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Prostaglandin FP receptor inhibitor reduces ischemic brain damage and neurotoxicity

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Abstract

Bioactive lipids such as the prostaglandins have been reported to have various cytoprotective or toxic properties in acute and chronic neurological conditions. The roles of PGF_{2α} and its receptor (FP) are not clear in the pathogenesis of ischemic brain injury. Considering that this G-protein coupled receptor has been linked to intracellular calcium regulation, we hypothesized that its blockade would be protective. We used FP antagonist (AL-8810) and FP receptor knockout (FP^{-/-}) mice in *in vivo* and *in vitro* stroke models. Mice that were treated with AL-8810 had 35.7 ± 6.3% less neurologic dysfunction and 36.4 ± 6.0% smaller infarct volumes than did vehicle-treated mice after 48 hours of permanent middle cerebral artery occlusion (pMCAO); FP^{-/-} mice also had improved outcomes after pMCAO. Blockade of the FP receptor also protected against oxygen-glucose deprivation (OGD)-induced cell death and reactive oxygen species formation in slice cultures. Finally, we found that an FP receptor agonist dose dependently increased intracellular Ca²⁺ levels in cultured neurons and established that FP-related Ca²⁺ signaling is related to ryanodine receptor signaling. These results indicate that the FP receptor is involved in cerebral ischemia-induced damage and could promote development of drugs for treatment of stroke and acute neurodegenerative disorders.

Keywords

cerebral ischemia; inflammation; receptor; stroke; oxygen glucose deprivation

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Disclosures

None.

Introduction

We and others have described that the bioactive lipids, such as the prostaglandins, might affect neuronal outcomes after a variety of brain insults, including hypoxia, inflammation, and excitotoxicity (Dore et al., 2003). Therefore, we have focused our research on the unique properties of the prostaglandins and their receptors (Doré, 2006). Cyclooxygenases are the rate limiting enzymes for the conversion of arachidonic acid to prostaglandin PGH_2 , which is metabolized by specific prostaglandin synthases to at least five structurally related bioactive lipid molecules, including PGE_2 , PGD_2 , $\text{PGF}_{2\alpha}$, PGI_2 , and thromboxane A_2 (Doré, 2006). $\text{PGF}_{2\alpha}$, which is synthesized from PGH_2 via PGF synthase, plays a major role in initiation of parturition, renal function, control of cerebral blood flow autoregulation in newborn piglets (Chemtob et al., 1990), contraction of arteries, and myocardial dysfunction (Takayama et al., 2005). Recently, a report suggested that FP signaling can facilitate idiopathic pulmonary fibrosis (Oga et al., 2009). Although the critical biological functions of $\text{PGF}_{2\alpha}$ were reported to be mediated through activation of the FP receptor, the effect and mechanism of FP activation are not well understood in ischemic stroke, especially in regard to stroke-related Ca^{2+} signaling. Based on reports of the Prostaglandin F (FP) receptor's presence in the central nervous system and its physiologic properties in regards to stroke and Ca^{2+} signaling (Abramovitz et al., 1994; Kitanaka et al., 1994; Saleem et al., 2009a), we hypothesize that activation of the FP receptor subsequent to injury contributes to excitotoxic and ischemic damage. Therefore, our goal is to investigate the role of the FP receptor in the brain and in neuronal cells and to further address relevant signaling pathways.

We have established the importance of the PGE_2 EP1 receptor in ischemic models (Ahmad et al., 2008). Based on its similarity to the EP1 receptor in terms of amino acid sequence and cell signaling, we hypothesize that the FP receptor might contribute to excitotoxic and ischemic brain damage similar to that caused by the EP1 receptor. We speculate that the mechanism of injury might stem from dysregulation of Ca^{2+} homeostasis. Therefore, we studied the role of the FP receptor in a mouse model of permanent middle cerebral artery occlusion (pMCAO) and in ischemia-related Ca^{2+} signaling in cultured neurons.

