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Kinetic Changes of COX-2 Expression during Reperfusion Period after Ischemic Preconditioning Play a Role in Protection Against Ischemic Damage in Rat Brain

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A brief ischemic insult induces significant protection against subsequent massive ischemic events. The molecular mechanisms known as preconditioning (PC)-induced ischemic tolerance are not completely understood. We investigated whether kinetic changes of cyclooxygenase (COX)-2 during reperfusion time-periods after PC were related to ischemic tolerance. Rats were given PC by occlusion of middle cerebral artery (MCAO) for 10 min and sacrificed after the indicated time-periods of reperfusion (1, 2, 4, 8, 12, 18 or 24 h). In PC-treated rats, focal ischemia was induced by occlusion of MCA for 24 h and brain infarct volume was then studied to determine whether different reperfusion time influenced the damage. We report that the most significant protection against focal ischemia was obtained in rats with 8 h reperfusion after PC. Administration of indomethacin (10 mg/kg, oral) or rofecoxib (5 mg/kg, oral) 48 h prior to PC counteracted the effect of PC. Immunohistochemical analysis showed that COX-2 and HO-1 protein were induced in PC-treated rat brain, which was significantly inhibited by rofecoxib. Taken together, we concluded that the kinetic changes of COX-2 expression during the reperfusion period after PC might be partly responsible for ischemic tolerance.

Key Words: Ischemic preconditioning, Stroke, Heme oxygenase, Cyclooxygenase

INTRODUCTION

Neuronal death in the acute phase after transient focal ischemia is the underlying cause of neurological dysfunction that is often reported in stroke sufferers. No satisfactory therapies that limit neuronal damage and neurological dysfunction after stroke in humans are currently available. While the exact molecular mechanisms that govern stroke-induced neuronal death are not yet known, massive inflammation is implicated in secondary ischemic brain damage (Zhang and Stanimirovic, 2002; Xu et al, 2005; Khan et al, 2005). Investigations using genetically altered cyclooxygenase (COX) knockout mice provided novel information about COX-2. In 2002, Ray and colleagues reported the risk of serious coronary heart disease of COX-2 selective non-steroidal anti-inflammatory drugs. COX-2 is thus regarded as a cardioprotective protein that alleviates ischemia/reperfusion injury and mediates the late phase of preconditioning (PC) (Shinmura et al, 2002). PC is an endogenous neuroprotective mechanism by which a sublethal ischemic event confers tolerance to subsequently lethal ischemia. Although the molecular mechanisms of PC are not fully understood, this phenomenon was observed in multiple organs including the brain, in various species of mammals (Edwards et al, 2000; Kirino, 2002; d Stenzel-Poore et al, 2004). Recent studies showed that PC induced by a 10 min transient middle cerebral artery occlusion (MCAO) in adult rats significantly prevents the infarction and neuronal death caused by a 60 min MCAO induced 3 days after PC (Dhodda et al, 2004). This prompted us to ask following questions. What happens during the 3 days after PC in the brain and how is this related to neuronal protection against a 60 min MCAO? If changes in the level of COX-2 protein occur during that period of time and this acts as a signal for new protein(s) synthesis, such as HO-1. We report that kinetic changes of COX-2 expression occurred during the reperfusion period after PC reaching its maximum at 8 h, which correlated with maximum protection of the brain against 24 h MCAO-induced injury. The COX-inhibitors were also shown to completely counteract the beneficial effects of PC and increased infarct volume against focal ischemia.

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ABBREVIATIONS: ANOVA, One-way analysis of variance; COX-2, Cyclooxygenase; HO-1, Heme oxygenase-1; MCAO, Middle cerebral artery occlusion; NMDA, N-methyl-D-aspartate; PC: Preconditioning; SDS, Sodium dodecyl sulfate; TTC, Triphenyltetrazolium chloride; PBS, Phosphate buffered saline.

