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Selective Blockade of PGE_2 EP1 Receptor Protects Brain against Experimental Ischemia and Excitotoxicity, and Hippocampal Slice Cultures against Oxygen-Glucose Deprivation

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Cyclooxygenase-2 (COX-2) enzyme increases abnormally during excitotoxicity and cerebral ischemia and promotes neurotoxicity. Although COX-2 inhibitors could be beneficial, they have significant side effects. We and others have shown that the EP1 receptor is important in mediating PGE_2 toxicity. Here, we tested the hypothesis that pretreatment with a highly selective EP1 receptor antagonist, ONO-8713, would improve stroke outcome and that post-treatment would attenuate NMDA-induced acute excitotoxicity and protect organotypic brain slices from oxygen-glucose deprivation (OGD)-induced toxicity. Male C57BL/6 mice were injected intracerebroventricularly with ONO-8713 before being subjected to 90-min middle cerebral artery occlusion (MCAO) and 96-h reperfusion. Significant reduction in infarct size was observed in groups given 0.1 (25.9 ± 4.7%) and 1.0 nmol (27.7 ± 2.8%) ONO-8713 as compared with the vehicle-treated control group. To determine the effects of ONO-8713 post-treatment on NMDA-induced excitotoxicity, mice were given a unilateral intrastriatal NMDA injection followed by one intraperitoneal injection of 10 μg/kg ONO-8713, 1 and 6 h later. Significant attenuation of brain damage (26.6 ± 4.9%) was observed at 48 h in the ONO-8713-treated group. Finally, brain slice cultures were protected (25.5 ± 2.9%) by the addition of ONO-8713 to the medium after OGD. These findings support the notion that the EP1 receptor propagates neurotoxicity and that selective blockade could be considered as a potential preventive and/or therapeutic tool against ischemic/hypoxic neurological conditions.

Keywords: Antagonism; Hippocampal slice cultures; Middle cerebral artery occlusion; ONO-8713; Oxygen glucose deprivation; Prostaglandin

INTRODUCTION

Cyclooxygenase (COX)-1 and COX-2 are the rate limiting enzymes associated with the generation of five primary bioactive prostanoids. However, under pathological conditions associated with the excessive activation of NMDA receptors, the enzymatic activity of COX-2 (the inducible isoform) is highly up-regulated, as is the generation of the prostanoids

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(Adams *et al.*, 1996; Takadera and Ohyashiki, 2006). Given the potential effects of COX-2 inhibitors in minimizing inflammation, these inhibitors were also considered to have the potential to minimize neurodegeneration. However, recent reports show that these COX-2 inhibitors may carry potential side effects such as cardiac complications (Bresalier *et al.*, 2005a; Graham *et al.*, 2005; Baron *et al.*, 2008). Therefore, an alternative therapeutic approach would be important to investigate the effect of agonists and antagonists of prostanoid receptors in modulating neurological conditions, including stroke.

Prostaglandin E_2 (PGE_2) is one of the prostanoids that is synthesized by the enzymatic action of COX on arachidonic acid. PGE_2 exerts beneficial as well as harmful effects via the trans-membrane G-protein-coupled receptors designated EP1, EP2, EP3, and EP4. Previous studies from various labs including our own have established that EP1 increases intracellular Ca^{2+} level, whereas EP2 and EP4 up-regulate cAMP level; EP3 can either increase Ca^{2+} level or decrease cAMP (Narumiya *et al.*, 1999; Kobayashi and Narumiya, 2002; McCullough *et al.*, 2004; Echeverria *et al.*, 2005; Ahmad *et al.*, 2006a; Kawano *et al.*, 2006). We have observed that those prostanoid receptors that lead to increased Ca^{2+} are toxic, whereas those that regulate cAMP are protective.

PGE_2 has been investigated extensively for its role in various physiological responses (Stock *et al.*, 2001; Nakayama *et al.*, 2004a; Oka, 2004). Although the actions of PGE_2 through the EP1 receptor are well documented for many disease conditions, the role of the EP1 receptor in stroke and excitotoxicity has been shown only recently by us and others (Ahmad *et al.*, 2006b; Kawano *et al.*, 2006). We have shown that EP1 receptor activation increases brain damage after NMDA toxicity, whereas its inhibition leads to the prevention of NMDA-induced brain damage (Ahmad *et al.*, 2006b). Similarly, genetic deletion of EP1 receptor protects mice from stoke and NMDA-induced brain damage. Furthermore, parallel work from Dr. Iadecola's group showed that EP1 activation impaired the Na^{+}-Ca^{2+} exchange necessary for Ca^{2+} homeostasis and exacerbated brain damage caused by excitotoxicity and brain ischemia (Kawano *et al.*, 2006).