Materials and Methods

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee. All mice were maintained and housed in the vivarium under controlled conditions ($23 \pm 2^\circ\text{C}$; 12 hours light/dark periods) with access to food and water *ad libitum*. Adult male WT mice and $\text{FP}^{-/-}$ C57BL/6 mice were used at 8–10 weeks of age (20–25 g) in this study.

pMCAO, neurologic function, and infarct size determination

The pMCAO procedure was carried out as previously described (Saleem et al., 2009c). Mice were anesthetized with 250 mg/kg Avertin (Sigma), administered intraperitoneally, and a 2.0-mm burr hole was made just over the middle cerebral artery, visible through the temporal bone. The main trunk of the distal part of middle cerebral artery was occluded with a bipolar coagulator. Successful occlusion was defined as 80% decrease in blood flow and was confirmed by laser-Doppler flowmetry. Body temperature was maintained at $36.5\text{--}37.5^\circ\text{C}$. To evaluate neurologic deficit in the mice, we applied a piece of adhesive tape to each paw and recorded the time required for the mouse to remove each piece. A cut-off time of 120 seconds was used. Mice were trained daily before surgery and subsequently tested 24 hours before and 24 and 48 hours after surgery. After the post-surgical test, mice were sacrificed for infarct volume analysis. Brains were harvested, sliced into five 2-mm thick

coronal sections, mounted onto slides, and stained with 1% triphenyltetrazolium chloride (TTC). The infarct area was translated into a percentage of the contralateral hemisphere of the same brain with correction for swelling (Saleem et al., 2009c).

Mouse hippocampal organotypic slice cultures

Hippocampi from 7–8-day-old C57BL/6 mouse pups were dissected out, and 350- μ m sections were obtained with a Vibratome and stored in Hibernate solution (BrainBits) bubbled with 95% O₂/5% CO₂ (Ahmad et al., 2008). Slices were transferred onto membrane inserts in plates containing Neurobasal medium with B27 and GlutaMAX-I (Invitrogen). On day 13, the medium was replaced with medium containing propidium iodide (PI; 4.5 μ g/mL).

Oxygen-glucose deprivation (OGD) and measurement of neuronal death and reactive oxygen species (ROS) formation

Anoxia was induced by subjecting slices to oxygen-glucose deprivation (OGD) for 1 hour in deoxygenated glucose-free Hibernate A solution and a 37°C chamber with anoxic gas (5% CO₂, 95% N₂) (Saleem et al., 2009b; Zhou et al., 2008). After OGD, slices were treated with 0.1–10 μ M AL-8810 (Cayman Chemical Co.). Slices were imaged for PI fluorescence 24 hours after OGD. To obtain maximal PI fluorescence, they were stimulated for 24 hours with a lethal dose of NMDA (100 μ M) and photographed. To measure cell death, mean PI fluorescence in the CA1 subregion of each slice was quantified. Sequential fluorescence was measured before OGD stimulation to determine basal levels of neuronal death (F_{basal}); 24 hours after stimulation with OGD (F_{OGD}); and after a final exposure to 100 μ M NMDA overnight (F_{max}). The percentage of cell death was calculated by the formula: $(F_{\text{OGD}} - F_{\text{basal}}) / (F_{\text{max}} - F_{\text{basal}}) \times 100\%$. During OGD, we exposed the slice cultures to 5 μ M dihydroethidium, an indicator of ROS. After OGD, the medium was changed and slices were incubated for 2 hours. Dihydroethidium intensity in the CA1 region was then evaluated.

