FULL PAPER *Anatomy*

Species-Difference of Cyclooxygenase-2 in the Hippocampus of Rodents

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ABSTRACT. Cyclooxygenase (COX) generates free radicals and it is important in inflammatory response. In this study, we observed the immunoreactivity in mice (ICR and C57BL/6 strain), rats and gerbils. In these animals, COX-2 immunoreactivity was mainly detected in pyramidal cells of the hippocampal CA2/3 region and in granule cells of the dentate gyrus. COX-2 immunoreactivity in the CA2/3 region was the highest in ICR mice, while in gerbils COX-2 immunoreactivity was the lowest; COX-2 immunoreactivity in the dentate gyrus was the highest in rats and the lowest in gerbils. The protein levels of COX-2 were similar to the immunohistochemical data. COX-2 mRNA transcript was the highest in the gerbil and the lowest in the rat. In brief, COX-2 protein, not mRNA, in the hippocampus is generally higher in mice (ICR and C57BL/6 strain) than rats and gerbils.

KEY WORDS: cyclooxygenase-2, gerbil, hippocampus, mouse, rat.

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Cyclooxygenase (COX) converts membrane-derived arachidonic acid to prostaglandins and generates free radicals [16]. Two distinct isoforms of COX have been characterized, a constitute form, COX-1 and a mitogen-inducible form, COX-2 [21]. It is now believed that COX-2 is of primary importance in inflammatory response [2, 20].

It has been reported that basal levels of COX-2 expression are observed in neurons in the hippocampus as well as in the neocortex [6, 19], although its function has not been reported. In physiological conditions, N-methyl-*D*-aspartate synaptic activity rapidly induces COX-2 expression [1, 4, 5]. There are many reports that the expression of COX-2 is increased by traumatic brain injury, ischemia or kainate-induced neuronal damage [9, 13, 15]. The COX-2 overexpression potentiates excitotoxicity [7, 14]. In addition, these increases of neuronal injury by COX-2 overexpresssion are reversed by selective COX-2 antagonist [22].

Although a number of reports have focused on COX-2-immunoreactive neurons in the hippocampus in various brain injuries, comparative studies on COX-2 immunoreactivity in physiologically normal conditions have not been conducted in the hippocampus of rodents. Therefore, in the present study, the authors investigated the differences of COX-2 immunoreactivity, its protein and mRNA levels in the hippocampus of the mouse, rat and gerbil to show the differences of COX-2 among these rodents.

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MATERIALS AND METHODS

Experimental animals: Male ICR, C57BL/6 mice, Sprague-Dawley (SD) rats and Mongolian gerbils (*Meriones unguiculatus*) were obtained from the Experimental Animal Center, Hallym University, Chuncheon, South Korea. All animals were used at 3 months of age and 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 free access to food and water. The procedures for handling and caring for the animals adhered to the guidelines that are in compliance with the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85–23, 1985, revised 1996), and they were approved by the Institutional Animal Care and Use Committee (IACUC) at Hallym's Medical Center. All of the experiments were conducted to minimize the number of the animals used and the suffering caused by the procedures used in the present study.

Immunohistochemistry for COX-2: For COX-2 immunohistochemistry, seven animals in each group were anesthetized with intraperitoneal injection of chloral hydrate (50 mg/kg) and perfused transcardially 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. Thereafter frozen tissues were serially sectioned on a cryostat (Leica, Wetzlar, Germany) into 30-μm coronal sections, and they were then collected into six-well plates containing PBS.

Immunohistochemistry was performed under the same conditions in mice, rats and gerbils in order to examine

whether the degree of immunohistochemical staining was accurate. 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 antibody (1:200, Cat.# 160106 and 160126, Cayman, Ann Arbor, MI, U.S.A.) overnight at room temperature and subsequently exposed to biotinylated goat anti-rabbit IgG and streptavidin peroxidase complex (diluted 1:200, Vector, Burlingame, CA, U.S.A.). They were then visualized with reaction to 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.

A negative control test was carried out using pre-immune serum instead of primary antibody in order to establish the specificity of the immunostaining. The negative control resulted in the absence of immunoreactivity in all structures.

