Increased Neuronal Injury in Transgenic Mice with Neuronal Overexpression of Human Cyclooxygenase-2 is reversed by Hypothermia and Rofecoxib Treatment

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Abstract: Cyclooxygenase-2 (COX-2) is up-regulated during ischemia. However, the role of COX-2 in neuronal injury is still unclear. In this study we tested whether neuronal overexpression of human COX-2 in a transgenic mouse model potentiates neuronal injury after global ischemic insult. Further, we tested whether the neuronal injury could be ameliorated by intra-ischemic mild hypothermia (33-34°C) alone or in combination with diet treatment of rofecoxib, a COX-2 specific inhibitor. Global ischemia with intra-ischemic normothermia (36-37°C) resulted in significantly higher neuronal damage in the CA1 region of hippocampus of transgenic mice than in wild type controls, confirming a deleterious role of COX-2 in ischemic neuronal damage. Hypothermia significantly reduced neuronal damage in both transgenic mice and wild type controls to the same extent, suggesting that the aggravating effect of COX-2 could be largely eliminated by hypothermia. When hypothermia was combined with rofecoxib treatment, neuronal damage was further reduced in response to global ischemia. The results suggest that COX-2 inhibition by prophylactic treatment with rofecoxib coupled with hypothermia at the time of acute stroke insult could be an effective therapeutic approach in early stages of stroke treatment in high risk patients.

Key Words: COX-2, ischemia, hypothermia, rofecoxib, stroke.

INTRODUCTION

Cyclooxygenase (COX) converts membrane-derived arachidonate to prostaglandins and generates free radicals (Pasinetti, 1998). COX has two isoforms: while COX-1 is largely constitutive, and is involved in maintaining normal cell function (Williams and DuBois, 1996), COX-2 is inducible by inflammatory signals such as cytokines and lipopolysaccharides (Cao *et al*., 1995), and has been implicated in neurodegeneration due to ischemia (Nogawa *et al*., 1997), trauma (Resnick *et al*., 1998) and chronic neurological diseases such as Alzheimer's disease (Pasinetti, 1998).

In global ischemia, which occurs in the event of cardiac arrest, transient cessation of blood flow to the brain results in neuronal death in vulnerable brain regions such as hippocampus (Abe *et al*., 1995). The mechanism(s) of neuronal death has been attributed to glutamate neurotoxicity, energy failure, oxidative stress, inflammation, and apoptotic gene expression (Rothman and Olney 1986; Lo *et al*., 2003). Evidence suggests that COX-2 is involved in ischemic neuronal death. COX-2 mRNA expression is rapidly elevated in ischemic brain (Planas *et al*., 1995; Collaco-Moraes *et al*., 1996; Nogawa *et al*., 1997; Koistinaho *et al*., 1999) and persists in injured neurons (Nogawa *et al*., 1997). COX-2 enzymatic products such as prostaglandin E2 (PGE2) exacerbate neuronal injury (Pasinetti, 1998), inhibit astrocytic glutamate uptake (Bezzi *et al*., 1998), thus potentiating glutamate neurotoxicity (Kelley *et al*., 1999; Hewett *et al*., 2000; Iadecola *et al*., 2001; Kunz and Oliw, 2001). PGE2 physically associates with Bax, triggering apoptotic-like changes followed by association with mitochondria (Lalier *et al*., 2007). COX-2 may worsen oxidative injury alone (Pasinetti, 1998) or act synergistically with inducible nitric oxide synthase (iNOS) (Nogawa *et al*., 1998). Pharmacological COX-2 inhibition or gene knockout alleviate glutamate neurotoxicity (Hewett *et al*., 2000; Iadecola *et al*., 2001; Kunz and Oliw, 2001), and reduce ischemic injury (Nogawa *et al*., 1997; Nakayama *et al*., 1998). Our previous studies have shown that COX-2 overexpression potentiated excitotoxicity (Kelley *et al*., 1999; Mirjany *et al*., 2002).