Use of various EP1 receptor antagonists has been reported to minimize or prevent certain pathological events (Oka *et al.*, 1998a; Omote *et al.*, 2002; Walch *et al.*, 2003; Matsuo *et al.*, 2004; Nakayama *et al.*, 2004b). The intriguing finding that the EP1 receptor contributes to the propagation of brain damage in excitotoxicity and stroke has prompted researchers to develop and test clinically active drugs that are selective for the EP1 receptor, to better understand its physiological and pathological effects. The neuroprotective role of SC51089, a selective EP1 antagonist, in stroke and excitotoxicity has been reported (Abe *et al.*, 2008; Zhou *et al.*, 2008). The relatively new compound ONO-8713 is a highly selective EP1 antagonist with relative affinities of 0.3 nM for EP1 and >1000 nM for all other prostaglandin receptors (Watanabe *et al.*, 2000). We have shown that pretreatment with ONO-8713 attenuates NMDA-induced brain damage in mice (Ahmad *et al.*, 2006b). To extend our previous finding, here we investigated whether pretreatment with ONO-8713 also can minimize stroke outcome. Furthermore we tested whether systemic administration of ONO-8713 1 h after NMDA injection can minimize excitotoxicity in mice. Finally, to determine whether post-treatment is neuroprotective in ischemia, we subjected mouse hippocampal slice cultures to oxygen-glucose deprivation (OGD) as an *in vitro* model of brain ischemia and then treated them with ONO-8713.

MATERIALS AND METHODS

Animals and drugs

Male C57BL/6 mice (8-10 weeks old; 20-25 g) were obtained from Charles River Laboratories, Inc. (Wilmington, MA). All animal protocols were approved by the Johns Hopkins University Animal Care and Use Committee. The animals were permitted free access to water and food before and after surgery. ONO-8713 was provided by one of the authors (T.M.).

Intracerebroventricular (ICV) Pretreatment with ONO-8713

We have previously shown that ICV pretreatment with ONO-8713 attenuates NMDA-induced excitotoxic brain damage; therefore in this study, we wanted to know if the same dose and mode of drug delivery could also protect the brain against middle

cerebral artery occlusion (MCAO)-induced transient ischemia. After recording the weight and rectal temperature, we anesthetized the mice and placed them on a stereotaxic stand for intracerebral microinjections, as described earlier (Ahmad *et al.*, 2006b). Briefly, mice were given a single injection of 0.1, 1.0, or 10.0 nmol ONO-8713 or vehicle in a volume of 0.2 μl in the right cerebral ventricle. After the injection, the hole was closed with bone wax, and the overlying skin was sutured. Mice then were immediately subjected to MCAO.

MCAO and Reperfusion

Throughout the MCAO procedure, body temperature (rectal) of the mice was monitored and maintained at 37.0 ± 0.5°C by a heating pad. Anesthesia was maintained by a continuous flow of halothane (1-1.5%) in oxygen-enriched air via a nose cone. Relative cerebral blood flow (CBF) was monitored with laser-Doppler flowmetry (Moor Instruments, Devon, England) by a flexible fiber optic probe. Following the protocol described previously (Ahmad *et al.*, 2006c), MCAO was carried out under aseptic conditions with a silicone-coated nylon monofilament. Proper MCAO induction was achieved when CBF decreased by more than 80% from the baseline. Mice in which the CBF did not decrease by more than 80% and mice that died during surgery were excluded from the groups. The numbers of mice excluded from the vehicle-, 0.1-, 1.0-, and 10.0-nmol groups were 5, 3, 4, and 5, leaving 10, 11, 11, and 10, mice, respectively, for analysis. During the 90 min of occlusion, the incision was sutured, anesthesia was discontinued, and the animals were transferred to a temperature-controlled chamber to maintain their body temperature. To achieve the reperfusion, the mice were briefly re-anesthetized with halothane, and the filament was withdrawn. After the incision was sutured, the mice were returned to the temperature-controlled chamber for 2 h and then transferred to their home cages and allowed to survive for 4 days.

Quantification of Infarct Volume

Four days after MCAO, mice were deeply anesthetized, and their brains were harvested and sliced coronally into five 2-mm-thick sections. The sections were incubated with 1% 2,3,5-triphenyl-tetrazolium chloride (TTC) in saline for 20 min at 37°C. Macrographs were obtained (SigmaScan Pro, SPSS, Port Richmond, CA) and the area of infarcted brain, recognized by the lack of TTC staining, was measured on the rostral and caudal surfaces of each slice and numerically integrated across the thickness of the slice to obtain an estimate of infarct volume. Volumes from all five slices were summed to calculate total infarct volume over the entire hemisphere and expressed as a percentage of the volume of the contralateral structure. Infarct volume was corrected for swelling by comparing the volumes in the ipsilateral and contralateral hemispheres.