METHODS

Animals and surgical procedures

Adult male Sprague Dawley rats (180~200 g; Samtako, Korea) were used in the study. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, US Department of Health and Human Services Publication [NRC, 1996]. Transient MCA occlusion was conducted as described previously (Planas, 1999). In brief, rats were anesthetized with rumpun and ketamine, and placed in an operating blanket fitted with a nose cone to administer 2% halothane anesthesia. The left common carotid artery was exposed, and the external carotid artery and pterugopalatine arteries were ligated. A 3-0 monofilament nylon suture with a round tip was introduced through an external carotid artery stump and gently advanced to the internal carotid artery as far as to the anterior cerebral artery. The entire procedure was completed within 20 min, and if the time was exceeded or any step was delayed, the rat was excluded from the data. PC was conducted by insertion of a silk thread to the artery for 10 min. After the wound was sutured, the rats were allowed to recover from anesthesia before returning to the cage with free access to rat chow and water. The PC-treated rats were sacrificed after the indicated time of reperfusion was completed (1~24 h). During the MCA occlusion, body temperature (37~38°C) was maintained at physiological levels. Sham operated rats underwent the same procedures except the occlusion.

Measurement of volume of infarct size

The brains were quickly removed and sectioned coronally into seven slices each with a 2-mm thickness. The brain slices were incubated for 30 min in a 2% solution of TTC (Triphenyltetrazolium chloride) at 37°C and fixed by immersion in 10% phosphate-buffered formalin solution. Seven brain sections per animal, stained with TTC, were recorded with a color video camera attached to a computer 48 hours after formalin fixation in order to measure lesion areas. Areas not stained red with TTC, which were considered to be lesion areas, were calculated using a video image analysis system (Leica-Q500 IW). The volume of the infarct was calculated by measuring infarct areas on separate slices, multiplying these areas by 2 mm-slice (the thickness of each slice), and summing all the values. The brain infarct size was significantly reduced in rats that were allowed 8 h reperfusion after PC. Therefore, indomethacin (10 mg/kg, oral) or refecoxib (5 mg/kg) was administered 48 h before PC and allowed 8 h of reperfusion to determine whether COX enzyme activity was related with neuronal damage.

Western blot analysis

The brain were quickly removed and placed in lysis buffer containing protease inhibitor, homogenized and then centrifuged. After quantitating the protein levels, each sample was boiled in loading buffer and loaded onto gradient sodium dodecyl sulfate (SDS)-polyacrylamide gels. Following electrophoresis, the proteins were transferred onto an immobilon-polyvinylidine fluoride membrane. Membranes were blocked with TBS-T containing 5% (w/v) skim milk powder for 1 h at room temperature. The membrane was incubated with primary antibodies against COX-2, HO-1 or β-actin at a dilution of 1 : 1,000 overnight at 4°C. The proteins were detected with horseradish peroxidase-conjugated secondary antibody (1 : 5,000 dilutions in TBS-T containing 5% skim milk powder, 1 h, room temperature) and were visualized by enhanced chemiluminescence.

Immunohistochemistry

Brains were perfusion-fixed with 4% (w/v) paraformaldehyde solution in phosphate buffered saline (PBS), removed and post-fixed overnight in the same fixative prior to vibratome sectioning. The 33-μm coronal sections were mounted on 2% gelatin-coated slides and then dried on a slide warmer at 50°C for 30min. Sections containing the hippocampus were immunostained for COX-2 or HO-1. Briefly, the sections were incubated overnight at 4°C with COX-2 or HO-1 antibody (1 : 500) in PBS (pH 7.4) with 0.2% Triton. After washing with PBS containing 0.2% Triton, the secondary anti-mouse IgG antibody was applied for 90 min at room temperature. After further washing, ABC Elite (Vector Laboratories, Burlingame, USA) was applied for 90 min, then the slides were rinsed with PBS followed by Tris buffer (pH 7.6), and reacted with DAB (0.1 mol/L Tris, 0.04% DAB, 0.033% H_2O_2); the reaction was stopped with phosphate buffer. The slides were dehydrated, cleared and cover-slipped for analysis.

Statistics

All data are expressed as mean±SD unless otherwise indicated. Comparisons of parameters among the four groups were made by one-way analysis of variance (ANOVA) for repeated measure, followed by Scheffé test. A probability value <0.05 indicated statistical significance.