Western blot analysis for COX-2: To confirm differences in COX-2 levels in the hippocampus of mice, rats and gerbils, five animals in each group were sacrificed and used for western blot analysis. After sacrificing them and removing the brain, the hippocampus was then dissected with a surgical blade. The tissues were homogenized in 50 mM PBS (pH 7.4) containing ethylene glycol bis (2-aminoethyl Ether)-N,N,N,,N, tetraacetic acid (EGTA, pH 8.0), 0.2% nonidet P-40, 10 mM ethylendiamine tetraacetic acid (EDTA, 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 (PMSF) and 1 mM dithiothreitol (DTT). After centrifugation, the protein level was determined in the supernatants using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL, U.S.A.). Aliquots containing 50 μg of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto a 5% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Crop, East Hills, NY, U.S.A.). To reduce background staining, the membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with rabbit anti-COX-2 antiserum (1:500), peroxidase-conjugated rabbit anti-goat IgG (Sigma) and an ECL kit (Pierce Chemical).

RNA analysis for COX-2: To confirm differences in COX-2 mRNA levels in the hippocampus of mice, rats and gerbils, five animals in each group were sacrificed and used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Total RNA was extracted from hippocampus by using Pure Link™ RNA mini kit (Invitrogen Co., CA, U.S.A.) according to the manufacturer's instruction. RNA was quantified using NanoVue spectrophotometer (GE healthcare Co., NJ, U.S.A.). First-strand cDNA was synthesized using the AccuPower RT Premix (Bioneer Co., Daejeon, Korea) according to the manufacturer's instructions. This was designed to convert 1 μg of total RNA into 20 μl of first strand cDNA using oligo dT20 primer. Quantitative real time PCR reactions were performed using SYBR Green dye for COX-2 in ABI StepOne Real Time PCR instrument (Applied Biosystems Co., Cheshire, U.K.). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous reference control for all transcripts. The sequences of probes, forward and reverse primers (Bioneer Co.) were as follows.

COX-2 mice
Forward: 5'-AGA AGG AAA TGG CTG CAG AA-3'
Reverse: 5'-GCT CGG CTT CCA GTA TTG AG-3'
COX-2 rat
Forward: 5'-GCA CAA ATA TGA TGT TCG CAT TC-3'
Reverse: 5'-CAG GTC CTC GCT TCT GAT CTG-3'
COX-2 gerbil
Forward: 5'-GCC GTC GAG TTG AAA GCC CTC TAC A-3'
Reverse: 5'-CCC CGA AGA TGG CGT CTG GAC-3'
GAPDH mouse
Forward: 5'-GAC GGC CGC ATC TTC TTG T-3'
Reverse: 5'-CAC ACC GAC CTT CAC CAT TTT-3'
GAPDH rat
Forward: 5'-GCA AGA GAG AGG CCC TCA G-3'
Reverse: 5'-TGT GAG GGA GAT GCT CAG TG-3'
GAPDH gerbil
Forward: 5'-AAC GGC ACA GTC AAG GCT GAG AAC G-3'
Reverse: 5'-CAA CAT ACT CGG CAC CGG CAT CG-3'

Data analysis: All measurements were performed in order to ensure objectivity in blind conditions, by 2 observers for each experiment, carrying out the measures of experimental samples under the same conditions.

In order to quantitatively analyze COX-2 immunoreactivity, the corresponding areas of the hippocampus were measured from 25 sections per animal. Images of all COX-2-immunoreactive structures were taken from 3 layers (strata oriens, pyramidale and radiatum in the hippocampus proper; molecular, granule cell and polymorphic layers in the dentate gyrus) through an AxioM1 light microscope (Carl Zeiss, Göttingen, Germany) equipped with a digital camera (Axiocam, Carl Zeiss) connected to a PC monitor. Video images were digitized into an array of 512 × 512 pixels corresponding to a tissue area of 140 × 140 μm (40 × primary magnification). Each pixel resolution was 256 gray levels. The density of all COX-2-immunoreactive structures was evaluated on the basis of a optical density (OD), which was obtained after the transformation of the mean gray level using the formula: OD = log (256/mean gray level). The OD of background was taken from areas adjacent to the measured area. After the background density was subtracted, a ratio of the optical density of image file was calibrated as % (relative optical density) using Adobe Photoshop version 8.0 and then analyzed using NIH Image 1.59 software.