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Hypothermia affects a variety of cellular activities (Inamasu and Ichikizaki, 2002), and has been shown to be protective against ischemia in both animal experiments (Tsuchiya *et al*., 2002) and clinical trials (Inamasu and Ichikizaki, 2002; Sterz *et al*., 2003). Hypothermia in combination with drug therapy has been shown to be more effective than single treatment regimen (Schmid-Elsaesser *et al*., 1999). Rofecoxib [4-[4-(methylsulfonyl)phenyl]-3-phenyl-2(5H)-furanone] is a selective COX-2 inhibitor and prevents hippocampal neuronal injury in an animal model of excitotoxic neurodegeneration (Hewett *et al*., 2006). In this study, we set to test whether hCOX-2 transgenic mice potentiates ischemic neuronal injury. Further, we tested whether COX-2 inhibition could provide added protection in addition to hypothermia. Our data support a deleterious role of COX-2 in ischemic neuronal injury and COX-2 inhibition in combination with hypothermia may promise a more effective approach in clinical stroke therapy.

MATERIALS AND METHODS

Animals

All procedures involving animals met the guidelines described in the *NIH Guide for the Care and Use of Laboratory Animals* and had been approved by the Animal Facility of the Mount Sinai School of Medicine. Transgenic mice with neuronal overexpression of human COX-2 were established as previously described (Kelley *et al*., 1999). Offspring generated from inbred homozygous hCOX-2 mice were used in this study. All mice used were male and 2-4 months of age. These hCOX-2 transgenic mice showed no signs of developmental abnormality, no apparent differences in body weight, gross behavior compared to wild type mice with the same genetic background (Kelley *et al*., 1999). Wild type mice with the same genetic background (B6C3, F1) served as controls (Taconic Farms, Germantown, NY, USA).

Global Ischemia Surgery

Occlusion of bilateral common carotid arteries was employed to model global ischemia. Rectal temperature was monitored during surgery. In preliminary experiments, 10 min occlusion caused minimal neuronal damage in pyramidal layers of the hippocampus (not shown). Subsequently, 20 min occlusion was adopted in our study. Mice were anesthetized by intraperitoneal injection (20ml/kg) of Avertin (1.25% tribromoethanol) and fixed in a supine

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position. After a midline skin cut in the neck, bilateral common carotid arteries were carefully isolated. Rectal temperature was monitored using a point thermometer (Fisher, Morris Plains, NJ, USA) during surgery. During anesthesia, rectal temperature falls to 30-33°C. Intra-ischemic hypothermia (33-34°C) or normothermia (36-37°C) was achieved with a heating lamp. Once the desired temperature was reached, two micro clips (Fine Science Tools, Foster City, CA, USA) were applied to the common carotid arteries to block blood flow. After withdrawal of the clips, the wound was closed. Mice were allowed to recuperate in a warm (30°C) humidified box for 2hr before returning to their cages. Sham control mice were treated the same way except that no clips were applied.

Mice were sacrificed 3 days after surgery by cervical dislocation and the brains were frozen in 2-methyl butane pre-cooled on dry ice. Cryosections (10μm) at the level of mid hippocampus (Bregma - 2.2mm) were made and stored at -80°C until use.

Apoptotic Neuronal Damage Assessment

Terminal deoxynucleotidyl transferase nick end labeling (TUNEL) method was used to identify apoptotic neurons according to the manufacturer's instruction (ApopTag kit, Intergen, Purchase, NY, USA). In brief, frozen brain sections were fixed in 1% paraformaldehyde in phosphate buffered saline (PBS) for 10min, and postfixed in ethanol: acetic acid mix (2:1) at -20°C for 5 min. After treatment with 3% H_20_2 in PBS for 5min to block endogenous peroxidase activity, slides were incubated with TdT enzyme in a humidified chamber at 37°C for one hour, followed by anti-digoxigenin conjugate for 30min, and TUNEL labeled cells were visualized using DAB substrate kit (Vector, Burlingame, USA). Slides were counter-stained with methyl green to aid microscopic inspection. To minimize the variations between individual animals, neuronal damage was assessed by scoring the percentage of TUNEL-positive neurons in the CA1 region of hippocampus as previously reported (Kelly *et al*., 2001). The slide showed most damage was scored, with 0, no death; 1, <30%; 2, 30-65%; 3, >65%.