RESULTS

Kinetic changes of COX-2 expression during reperfusion after PC

To evaluate the possibility of changes in the expression level of COX-2 depending on reperfusion time after PC, rats were preconditioned by occlusion of MCA for 10 min and reperfused at the indicated times shown in Fig. 1 upper panel. After reperfusion for the indicated times, rats were killed and COX-2 expression was analyzed by Western blotting. As shown in Fig. 1 lower panel, COX-2/β-action ratio was changed as the time of reperfusion varied, where maximum expression was shown at 1 h and 8 h reperfusion. It should be noted that COX-2 was expressed constitutively in the rat brain.

No inflammatory sign shown in brain after 8 h reperfusion post PC

Since COX-2 is expressed normally in the rat brain and 8 h reperfusion significantly increased COX-2 expression levels after 10 min PC, we wanted to know whether maximally induced COX-2 induced any inflammation or injury in the rat brain. As shown in Fig. 2, no inflammatory sign was observed in rat brains that were reperfused 8 h after PC. This implied that adequate amounts of COX-2 expression without inflammation might be beneficial

Preconditioning significantly decreased the infarction

Next, we compared the infarct volume, as an indicator of brain injury, by allowing reperfusion with different time-periods after PC. Also permanent ischemia (MCAO, 24 h) was induced right after designated reperfusion time periods. As shown in Fig. 3, brain damage in the rats was significantly diminished under conditions of a reperfusion time of 8 h after PC. About 33% of the whole brain was damaged when MCA was occluded for 24 h. However, the damage was proportionately decreased as the reperfusion time increased until 8 h after 10 min PC.

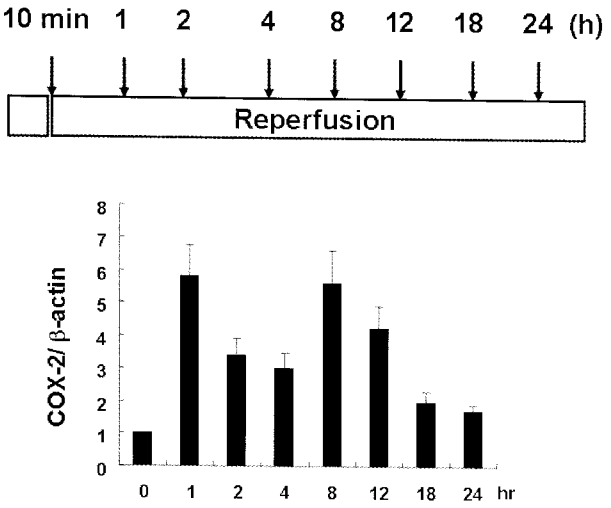

Fig. 1. Time course of COX-2 expression by Western blot analysis in rat brain subjected to MCA occlusion for 10 min. The results were presented as ratio of COX-2/β-actin, in which the amount of constitutively expressed COX-2/β-actin at 0 h was designated as 1. It should be noted that preconditioning increases COX-2 expression which varied depending on the reperfusion.

COX-inhibitors abrogates beneficial effect of PC

Next, we tested whether COX-2 expression was really important for protection of the brain against severe ischemia. To accomplish this, indomethacin (10 mg/kg) or rofecoxib (5 mg/kg) was administered 48 h prior to PC, and allowed to reperfuse for 8 h. As shown in Fig. 4A, brain damage was inflicted by these COX-inhibitors, although no damage was demonstrated in 8 h reperfused rat brain after PC. This indicated that COX-2 expression was necessary for presenting the beneficial effects of PC. Furthermore selective COX-2 inhibitors were more detrimental than nonselective COX-inhibitors, indicating that COX-1 played some role in the protective action as well.