Acute intrastriatal Injection of NMDA and post-Treatment with ONO-8713

To induce acute excitotoxicity, 15 nmol of NMDA in a volume of 0.3 μl was injected slowly into the striatum. The needle was left in place for 5 min and then retracted slowly. The hole was blocked with bone wax and the skin was sutured. After the surgical procedure, mice were placed in a thermoregulated chamber and transferred to their home cages after recovery from anesthesia. Throughout the experimental procedure, rectal temperature of the mice was monitored and maintained at 37.0 ± 0.5°C. Because we observed a neuroprotective effect after ICV pretreatment with ONO-8713, here we investigated whether a similar effect could be achieved with systemic injection of ONO-8713. At 1 and 6 h after the NMDA injection, the mice were given an intraperitoneal (i.p.) injection of 10 μg/kg ONO-8713 (n=5) or vehicle (n=7). Mice were allowed to survive for 48 h after the NMDA injection. No mortality was observed in either treatment group.

Quantification of the Excitotoxic Lesion Volume

Weight and rectal temperature were recorded, and mice were deeply anesthetized with pentobarbital. Mice were then transcardially perfused with cold PBS, followed by 4% paraformaldehyde in PBS. Brains were harvested, post-fixed in 4% paraformaldehyde for 24 h, equilibrated in 30% sucrose, and then snap frozen in pre-cooled 2-methylbutane. Sequential brain sections (25 μm) obtained on a cryostat were stained with Cresyl Violet to estimate lesion volume (Ahmad *et al.*, 2006a).

Preparation of Organotypic Mouse Hippocampal Slice Cultures

Hippocampal organotypic cultures were prepared according to previously published protocols (Stoppini *et al.*, 1991; Kawano *et al.*, 2006) with slight modification, as described below. Hippocampi from 7-8-day-old C57BL/6 mouse pups were dissected out aseptically, and 350-μm coronal sections were cut on a Vibratome (series 1000; Vibratome, St. Louis, MO) and kept in Hibernate A solution (BrainBits, Springfield, IL) bubbled with 95% O_2 and 5% CO_2. Slices were transferred onto 30-mm Millicell membrane inserts with 0.4-μm pore size (Millipore, Bedford, MA) in six-well plates. Cultures were maintained for 13 days in Neurobasal A medium (NBA; Invitrogen, Carlsbad, CA) containing B27 supplement and 2 mM Glutamax I (Invitrogen, Carlsbad, CA) in a humidified atmosphere with 5% CO_2 at 37°C; the medium was changed twice weekly. On day 13, the medium was replaced with fresh medium containing the fluorescent vital dye propidium iodide (PI; 5 μg/ml; Sigma, St. Louis, MO). After 24 h, the PI fluorescence was measured to confirm that slices were viable and healthy. PI enters into dying cells only, binds to nucleic acid, and causes the injured cells to fluoresce.

Oxygen Glucose Deprivation and post-Treatment with ONO-8713

Anoxia was induced by subjecting the brain slices to OGD for 1 h. Slice cultures were rinsed with normal medium, equilibrated for 30 min in the incubator, and then rinsed twice with warm, deoxygenated, glucose-free Hibernate A solution. The cultures were then transferred to an air-tight chamber, and anoxic gas (5% CO_2, 95% N_2) was flushed into the chamber for 1 h at 37°C. After OGD treatment, the slices were transferred to normal culture medium with or without 1 μM ONO-8713 and incubated for 24 h. Cultures were imaged for PI fluorescence 24 h after OGD, and the maximal PI fluorescence was obtained by stimulating the cultures for 24 h with a lethal amount of NMDA (100 μM).

Measurement of Neuronal Death in Organotypic Mouse Hippocampal Slice Cultures

Mean PI fluorescence in the CA1 sub-region of each hippocampal slice was quantified to determine the neuronal death. Sequential fluorescence was measured at (1) t=0 (F_{basal}), before OGD stimulation to measure basal levels of neuronal death; (2) t=24 h (F_{OGD}), 24 h after stimulation with OGD; and (3) t=max (F_{max}), after a final exposure of slices to a lethal amount of 100 μM NMDA overnight, to measure maximum fluorescence. Slices were imaged with a Nikon inverted fluorescence microscope equipped with a light-intensifying SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI) and SPOT Advanced software. The same camera settings were used throughout the experiments. Each experiment was carried out in triplicate, with n = 6-10 sections per condition per experiment. Digital images of PI staining at three different time points (before and after OGD and after NMDA treatment) were outlined to define the same CA1 region and mean fluorescence intensity obtained with corresponding time points: F_{basal}, F_{OGD}, and F_{max}. The percent cell death was calculated from the formula $(F_{OGD}-F_{basal}) / (F_{max}-F_{basal}) \times 100\%$.