Statistical analysis: The data were elevated by a one-way ANOVA SPSS program and comparisons among groups

were made Duncan's multiple-range test. Statistical significance was considered at $P<0.05$.

RESULTS

COX-2 immunoreactivity: In all the species, COX-2 immunoreactivity was mainly detected in the hippocampal CA2/3 region and dentate gyrus (Fig. 1). In addition, there were no significant differences between two antibodies used in this study (data not shown). Generally, COX-2 immunoreactivity was high in the CA2/3 region, and intermediate in the dentate gyrus, and low in the CA1 region (Figs. 1, 2 and 3). COX-2 immunoreactivity in all subregions was similar between ICR and C57BL/6 mice, but COX-2 immunoreactivity was low in C57BL/6 mice compared to that in the ICR mice (Figs. 1, 2 and 3).

In the CA1 region, COX-2 immunoreactivity was mainly detected in pyramidal cells of the stratum pyramidale: The COX-2 immunoreactivity was the highest in the mouse and the lowest in the gerbil (Figs. 2A-2D and 3).

COX-2 immunoreactivity in the CA2/3 region was also detected in pyramidal cells of the stratum pyramidale (Fig. 2E-2H). Strong COX-2 immunoreactivity was detected in many pyramidal cells in the ICR and C57BL/6 mice, showing that the immunoreactivity in the CA2/3 region was the highest among all the species; COX-2 immunoreactivity in the rat and gerbil was lower than that in the mice, showing that the immunoreactivity in the rat was slightly higher than that in the gerbil (Fig. 3).

In the dentate gyrus, many cells in the granule cell layer and some cells in the polymorphic layer showed COX-2 immunoreactivity (Fig. 2I-2L). The COX-2 immunoreactivity was the highest in the rat and the lowest in the gerbil (Fig. 3). In the rat, COX-2-immunoreactive processes are found in the molecular layer (Fig. 2K).

COX-2 protein levels: Western blot findings in the hippocampus of mice, rats and gerbils coincided with immunohistochemical changes (Fig. 4). COX-2 protein level in the hippocampal homogenates was the highest in the mice and lowest in the gerbil.

COX-2 mRNA levels: qRT-PCR in the hippocampus of mice, rats and gerbils was contradictory to immunohistochemical changes (Fig. 5). COX-2 mRNA level in the hippocampal homogenates was the highest in the gerbil and the lowest in the rat.

DISCUSSION

In this study, we observed the COX-2 immunoreactivity and protein levels in the hippocampus in mice, rats and gerbils. COX-2 immunoreactivity was mainly detected in pyramidal cells of the CA2/3 region and in granule cell of the dentate gyrus in all the animals. This result is supported by previous studies that endogenous COX-2 expression is