Rofocoxib reduces COX-2 as well as HO-1 protein in rat brain

As shown in Fig. 5, COX-2 (Fig. 5A) and HO-1 (Fig. 5B) proteins were successfully stained with corresponding antibodies in PC rats. In rats that were pretreated with rofecox-

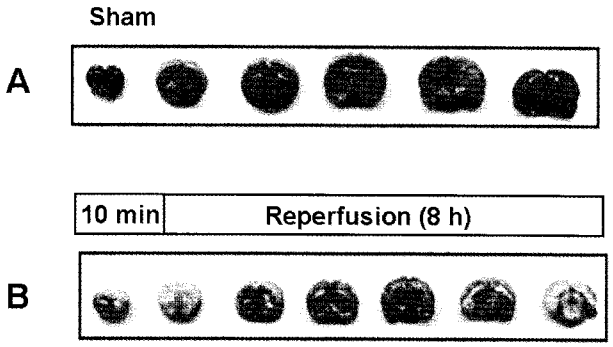

Fig. 2. A representative photograph of neuronal damage between sham-operated (A) and ischemic preconditioned rat brain (B), in which 8 h reperfusion was performed after 10 min MCA occlusion.

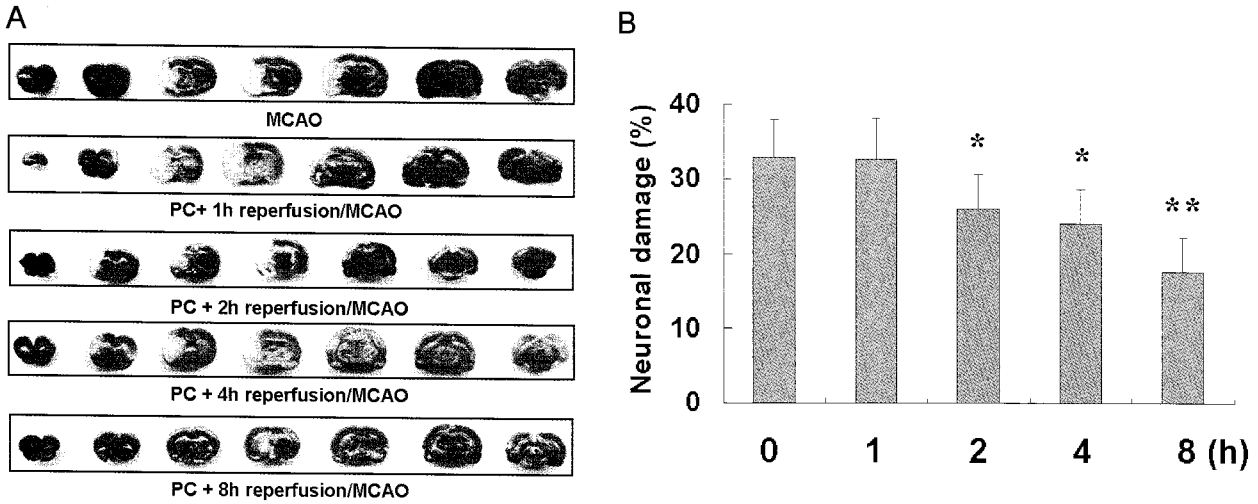

Fig. 3. Size of brain infarction according to reperfusion time after PC against focal ischemia (24 h, MCAO). (A). Percentile of neuronal damage (%) occurred with different time of reperfusion after ischemic preconditioning against focal ischemia (B).