Statistical Analysis

The brain sections were imaged and analyzed with SigmaScan Pro 5.0 software (Systat, Inc., Point Richmond, CA). Statistical analysis was performed by Student's *t*-test and *P* values of <0.05 were considered to be significant. All values are expressed as mean ± standard error of the mean (SEM).

RESULTS

ONO-8713 pretreatment reduces infarct volume.

The 0.1 and 1.0 nmol doses of ONO-8713 significantly ($P < 0.05$; FIG. 1B) reduced the infarct volume by 25.9 ± 4.7% and 27.7 ± 2.8%, respectively, compared to control volumes, whereas the effect of 10 nmol was not significant. Representative images of the TTC-stained sections from vehicle-treated and 1.0 nmol-treated mice are shown in FIG. 1A. No significant difference in the CBF was observed between the vehicle-treated and ONO-8713-treated mice.

ONO-8713 post-treatment attenuates NMDA-induced brain damage.

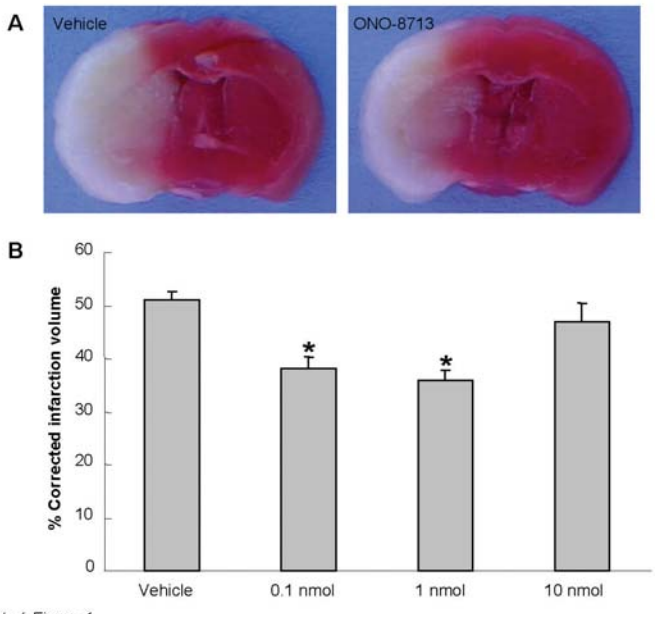

FIGURE 1 Effect of ONO-8713 pretreatment on MCAO-induced brain damage. Mice were treated with 0.1, 1.0, or 10 nmol of the EP1 antagonist ONO-8713 and subjected to 90-min MCAO. At 96 h, the mice were sacrificed and brain sections were stained with TTC to analyze the brain infarction. (**A**) Representative photographs of coronal sections of brains from vehicle-treated (left) and ONO-8713 (1.0 nmol)-treated (right) mice showing infarction due to MCAO. (**B**) Analysis of the TTC-stained brain sections revealed that 0.1 and 1.0 nmol ONO-8713 rescued the brain from ischemia, whereas 10 nmol ONO-8713 had no protective effect. **P* <0.01, when compared with vehicle-treated mice.

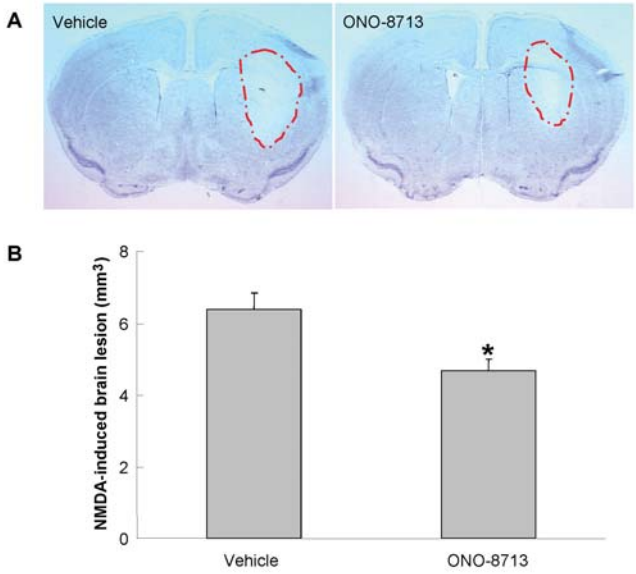

FIGURE 2 Effect of ONO-8713 post-treatment on NMDA-induced brain lesion. Anesthetized mice were given a single intrastriatal injection of 15 nmol NMDA (in 0.3 μl) to induce acute excitotoxicity. After 1 h and 6 h, mice were given a 10 μg/kg injection of ONO-8713 i.p. Mice were allowed to survive for 48 h, and brain sections were stained with cresyl violet to analyze the brain lesion. (**A**) Representative photographs of coronal sections of the brains from vehicle-treated (left) and ONO-8713-treated (right) mice; areas of brain injury are encircled by dashed lines. (**B**) Analysis of the brain sections revealed a significant neuroprotective effect from ONO-8713 post-treatment. **P* <0.05, when compared with the vehicle-treated group.