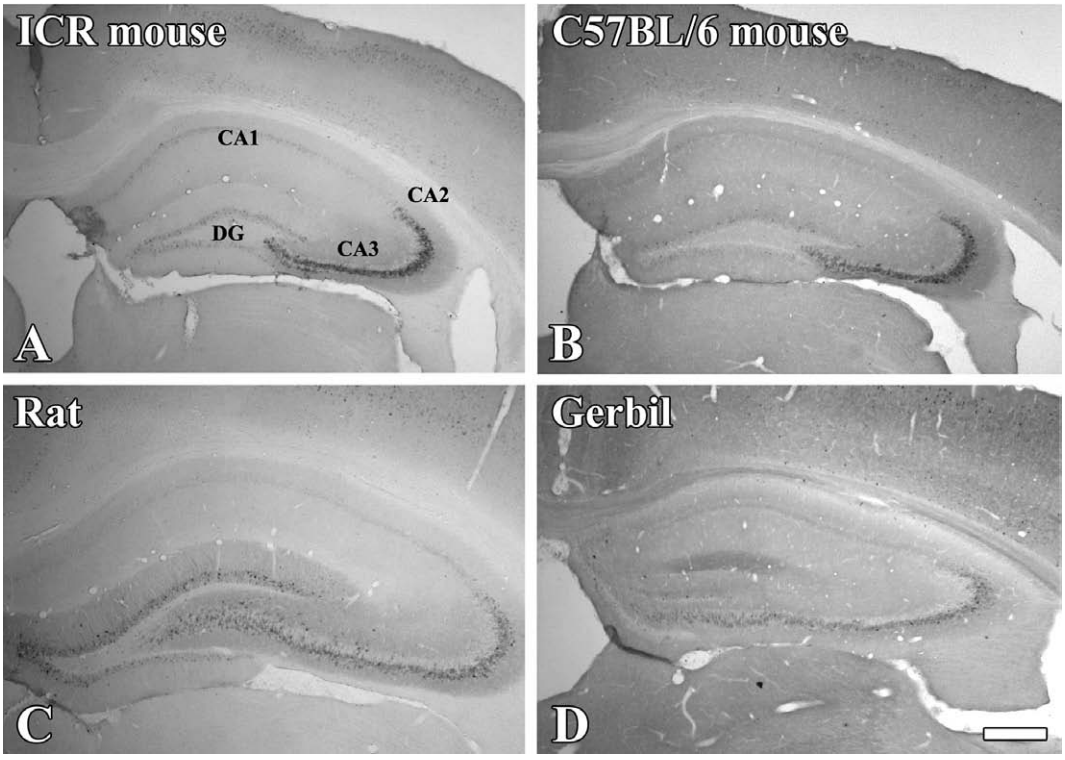

Fig. 1. Immunohistochemistry for COX-2 in the hippocampus of ICR (A), C57BL/6 mice (B), rats (C) and gerbils (D). COX-2 immunoreactivity is mainly detected in the hippocampal CA3 region of all species. However, the COX-2 immunoreactivity is significantly difference among the species. Bar=800 μm.