Rofecoxib Treatment

Mice were fed with rofecoxib (0.01%) (Merck Frosst, Montreal, Canada) diet or control diet starting one month before global ischemia or sham treatment till sacrifice. Rofecoxib intake per mouse reaches approximately 20mg/kg/day; this dose appears to be the maximum tolerable dose (MTD) defined as the highest dose that causes no more than 10% body weight change as compared to those fed with control diet. It is also defined as the dose not inducing mortality or external clinical signs of toxicity that would be predicted to shorten the natural life span of the animal.

Prostaglandin (PGE2) Assay

PGE2 content in the brain was measured by enzyme-linked immunosorbent assay kit (Cayman, Ann Harbor, MI, USA) according to the manufacturer's instructions. Briefly, fresh brain hemisphere was weighed, and homogenized in 0.1M PBS (containing 1mM EDTA and 10μM indomethacin). The homogenate was then mixed with an equal volume of ethanol and centrifuged. The supernatant fluid was diluted with 50 mM acetate buffer and purified through an affinity column (Cayman, Ann Harbor, MI, USA). After the column was equilibrated with column buffer (0.1M PBS, 7.7mM NaN3, 0.5 M NaCl), the supernatant was eluted, evaporated, re-dissolved in enzyme-linked immunosorbent assay buffer, and assayed according to the manufacturer's instructions. PGE2 content was expressed as ng/g tissue.

Vasculature Variation

Mouse brain was perfused through the heart with 1% carbon black (containing 1% Triton X-100 and 1% gelatin), and brain vasculature was examined under dissecting microscope. The posterior communicating artery (Pcom) on each side, which connects forebrain and hindbrain circulation, was assessed according to Yang *et al*. (1997). Under dissecting microscope, the diameter of the Pcom was examined and expressed as the percentage of the diameter of the basilar artery.

Statistics

All values expressed as mean ± SEM. One-way ANOVA was used to test significance among groups, and Newman-Keul post-test was used for pair comparison. When appropriate, t-test was also used. Significance level was set at $p<0.05$.

RESULTS

Hypothermia in Combination with Rofecoxib Prevents Neuronal Death Apoptosis

Under normothermia conditions, global ischemia potentiated apoptotic neuronal damage in hCOX-2 transgenic mice relative to the wild type (WT) control group ($p<0.05$) (Fig. **1A**). We found that hCOX-2 expression in neurons significantly potentiated apoptotic cell injury by TUNEL assay in the CA1 subdivision of the pyramidal cell layer of the hippocampal formation, in particular, the granule layer of the dentate gyrus, 72 hr after global ischemia, relative to the WT lesioned group (Fig. **1A**; Fig. **1B** panels **C**, **D** versus **A**, **B**).

When hypothermia was induced in transgenic hCOX-2 ($p<0.05$) or WT ($p<0.05$) mice intraischemically, a significant attenuation of apoptotic damage relative to normothermia was observed by TUNEL assay. However, despite hypothermia, hCOX-2 overexpressing transgenic mice had a relatively higher apoptotic neuronal damage compared to WT mice ($p<0.05$) (Fig. **1A**; Fig. **1B** panels **E**, F versus **C**, **D**) ($P<0.05$).