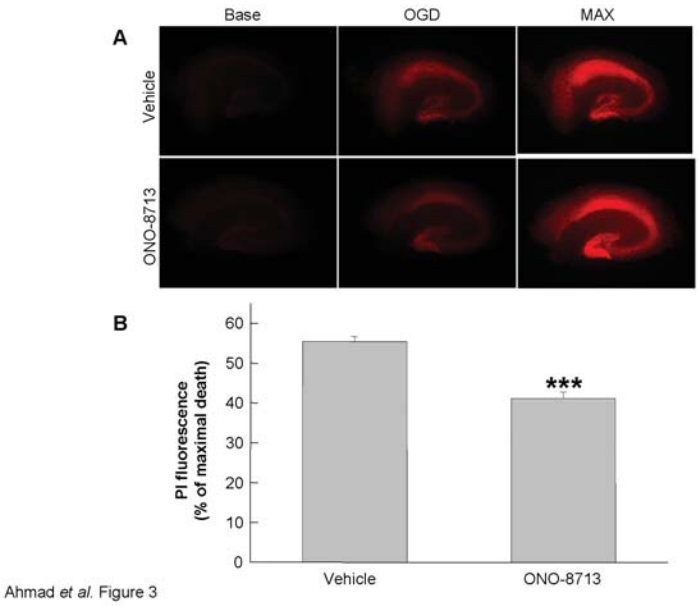

FIGURE 3 Effect of ONO-8713 on OGD-induced cell death in the CA1 region of cultured hippocampal slices. Organotypic mouse hippocampal slice cultures were placed in an air-tight chamber that was flushed with anoxic gas (5% CO_2, 95% N_2) for 1 h at 37°C. Then the cultures were transferred to normal culture medium with or without 1 μM ONO-8713 and incubated for 24 h. The PI fluorescence was obtained at 24 h after OGD, and the maximal PI fluorescence was obtained by stimulating the cultures for 24 h with a lethal amount of NMDA (100 μM). (**A**) Fluorescence images (4X magnification) showing PI staining in hippocampal slices subjected to OGD for 1 h. Images were taken before OGD (Base), 24 h after OGD (OGD), and 24 h after NMDA incubation (MAX). (**B**) Histogram representing CA1 neuronal damage measured by PI fluorescence intensity. The neuronal damage was significantly lower in ONO-8713-treated slices (n=27) than in vehicle-treated slices (n=24) after OGD-induced injury. ***P <0.001 as compared with the vehicle-treated group.

The systemic i.p. injection of 10 μg/kg ONO-8713 significantly (P <0.05) reduced the NMDA-induced brain damage. FIG. 2A shows representative images of the NMDA + vehicle group (left) and NMDA + ONO-8713 group (right). Analysis of the stained sections revealed a significant decrease (P <0.05; 26.6 ± 4.9%) in the lesion volume of the ONO-8713 post-treated groups (FIG. 2B).

ONO-8713 post-treatment reduces neuronal damage in the CA1 region of hippocampal slices following OGD.

To study the effect of ONO-8713 on an *in vitro* model of ischemia, mouse organotypic hippocampal slices were exposed to 1 μM ONO-8713 for 24 h after OGD (FIG. 3). Cell death analysis indicated a significant decrease in the cell death after OGD in the ONO-8713-treated group (41.0 ± 2.0%; P <0.001, n=27 slices) as compared with that of the vehicle-treated group (55.0 ± 1.0%; n=24 slices). These results further support the hypothesis that inhibition of the EP1 receptor reduces the OGD-induced cell death in hippocampal slices.

DISCUSSION

We have previously shown that ICV pretreatment of mice with 1 and 10 nmol EP1 receptor antagonist ONO-8713 results in attenuated NMDA-induced brain damage, whereas pretreatment with 10 nmol of the EP1 agonist ONO-DI-004 augments the NMDA-induced lesion volume (Ahmad *et al.*, 2006b). In this study we tested whether pretreatment with the same dose of ONO-8713 would attenuate brain infarction caused by MCAO. The data reveal that brain damage was significantly attenuated with the 0.1 and 1.0 nmol doses of ONO-8713. We further examined whether this drug is capable of preventing/minimizing the NMDA-induced brain damage when applied systemically after the onset of the toxicity. Interestingly the NMDA-induced brain lesion was significantly

attenuated when ONO-8713 was injected i.p. at 1 and 6 h after the NMDA insult. Moreover to determine if ONO-8713 has a neuroprotective effect in an *in vitro* model of ischemia, we subjected organotypic hippocampal slices to OGD. The data revealed that treatment of slices with ONO-8713 after OGD minimized the OGD-induced cell death.