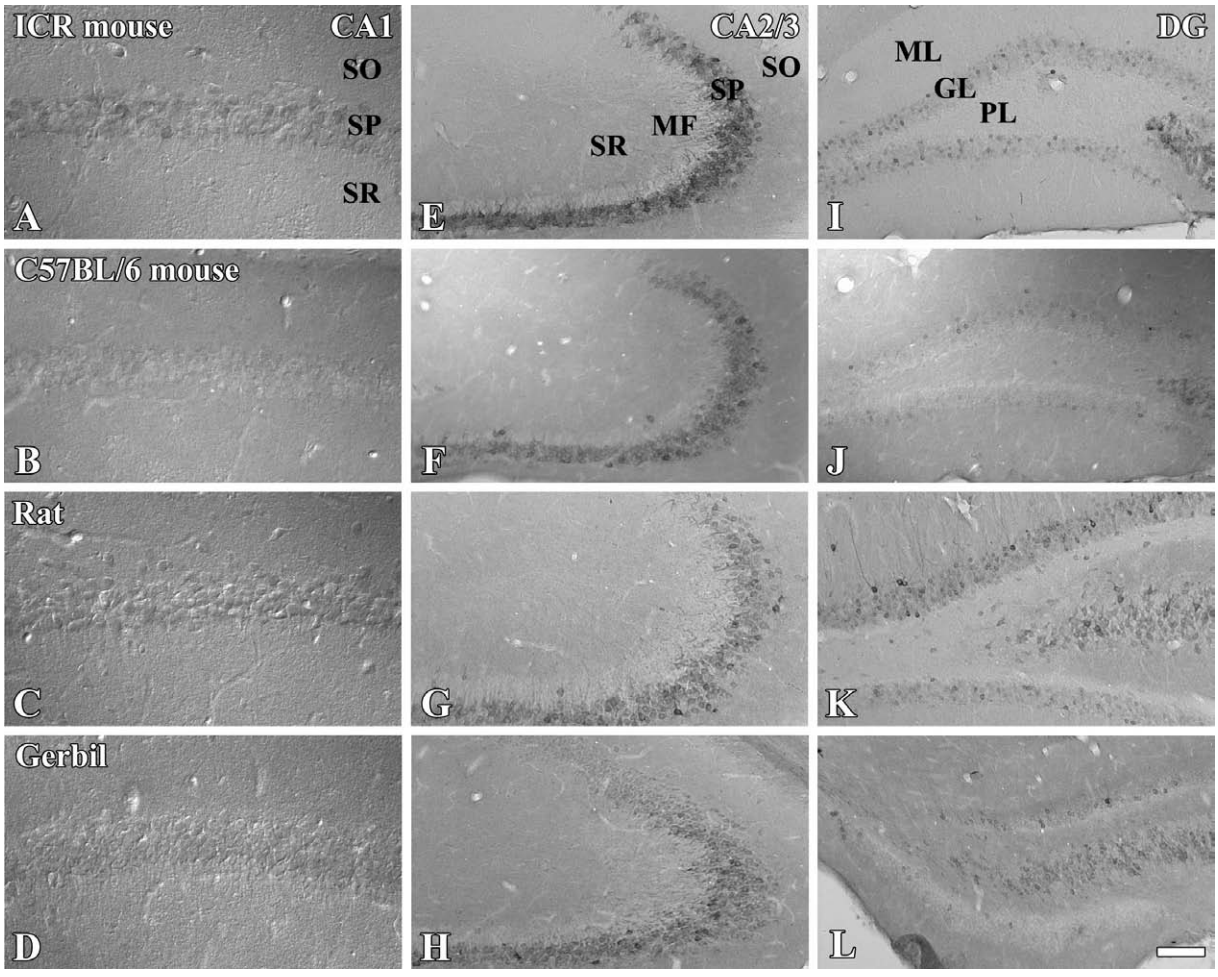

Fig. 2. Immunohistochemistry for COX-2 in the CA1 region (A-D), CA2/3 region (E-H) and dentate gyrus (I-L) in ICR (A, E and I), C57BL/6 (B, F and J) mice, rats (C, G and K) and gerbils (D, H and L). Weak COX-2 immunoreactivity is detected in the stratum pyramidale of the CA1 region in ICR (A) and C57BL/6 (B) mice. In the CA2/3 region, COX-2 immunoreactivity is found in the stratum pyramidale (SP) in all the species, showing the highest immunoreactivity in ICR (E) and C57BL/6 (F) mice. In the dentate gyrus, COX-2 immunoreactivity is mainly detected in the granule cell layer (GCL), showing the highest immunoreactivity in rats (K). ML, molecular layer; PL, polymorphic layer; SO, stratum oriens; SR, stratum radiatm. Bar=100 μm.

mainly detected in the hippocampal CA3 region of the rat [8, 11, 18]. However, Lee *et al.* did not detect COX-2 immunoreactive neurons in the hippocampal CA3 region of normal C57BL/6 mice [10]. However, Ristori *et al.* showed that COX-2 immunoreactivity was observed in all pyramidal cells including the CA3 region [17]. This discrepancy may be associated with the antibody they used.

In this study, we observed that the COX-2 immunoreactivity and protein levels were the highest in the mouse hippocampus and the lowest in the gerbil hippocampus. The differences of COX-2 basal levels in the CA3 region may be associated with the vulnerability of hippocampal CA3 neurons. It has been reported that neuronal COX-2 transgenic mice are more susceptible to kainic acid excitotoxicity [7]. This result is supported by previous studies that the extent of neuronal damage in gerbils induced by kainic acid was minor when compared with that of the rat [3, 12]. In addition, differential expression patterns for caspases and transcription factor c-fos were recently described in the rat and gerbil, following systemic administration of kainic acid [3]: similar distribution pattern of caspase and c-fos was observed in kainic acid treated gerbils and rats although minimized in gerbils.

Unlike immunohistochemistry and western blot study, COX-2 mRNA in the hippocampal homogenates was the highest in the gerbil, but the owest in the rat. Discrepancies between the mRNA and protein expression for COX-2 suggest broad alterations of post-transcriptional processes among these rodents.

