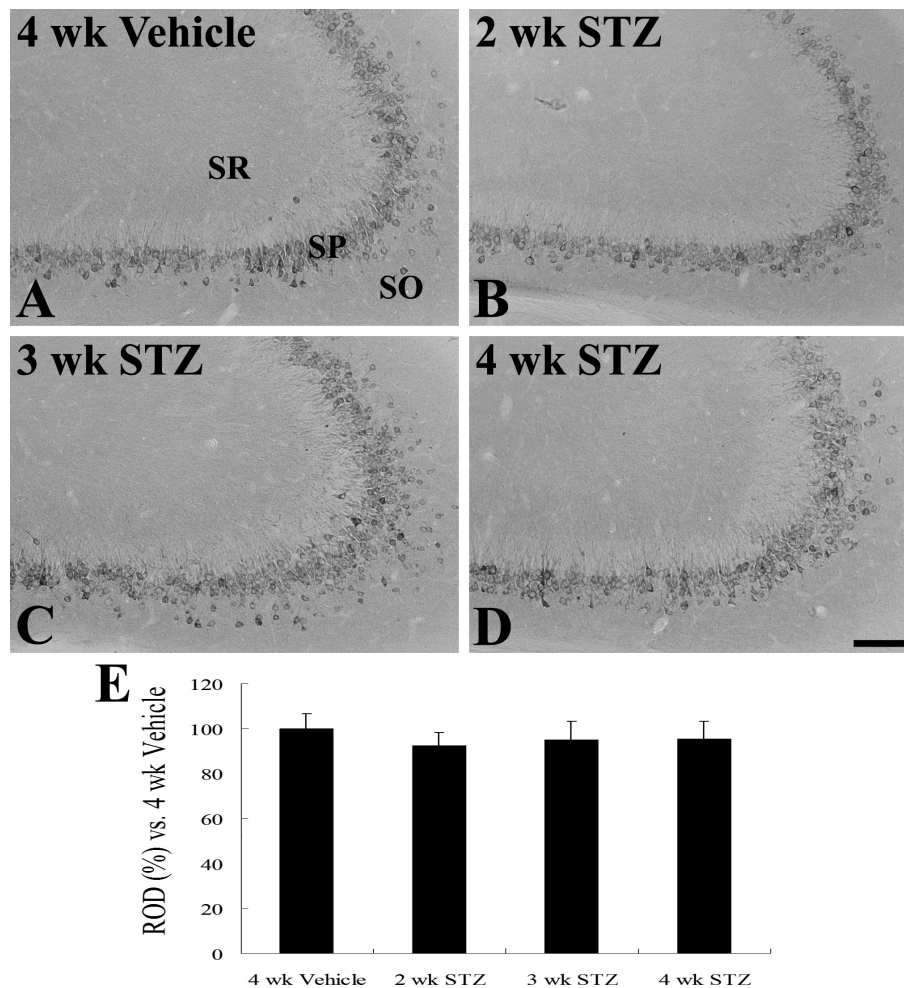
Fig. 3. Cyclooxygenase-2 (COX-2) immunoreactivity in the hippocampal CA3 region of groups at 4 weeks after vehicle treatment (A), and 2 (B), 3 (C) or 4 weeks (D) after STZ treatment. COX-2 immunoreactivity is found in the stratum pyramidale (SP) of the CA3 region. SO, stratum oriens; SR, stratum pyramidale. Scale bar=100 $\mu$m. E: Relative optical densities (ROD) as percentage values of COX-2 in the CA3 region of rats groups at 4 weeks after vehicle treatment, and 2, 3 or 4 weeks after STZ treatment. Differences among the means were analyzed statistically by one-way analysis of variance followed by Newman-Keuls method. There are no significant differences between groups. The bars indicate the SEM.

In this study, we investigated COX-2 immunoreactivity in the dentate gyrus and hippocampal CA3 region in diabetic rats. There were some differences in expression of COX-2 between vehicle-treated control and STZ-treated diabetic animals. COX-2 immunoreactivity was significantly increased in the dentate gyrus, not CA3 region, after STZ treatment, but after this period, the increase pattern was conversed.

The changes of COX-2 immunoreactivity in the dentate gyrus, not CA3 region may be associated with the neurogenesis occurred in the dentate gyrus. COX-2's action is involved with various prostaglandins [28], cell cycle proteins [23] and the NMDA receptor [19]. Still, COX-2's roles in neurogenesis are not fully elucidated and it can behave like a double-edged sword with positive or negative effect. One of

possible actions of COX-2 is on cell cycle proteins. COX-2 showed a positive effect on proliferation of neural progenitor cells and synaptic plasticity [28]. COX-2 inhibition is related with memory impairment and COX-2 knockouts showed reduced hippocampal neurogenesis [31]. In our previous study, we reported that STZ significantly increased latency time in a Morris water maze test and doublecortin immunoreactive neuroblasts were significantly decreased 2–3 weeks after STZ treatment [5]. However, in the present study, we observed the decrease of COX-2 immunoreactivity in the dentate gyrus at 4 weeks after STZ treatment. This result was coincided with previous study that doublecortin immunoreactive neuroblasts were increased at 4 weeks after STZ treatment [5]. It has been reported that neurogenesis is

transiently increased in brain damage such as ischemia [6, 14, 30, 33]. In type 2 diabetic rats, we reported that diabetes significantly reduced cell proliferation, neuroblast differentiation and COX-2 levels in the dentate gyrus [12, 13]. In addition, the blockade of COX-2 by celecoxib significantly ameliorated the reduction of neuroblast differentiation [13]. Our results strongly suggest that the action of COX-2 is related to neuroblast differentiation in the dentate gyrus.

Another possible action of COX-2 is the pro-inflammatory effect. In STZ-induced diabetic animals, insulin treatment resulted in reduced ischemia and reperfusion injury with decreased expression of nuclear factor-κB, COX-2 and inducible nitric oxide synthase [7]. In alcohol-induced brain damage, COX-2 was induced and its pattern was similar to that induced by excitatory amino acids [19], and COX-2 induction was also observed after seizure in rats [34]. Increased expression of COX-2 is reported to be related with reactive oxygen species and neuronal oxidative stress [22]. Alteration of the COX-2 pathway and oxidative stress was reported to being related with diabetic neuropathy [17]. In the present study, we also observed transient increase of COX-2 immunoreactivity in the dentate gyrus.

In conclusion, after STZ treatment, a type 1 diabetic-like state was induced in rats and COX-2 immunoreactivity was transiently increased in the dentate gyrus, not hippocampal CA3 region, 2–3 weeks after STZ treatment. These increases of COX-2 immunoreactivity may be associated with reduction of neural plasticity in the dentate gyrus.

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