Under toxic conditions, COX-2 over-expression leads to the generation of the prostanoids, which act through their respective receptors, depending on the nature of the toxicity and affinity of the respective prostanoids toward their receptors. Because prostaglandins are generally regarded as pro-inflammatory markers, it was originally proposed by researchers that COX-2 inhibitors could be novel therapeutic agents in minimizing brain damage (Nakayama *et al.*, 1998; Nagayama *et al.*, 1999; Doré *et al.*, 2003). However, data from some clinical studies show that COX-2 inhibitors might lead to complex cardiac problems (Bresalier *et al.*, 2005b; Graham *et al.*, 2005). Therefore we focused on the prostaglandins downstream of COX and chose PGE_2 as potential target to be investigated in stroke and excitotoxicity.

PGE_2 exerts its protective or toxic effects through its receptors, EP1-EP4. The role of EP1 receptor activation in various pathological conditions is well established (Oka *et al.*, 1998b; Kawahara *et al.*, 2001; Nakayama *et al.*, 2004a). It has been reported that intrathecal administration of PGE_2 causes allodynia through the EP1 receptor in conscious mice (Minami *et al.*, 1994). In a subsequent study, the same group showed that this induction of allodynia could be the result of enhanced nitric oxide production via EP1 receptor activation (Sakai *et al.*, 1998). Moreover, reports suggest that nitric oxide is involved in increasing the intracellular level of Ca^{2+} and generation of highly pro-oxidant radicals that disrupt the cellular functions (Manevich *et al.*, 2001; Urushitani *et al.*, 2001; Salvador Moncada, 2006; Florea and Blatter, 2008). Based on these and our current results, we believe that EP1 receptor activation augments the NMDA-induced Ca^{2+} dysregulation, whereas ONO-8713 inhibits the EP1 activity and reduces the intracellular overload of Ca^{2+} and the disruption of Ca^{2+} homeostasis, thus reducing toxicity.

Now data are emerging that implicate the role of EP1 receptor in propagating brain damage. Our previous findings that EP1 receptor activation by pretreatment with ONO-DI-004 propagates NMDA-induced brain damage, whereas pretreatment with the EP1 receptor antagonist, ONO-8713, minimizes brain damage in mice (Ahmad *et al.*, 2006b) are further supported by Kawano *et al.* (2006), who showed that EP1 receptor antagonism by the EP1 receptor inhibitor SC51089 decreases brain damage by attenuating Ca^{2+} dysregulation. In a model of *in vitro* brain ischemia, it was shown that antagonism of EP1 receptor by SC-19220 protects rat fetal neuronal cultures from OGD (Gendron *et al.*, 2005). Another study from Iadecola's group revealed that the EP1 receptor antagonist SC51089 protects brain from focal and global ischemia (Abe *et al.*, 2008) and hippocampal slices from OGD by regulating the PI3K/Akt survival pathway (Zhou *et al.*, 2008). The authors showed that SC51089 induced Akt activation and attenuated the mitochondrial translocation of the pro-apoptotic protein BAD. In addition to the evidence showing the role of EP1 activation and consequently downstream signaling in propagating neurotoxicity, studies have shown deleterious effects of the EP1 receptor in cerebral vasculature. We have previously shown that during MCAO, CBF increases significantly in $EP1^{-/-}$ mice as compared with the WT mice (Saleem *et al.*, 2007).

The accumulating evidence supports the neurotoxic potential of the EP1 receptor. To prevent or minimize this potential, it is important to develop and test drugs that are more selective toward this receptor. Therefore we examined the neuroprotective effect of the highly selective EP1 antagonist ONO-8713, not only in mouse models of MCAO and acute excitotoxicity, but also in an *in vitro* model of ischemic brain injury produced by OGD. The data show that ONO-8713 has a wide therapeutic window and can minimize brain damage even hours after the onset of toxicity. With this study we conclude that ONO-8713 has the therapeutic potential to minimize or prevent stroke in mice and could be considered for use as a therapeutic tool clinically.

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Potential Conflict of Interest

There are no conflicts of interest with this work.

References

Abe T, A Kunz, M Shimamura, P Zhou, J Anrather and C Iadecola (2008) The neuroprotective effect of prostaglandin E_2 EP1 receptor inhibition has a wide therapeutic window, is sustained in time and is not sexually dimorphic. *J. Cereb. Blood Flow Metab.* doi:10.1038/jcbfm.2008.88. In press.

Adams J, Y Collaco-Moraes and J de Belleroche (1996) Cyclooxygenase-2 induction in cerebral cortex: an intracellular response to synaptic excitation. *J. Neurochem.* **66**, 6-13.