In conclusion, COX-2 was differentially expressed in the hippocampal CA3 region and dentate gyrus in mice, rats and gerbils. These differences among the species may be associated with degree of vulnerability of hippocampal neurons, especially, CA3 pyramidal neurons.

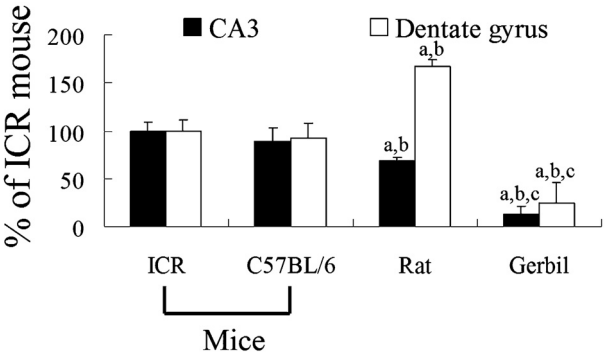

Fig. 3. Relative optical density as % of COX-2 immunoreactivity in the hippocampus in mice (ICR and C57BL/6 strain), rats and gerbils (n=7 per group; [a] P<0.05, significantly different from the ICR mouse, [b] P<0.05, significantly different from the C57BL/6 mouse, [c] P<0.05, significantly different from the rat. The bars indicate the means ± SEM.

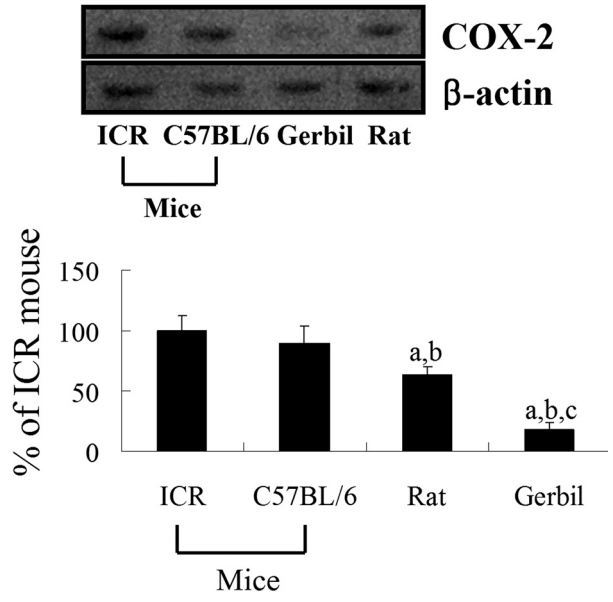

Fig. 4. Western blot analysis of COX-2 in the hippocampus derived from mice (ICR and C57BL/6 strain), rats and gerbils. The relative optical density as % of immunoblot band is also represented (n=5 per group; [a] P<0.05, significantly different from the ICR mouse, [b] P<0.05, significantly different from the C57BL/6 mouse, [c] P<0.05, significantly different from the rat). The bars indicate the means ± SE.

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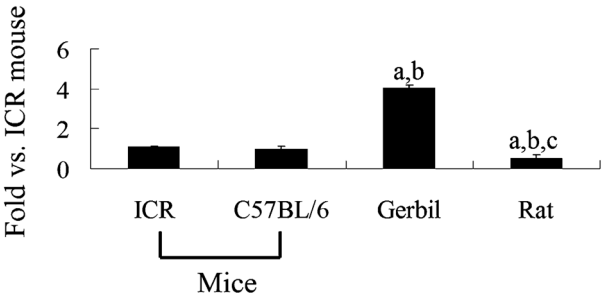

Fig. 5. Quantitative RT-PCR analysis of COX-2 in the hippocampus derived from mice (ICR and C57BL/6 strain), rats and gerbils (n=5 per group; [a] P<0.05, significantly different from the ICR mouse, [b] P<0.05, significantly different from the C57BL/6 mouse, [c] P<0.05, significantly different from the gerbil). The bars indicate the means ± SE.

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