Most interestingly, we found that hippocampal neuroprotection in hCOX-2 transgenic, and WT animals, was significantly potentiated by prophylactic treatment with the COX-2 inhibitor rofecoxib, delivered to mice in the diet for one month (Fig. **1A**; Fig. **1B** panels **G**, **H**). Assuming that the food intake in 2-3 month old mice used in our study is approximately 1-2 g per day, we calculated that the daily dose of Rofecoxib received in the treated group was 20mg/kg.

Brain PGE2 Levels

The expression of hCOX-2 transgene in all the transgenic mice used in this study was confirmed by in situ hybridization (not shown) as previously described (Kelley *et al*., 1999). To confirm the functional expression of hCOX-2 transgene, brain PGE2 content was measured. These mice showed about 2-3 fold higher brain PGE2 levels (48.2 ± 10.1 ng/g tissue) compared to wild type controls (19.5 ± 2.6 ng/g tissue). To examine the effectiveness of one month rofecoxib diet treatment, brain PGE2 levels were measured, and the results are shown in Fig. (**2**). Rofecoxib treatment significantly reduced PGE2 levels in the brains of wild type mice (10.8 ± 0.6 ng/g tissue) and transgenic mice (14.5±4.0 ng/g tissue). There is no significant difference between wild type and transgenic mice after drug treatment.

Variations in Vasculature

Differences in brain vasculature might lead to different outcome after ischemic injury. In the two-vessel global ischemia model used in this study, after blockade of common carotid arteries, the brain region originally innervated by inner carotid arteries can still get collateral blood supply from vertebral arteries by way of posterior communication artery. To examine whether there are differences in brain vasculature, hCOX-2 transgenic and wild type mice were injected intracardially with carbon black to reveal brain blood vessels. On average, the diameter of posterior communication artery is 30±11% of the basilar artery in hCOX-2 transgenic mice, comparable to wild type mouse (35±11%) (Fig. **3**). When the less developed side was compared, hCOX-2 transgenic mice (17±7 %) were also not significantly different compared to wild type mice (20 ± 8%).