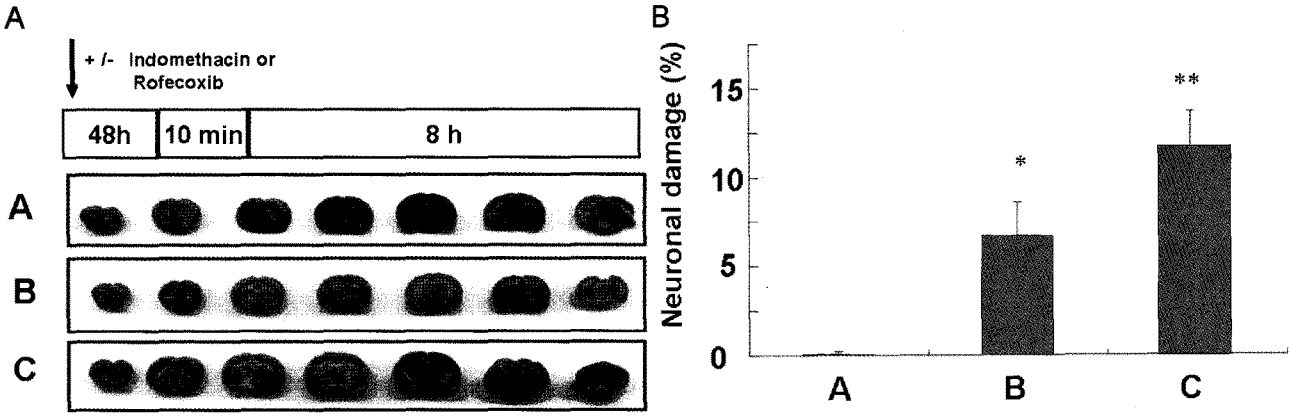

Fig. 4. A representative photograph of neural damage by COX-inhibitors in PC-treated rat brain. A. PC only, B. Indomethacin/PC, C. roferocoxib/PC (A) Percentile of neuronal damage (%) occurred with COX-inhibitors in PC-treated rat (B).

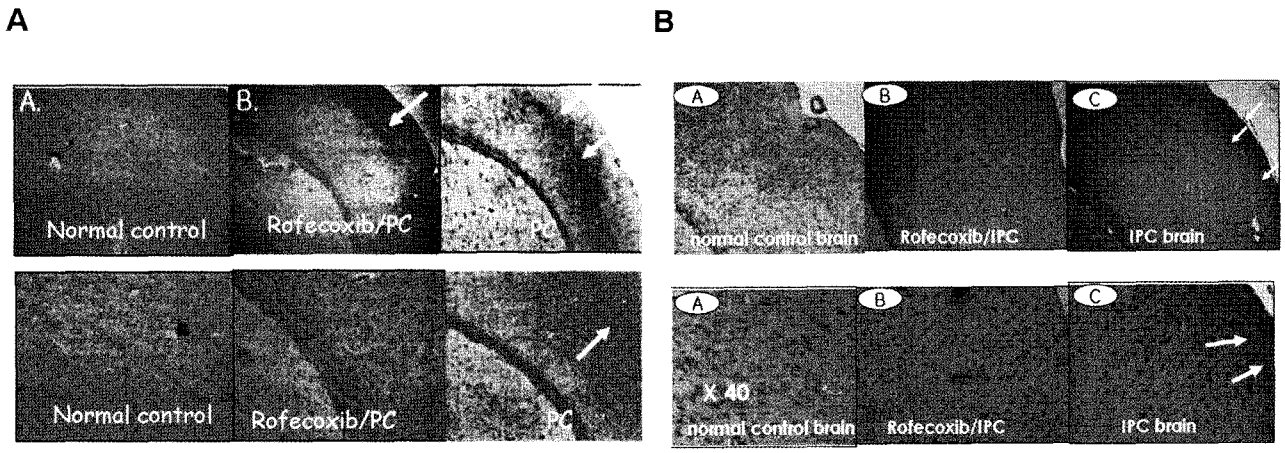

Fig. 5. Immunohisotochemical staining of COX-2 and HO-1 in the brain treated with or without rofecoxib in PC-treated rat. (A) COX-2 (B) HO-1.

ib, there was a significant reduction in the levels of COX-2 (Fig. 5A) as well as HO-1 protein (Fig. 5B) in the brain.

DISCUSSION

Neurons can be preconditioned by various procedures to resist ischemic events. PC is characterized by a brief episode of ischemia that renders the brain more resistant to subsequent longer ischemic events. This ischemic tolerance has been shown in numerous experimental models of cerebral ischemia. This phenomenon of ischemic tolerance has been confirmed in various animal models of forebrain ischemia and focal cerebral ischemia. In the brain, ischemic PC is mediated largely through the activation of the N-methyl-D-aspartate (NMDA) receptors via increases in the intracellular levels of calcium, and requires new protein synthesis. Requirements for the induction of tolerance depend, in part, on the experimental model, whether global or focal ischemiae, and the animal species studied.