Ahmad AS, H Zhuang, V Echeverria and S Doré (2006a) Stimulation of prostaglandin EP2 receptors prevents NMDA-induced excitotoxicity. *J. Neurotrauma* **23**, 1895-1903.

Ahmad AS, S Saleem, M Ahmad and S Doré (2006b) Prostaglandin EP1 receptor contributes to excitotoxicity and focal ischemic brain damage. *Toxicol. Sci.* **89**, 265-270.

Ahmad M, S Saleem, H Zhuang, AS Ahmad, V Echeverria, A Sapirstein and S Doré (2006c) 1-HydroxyPGE$_1$ reduces infarction volume in mouse transient cerebral ischemia. Eur. *J. Neurosci.* **23**, 35-42.

Baron JA, RS Sandler, RS Bresalier, A Lanas, DG Morton, R Riddell, ER Iverson and DL Demets (2008) Cardiovascular events associated with rofecoxib: final analysis of the APPROVe trial. *Lancet* **372**(9651), 1756-1764. Epub 2008 Oct 14.

Bresalier RS, RS Sandler, H Quan, JA Bolognese, B Oxenius, K Horgan, C Lines, R Riddell, D Morton, A Lanas, MA Konstam, JA Baron and the Adenomatous Polyp Prevention on Vioxx Trial I (2005) Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N. Engl. J. Med.* **352**, 1092-1102.

Doré S, T Otsuka, T Mito, N Sugo, T Hand, L Wu, PD Hurn, RJ Traystman and K Andreasson (2003) Neuronal overexpression of cyclooxygenase-2 increases cerebral infarction. *Ann. Neurol.* **54**, 155-162.

Echeverria V, A Clerman and S Doré (2005) Stimulation of PGE_2 receptors EP2 and EP4 protects cultured neurons against oxidative stress and cell death following β-amyloid exposure. *Eur. J. Neurosci.* **22**, 2199-2206.

Florea SM and LA Blatter (2008) The effect of oxidative stress on Ca^{2+} release and capacitative Ca^{2+} entry in vascular endothelial cells. *Cell Calcium* **43**, 405-415.

Gendron TF, E Brunette, JS Tauskela and P Morley (2005) The dual role of prostaglandin E_2 in excitotoxicity and preconditioning-induced neuroprotection. *Eur. J. Pharmacol.* **517**, 17-27.

Graham DJ, D Campen, R Hui, M Spence, C Cheetham, G Levy, S Shoor and WA Ray (2005) Risk of acute myocardial infarction and sudden cardiac death in patients treated with cyclo-oxygenase 2 selective and non-selective non-steroidal anti-inflammatory drugs: nested case-control study. *The Lancet* **365**, 475-481.

Kawahara H, A Sakamoto, S Takeda, H Onodera, J Imaki and R Ogawa (2001) A prostaglandin E_2 receptor subtype EP1 receptor antagonist (ONO-8711) reduces hyperalgesia, allodynia, and c-fos gene expression in rats with chronic nerve constriction. *Anesth. Analg*. **93**, 1012-1017.

Kawano T, J Anrather, P Zhou, L Park, G Wang, KA Frys, A Kunz, S Cho, M Orio and C Iadecola (2006) Prostaglandin E_2 EP1 receptors: downstream effectors of COX-2 neurotoxicity. *Nat. Med.* **12**, 225-229.

Kobayashi T and S Narumiya (2002) Function of prostanoid receptors: studies on knockout mice. *Prostaglandins Other Lipid Mediat.* **68-69**, 557-573.

Manevich Y, A Al-Mehdi, V Muzykantov and AB Fisher (2001) Oxidative burst and NO generation as initial response to ischemia in flow-adapted endothelial cells. *Am. J. Physiol. Heart Circ. Physiol.* **280**, H2126-H2135.

Matsuo M, N Yoshida, M Zaitsu, K Ishii and Y Hamasaki (2004) Inhibition of human glioma cell growth by a PHS-2 inhibitor, NS398, and a prostaglandin E receptor subtype EP1-selective antagonist, SC51089. *J. Neuro-Oncology* **66**, 285-292.

McCullough L, L Wu, N Haughey, X Liang, T Hand, Q Wang, RM Breyer and K Andreasson (2004) Neuroprotective function of the PGE_2 EP2 receptor in cerebral ischemia. *J. Neurosci.* **24**, 257-268.

Minami T, I Nishihara, R Uda, S Ito, M Hyodo and O Hayaishi (1994) Involvement of glutamate receptors in allodynia induced by prostaglandins E_2 and $F_{2\alpha}$ injected into conscious mice. *Pain* **57**, 225-231.

Nagayama M, K Niwa, T Nagayama, ME Ross and C Iadecola (1999) The cyclooxygenase-2 inhibitor NS-398 ameliorates ischemic brain injury in wild-type mice but not in mice with deletion of the inducible nitric oxide synthase gene. *J. Cereb. Blood Flow Metab.* **19**, 1213-1219.