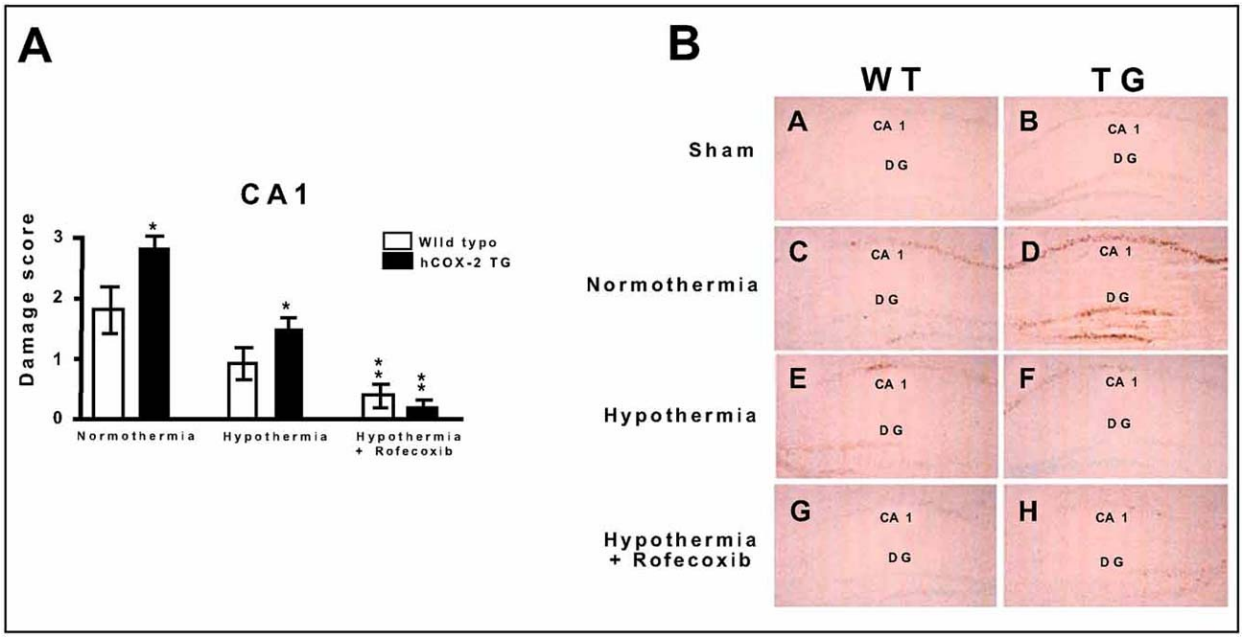

Fig. (1). hCOX-2 over-expression in the brain of hCOX-2 transgenics potentiates apoptotic neuronal death, which is prevented by hypothermia and prophylactic treatment with a COX-2 inhibitor. In **A**, the score was based on the area occupied by TUNEL positive cells in the CA1 subdivision of the hippocampal formation using 0–3 grading system (0 = no damage, 1 = <30% damage, 2 = 30–70% damage, 3 = >70% damage). Data are shown as Mean ± SEM, n=4-6 per group; * $p<0.05$ vs normothermia; ** $p<0.05$ vs hypothermia. No detectable TUNEL positive neurons were found in the sham vehicle group. In this study, TUNEL positive neurons (area) were quantified from 4-6 tissue sections per brain. In **B**, representative TUNEL histochemistry is shown in the different experimental groups as indicated. Abbreviations: CA1, hippocampal subdivision of the pyramidal neuron layer; DG, granular layer of the dentate gyrus. TUNEL positive cells are visible as brown immunopositive cells.

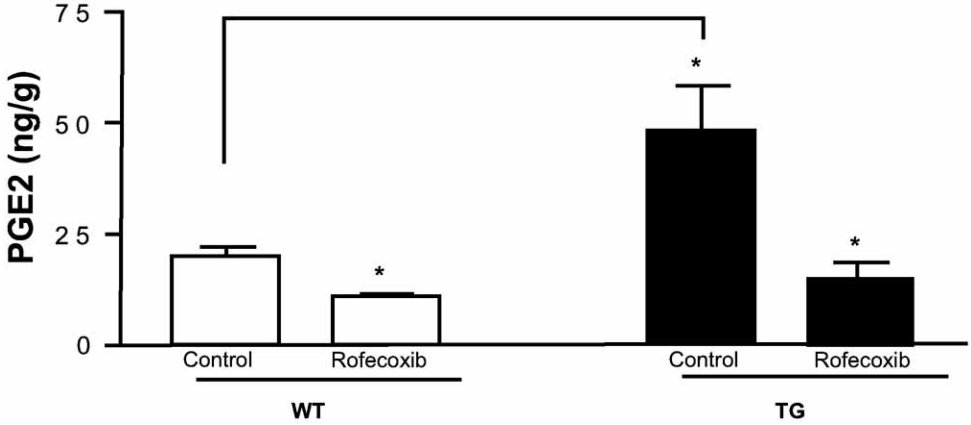

Fig. (2). Rofecoxib diet treatment effectively reduces brain PGE2 levels in both wild-type and transgenic mice. Brain PGE2 levels were assayed by ELISA after treating mice with rofecoxib diet or control diet for one month. Data expressed as mean ± SEM. Asterisks indicate significant difference (t-test, *, $p<0.05$). Without rofecoxib, PGE2 levels in the transgenic mice were significantly higher than in wild type mice. Rofecoxib significantly reduced PGE2 levels in wild type and hCOX-2 transgenic mice (n=5) compared to control diet treatment. There is no statistical difference between wild type and transgenic mice after rofecoxib treatment.