COX-2 is normally expressed throughout the brain in discrete populations of neurons and is regulated by glutamate receptors and glucocorticoids (Koistinaho et al, 1999). Interestingly, rats that were reperfused after PC resulted in varying levels of expression of COX-2 depending on the time-periods of reperfusion. When focal ischemia was given after PC, PC significantly prevented ischemic damages, but this depended on the degree of COX-2 expression during the reperfusion period. Kinetic changes of COX-2 expression were observed during the reperfusion period. Furthermore, the maximum expression of COX-2 occurred after 8 h reperfusion post PC, which correlates well with maximal protection against focal ischemia. This finding suggested that if the amount of COX-2 was expressed to the extent that inflammation did not occur, it might be potentially beneficial rather than detrimental In spite of the maximum COX-2 induction during the 8 h reperfusion period after PC, there was no sign of inflammation or neural damage. If no damage is induced even upon increased levels of COX-2 expression, this may be a sign of defense preparations in the brain for future ischemic attacks.

We confirmed that a 10 min MCAO (PC) did not show any sign of inflammation in the brain. This correlated with the report by Planas (1999). The timing, type of inflammatory mediator and the extent of its stimulation determine the balance between the 'bad' versus 'good' effects

of inflammation (Barone and Feuestein, 1999). Following a CNS insult, acute phase inflammation is believed to be detrimental, while the chronic phase inflammation might be essential for repair and regeneration. Some macrophage and microglial response after injury is necessary for scavenging the necrotic debris facilitating plasticity (Danton and Dietrich, 2003). However, excess infiltration of leukocytes into the brain is detrimental and therapies that prevent leukocyte infiltration during the acute phase after ischemia are neuroprotective (Yrjanheikki et al, 1999; Veldhuis et al, 2003; Yenari et al, 2005; Garau et al, 2005). Individual inflammatory mediators play a complex role in promoting protection as well as destruction after brain injury. For example, the complement system that is activated in the post-ischemic brain initiates local inflammatory responses that ultimately contribute to neuronal survival and tissue remodeling. However, anti-inflammatory drugs like minocycline and indomethacin are known to induce neuroprotection by preventing microglial activation and inflammation (Yrjanheikki et al, 1999; Monje et al, 2003). Although the exact molecular mechanisms and the complete spectrum of the beneficial effects of PC are still to be identified, upregulation of several neuroprotective cascades is thought to mediate PC-induced ischemic tolerance (Bernaudin et al, 2002; Truettner et al, 2002; Dhodda et al, 2004; Stenzel-Poore et al, 2004; Hong et al, 2004).

Induction of HO-1, a cytoprotective protein, was clearly shown in the PC rat brain, while rofecoxib-treated rat brain showed a significant inhibition of the induction of HO-1 by PC. At present, it is not known how COX-2 inhibitors influence HO-1 activity or expression, but recently we and others reported that the HO-1 inhibitor, SnPPIX, reduced COX-2 activity without changing the expression of COX-2 in vascular smooth muscle cells (Choi et al, 2008). Although it may be related to the common denominator for COX and HO-1, a heme molecule, the mechanism by which COX-2 and HO-1 mutually interact needs further study. The possibility of heme being involved in the mechanism, by which COX-2 selective inhibitor, rofecoxib, inhibits HO-1 expression in rat brain, also needs to be investigated.

In summary, we investigated the hypothesis that kinetic changes of COX-2 expression during reperfusion after ischemic PC may determine the extent of brain injury against severe ischemia. Indeed, we have clearly shown that PC protected brains from severe ischemia with different degrees depending on the reperfusion time. The maximal COX-2 induction was shown at 8 h reperfusion in 10 min PC. This seems to be important because 10 min MCAO did not induce any sign of inflammation. Furthermore, rofecoxib and indomethacin nullified the beneficial effects of PC. These findings strongly suggest that COX-2 induction played an important role in ischemic tolerance. We concluded that COX-2 expression after PC was an important and necessary factor for protection against severe ischemia, and the expression of COX-2 was shown to be dependent on the reperfusion time.

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