Nakayama M, K Uchimura, RL Zhu, T Nagayama, ME Rose, RA Stetler, PC Isakson, J Chen and SH Graham (1998) Cyclooxygenase-2 inhibition prevents delayed death of CA1 hippocampal neurons following global ischemia. *Proc. Natl. Acad. Sci. USA* **95**, 10954-10959.

Nakayama Y, K Omote, T Kawamata and A Namiki (2004a) Role of prostaglandin receptor subtype EP1 in prostaglandin E_2-induced nociceptive transmission in the rat spinal dorsal horn. *Brain Res.* **1010**, 62-68.

Nakayama Y, K Omote, T Kawamata and A Namiki (2004b) Role of prostaglandin receptor subtype EP1 in prostaglandin E_2-induced nociceptive transmission in the rat spinal dorsal horn. *Brain Res.* **1010**, 62-68.

Narumiya S, Y Sugimoto and F Ushikubi (1999) Prostanoid receptors: structures, properties, and functions. *Physiol. Rev.* **79**, 1193-1226.

Oka K, T Oka and T Hori (1998a) PGE_2 receptor subtype EP1 antagonist may inhibit central interleukin-1β-induced fever in rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **275**, R1762-R1765.

Oka K, T Oka and T Hori (1998b) PGE_2 receptor subtype EP1 antagonist may inhibit central interleukin-1β-induced fever in rats. *Am. J. Physiol.* **275**, R1762-R1765.

Oka T (2004) Prostaglandin E_2 as a mediator of fever: the role of prostaglandin E (EP) receptors. *Front. Biosci.* **9**, 3046-3057.

Omote K, H Yamamoto, T Kawamata, Y Nakayama and A Namiki (2002) The effects of intrathecal administration of an antagonist for prostaglandin E receptor subtype EP1 on mechanical and thermal hyperalgesia in a rat model of postoperative pain. *Anesth. Analg.* **95**, 1708-1712.

Sakai M, T Minami, N Hara, I Nishihara, H Kitade, Y Kamiyama, K Okuda, H Takahashi, H Mori and S Ito (1998) Stimulation of nitric oxide release from rat spinal cord by prostaglandin E_2. *Br. J. Pharmacol.* **123**, 890-894.

Saleem S, R Li, G Wei and S Doré (2007) Effects of EP1 receptor on cerebral blood flow in the middle cerebral artery occlusion model of stroke in mice. *J. Neurosci. Res.* **85**, 2433-2440.

Salvador Moncada JPB (2006) Nitric oxide, cell bioenergetics and neurodegeneration. *J. Neurochem.* **97**, 1676-1689.

Stock JL, K Shinjo, J Burkhardt, M Roach, K Taniguchi, T Ishikawa, HS Kim, PJ Flannery, TM Coffman, JD McNeish and LP Audoly (2001) The prostaglandin E_2 EP1 receptor mediates pain perception and regulates blood pressure. *J. Clin. Invest.* **107**, 325-331.

Stoppini L, PA Buchs and D Muller (1991) A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Meth.* **37**, 173-182.

Takadera T and T Ohyashiki (2006) Prostaglandin E_2 deteriorates *N*-methyl-D-aspartate receptor-mediated cytotoxicity possibly by activating EP2 receptors in cultured cortical neurons. *Life Sci.* **78**, 1878-1883.

Urushitani M, T Nakamizo, R Inoue, H Sawada, T Kihara, K Honda, A Akaike and S Shimohama (2001) N-methyl-D-aspartate receptor-mediated mitochondrial Ca^{2+} overload in acute excitotoxic motor neuron death: a mechanism distinct from chronic neurotoxicity after Ca^{2+} influx. *J. Neurosci. Res.* **63**, 377-387.

Walch L, E Clavarino and PL Morris (2003) Prostaglandin (PG) FP and EP1 receptors mediate $PGF_{2\alpha}$ and PGE_2 regulation of interleukin-1β expression in Leydig cell progenitors. *Endocrinology* **144**, 1284-1291.

Watanabe K, T Kawamori, S Nakatsugi, T Ohta, S Ohuchida, H Yamamoto, T Maruyama, K Kondo, S Narumiya, T Sugimura and K Wakabayashi (2000) Inhibitory effect of a prostaglandin E receptor subtype EP(1) selective antagonist, ONO-8713, on development of azoxymethane-induced aberrant crypt foci in mice. *Cancer Lett.* **156**, 57-61.

Zhou P, L Qian, T Chou and C Iadecola (2008) Neuroprotection by PGE_2 receptor EP1 inhibition involves the PTEN/AKT pathway. *Neurobiol. Dis.* **29**, 543-551.