DISCUSSION

COX-2 has been implicated in neuronal death in ischemia. In this study, we found increased neuronal damage in hCOX-2 transgenic mice in response to global ischemia. This data is consistent with a deleterious role of COX-2 in global ischemic neuronal death. Though rofecoxib is a selective COX-2 inhibitor and prevents hippocampal neuronal injury in an animal model of excitotoxic neurodegeneration, there are reports which shows that rofecoxib lacked significant protective effect on early neuronal cell death in the fluid percussion brain injury (FPI) model of traumatic brain injury (Kunz *et al*., 2006), failed to attenuate seizure-induced visuospatial learning deficits (Kunz *et al*., 2005), and does not slow cognitive decline in patients with mild-to-moderate Alzheimer's disease (Aisen *et al*., 2003). This study shows that the aggravating effect of COX-2 overexpression in a transgenic mouse model could be largely prevented by mild hypothermia in combination with prophylactic rofecoxib treatment.

The mechanism of ischemic neuronal death is complex and involves many of the known pathways such as excitotoxicity, energy failure, free radical generation, apoptotic cell death, with ex-

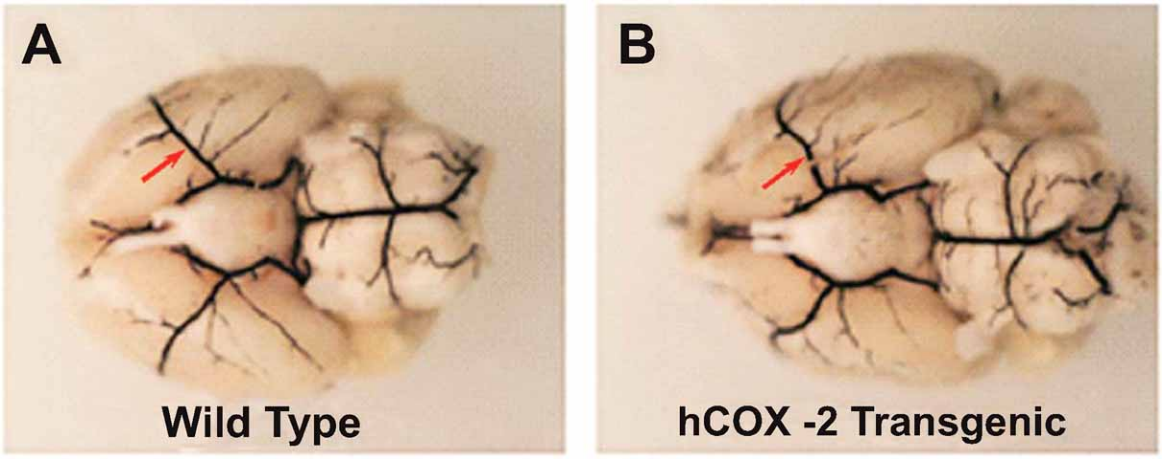

Fig. (3). Brain vasculature in wild type and hCOX-2 transgenic mice after ischemic injury. There was no difference in brain vasculature between wild type and hCOX-2 transgenic mice after ischemic injury. The arrow indicates left middle cerebral artery.

tensive overlapping and interaction (Lo *et al*., 2003). Glutamate excitotoxicity due to massive release of glutamate during ischemia is one of the major factors (Lipton, 1999). Interestingly, COX-2 activity is closely related to glutamate synaptic activity. In physiological conditions, NMDA synaptic activity rapidly induces COX-2 expression. In pathological conditions, such as seizure which is characterized by excessive synaptic activities, COX-2 induction is more dramatic (Yamagata *et al*., 1993; Kelly *et al*., 2001). Evidence suggests that COX-2 may aid or carry out part of the neurotoxic effect of glutamate. For example, PGE2 inhibits glutamate uptake in astrocytes (Bezzi *et al*., 1998) and leads to exacerbated neurotoxicity (Rothstein *et al*., 1993). COX-2/PGE2 may also lead to loss of expression of the endogenous CDK inhibitor $p16^{INK4}$ and the activation of cell cycle machinery that may facilitate neuronal death (Mirjany *et al*., 2002; Xiang *et al*., 2002). Paradoxically, COX-2 is also related to cell proliferation. COX-2 promotes cell survival by a mechanism linking increased expression of prosurvival genes coupled to inhibition of NO- and superoxide-mediated apoptosis (Chang *et al*., 2000).

Though the COX-2 inhibitor rofecoxib significantly protected healthy neurons in the hippocampal CA1 after ischemia (Candelario-Jalil *et al*., 2003), and ameliorates excitotoxic neuronal injury and, as such, may be a particularly promising therapeutical approach for the treatment of neurological diseases associated with overactivation of NMDA receptors (Hewett *et al*., 2006), few studies examined their efficacy in combination with hypothermia. In this study, we tested whether COX-2 inhibition could provide added protection in addition to hypothermia against global ischemia. Since PGE2, a COX-2 enzymatic product, enhances neuronal injury, we screened for PGE2 in normal and transgenic mice. We found an increase in expression of PGE2 in transgenic mice compared to the wild type mice, thus predicting that COX-2 is overexpressed in the transgenic mouse model. Since rofecoxib is known to inhibit COX-2 we hypothesized that the drug should decrease PGE2 in both wild type and transgenic mouse model. As expected COX-2 inhibition by rofecoxib diet treatment reduced PGE2 production in both control and transgenic mice.

Since mild hypothermia is known to be protective against ischemia, we explored for neuronal damage after global ischemia in both wild type and hCOX-2 transgenic mice at different temperature conditions. In normothermia (36-37°C) 75% of the transgenic mice showed severe neuronal damage compared to the wild type, showing that high temperature has a deleterious consequence on ischemia. Subsequently we subjected the mice to hypothermia (33-34°C) after global ischemia. Both the wild type and hCOX-2 transgenic mice had lesser neuronal damage, and there was no significant difference between wild type and transgenic mice. The results were consistent with previous studies showing that hypothermia is protective in ischemia (Lo *et al*., 2003). Hypothermia decreases metabolic stress (Astrup *et al*., 1981), decreases neurotransmitter release thus reducing excitotoxicity (Patel *et al*., 1994), inhibits microglia activation (Han *et al*., 2002), and reduces inflammation (Chatzipanteli *et al*., 2000). Deep hypothermia protects the neocortex and hippocampus from insult in an acute porcine model of hypothermic circulatory arrest (Ananiadou *et al*., 2007). Ohta *et al*. (2007) identified inflammatory genes like osteopontin, early growth response-1, or macrophage inflammatory protein-3 alpha upregulated during hypothermia and hypothermia is also known to reduce basal ganglia injury in some premature babies (George *et al*., 2007). We had observed a decrease in neuronal damage in both wild type and hCOX-2 transgenic mice after hypothermia alone (Fig. **1A**, **1B**). The attenuation of oxidative DNA damage and DNA damage-triggered pro-death signaling events may be an important mechanism underlying the neuroprotective effect of mild hypothermia against ischemic brain injury (Ji *et al*., 2007), whereas, Wang *et al*. (2005) observed that moderate hypothermia can prevent nerve cell apoptosis by a mechanism associated with BCL-2 and p53 genes. Kawamura *et al*. (2006) showed that hypothermia significantly attenuates the inflammatory response by its effect on multiple key mediators including cytokines, ICAM-1, and NF-kappaB.

As we had observed that rofecoxib and hypothermia *per se* reduces neuronal damage, we hypothesized that hypothermia in combination with rofecoxib treatment may further reduce neuronal death in both wild type and hCOX-2 transgenic mice compared to hypothermia alone. We observed less neuronal damage in wild type and transgenic animals when subjected to combination treatment. The results suggest that rofecoxib diet treatment coupled with hypothermia could be added to the clinical therapies in diseases such as stroke that involve combination of multiple approaches. Given the complexity of stroke evidenced by failure of individual drugs in clinical trials, approaches that involve multiple strategies may offer better outcome.

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