Neuronal cell cultures

Cerebral cortices were obtained from newborn pups (Zhen et al., In Press). The cortices were digested by 0.067% papain (Worthington) and 0.01% DNase and then triturated through pipette tips to dissociate the cells. Cells were plated onto 15-mm circular cover slips or in 24-well plates (0.5×10^6 cells per well) pre-coated with 0.01% poly-D-lysine and grown in Neurobasal medium, Glutamax, B27, and antibiotic-antimycotic solution as previously described (Saleem et al., 2008). In previous studies, our research group has demonstrated that this procedure produces cultures composed of 95% neurons and fewer than 5% astrocytes, based on MAP-2 immunostaining for neurons and GFAP staining for astrocytes (Echeverria et al., 2005). Experiments were performed after 10–15 days in vitro. For astrocyte cell cultures, the cortices were then dissociated by 0.25% trypsin+0.1% DNase digestion for 15 minutes. Cells (2×10^6) were plated onto 75-cm² flasks pre-coated with 0.1% collagen in MEM medium supplemented with 10% horse serum, 2% Glutamax, and 1% antibiotic-antimycotic (Invitrogen). The cell cultures were kept at 37°C in a humidified incubator with 95% air/5% CO₂. After 7–10 days, cells were subcultured into 24-well plates. Immunostaining with GFAP showed that these cultures were more than 98% astrocytes. All experiments were performed after 14–21 days in vitro.

OGD, cell viability assessment, and PGF_{2 α} ELISA in cultured cells

The cell cultures were exposed to OGD for 1 hour. First the neuronal or astrocyte cultures were rinsed with normal medium and equilibrated for 30 minutes at 37°C. Then they were rinsed with deoxygenated glucose-free MEM and transferred to an air-tight 37°C chamber.

Anoxic gas (5% CO₂, 95% N₂) was flushed into the chamber. After 1 hour, cultures were transferred to a normal incubator for reoxygenation. Cell survival was quantified with the lactate dehydrogenase (LDH) assay kit (Sigma) as described previously with some modifications (Saleem et al., 2007). At 24 hours after OGD, cell lysate and medium were collected, and LDH assay mixture was added to each sample. Absorbance was measured at a wavelength of 490 nm with a spectrophotometer (Bio-Tek). The percent cell viability was calculated by the formula: (cell lysis LDH/(medium LDH + cell lysis LDH)) × 100%. After OGD, neuronal cultures were also double-stained with 1 µg/mL Hoechst 33342 and 2 µg/mL PI to determine living or dead cells. OGD-induced PGF_{2α} production was determined by ELISA according to the manufacturer's instructions (Cayman Chemical), and absorbance was measured at a wavelength of 420 nm. LDH and PGF_{2α} ELISA experiments were repeated with three separate batches of cultures.

Ca²⁺ imaging

Intracellular Ca²⁺ imaging was performed as described in previous studies with some modifications (Kim et al., 2007). Cultured neurons were loaded with 3 µM fura-2AM in Tyrode solution [in mM: 138 NaCl, 2 CaCl₂, 4 KCl, 1 MgCl₂, 0.33 NaH₂PO₄, 10 HEPES, 10 glucose (pH=7.4)] at 37°C for 40 minutes. The excitation wavelengths were filtered through 340nm and 380-nm filters. Fluorescence intensity was collected with a cooled CCD digital camera (CoolSNAP, Photometrics) connected to a Nikon microscope. Intracellular Ca²⁺ concentration is presented as the ratio of collected fluorescence intensities of fura-2 excited at 340nm and 380nm, (Ratio_{340/380}). The Ratio_{340/380} was recorded every 5 seconds.

Statistical analysis

Data are expressed as means ± S.E.M. Statistical analysis was performed by one-way ANOVA followed by Bonferroni post hoc test or Student's *t*-test with Origin 8 (OriginLab). Significance was set at *P*<0.05.

Results

Blockade of FP receptor is protective in a stroke mouse model

To determine the effect of FP receptor inhibition in stroke, we injected mice i.v. with 1 or 10 mg/kg of the FP antagonist AL-8810 (Cayman Chemical) immediately after pMCAO. The cortical infarct volume was significantly less in the AL-8810-treated mice than in the vehicle-treated group 48 hours after pMCAO (Fig. 1A, B; *P*<0.05 [1 mg/kg], *P*<0.001 [10 mg/kg]). Mice treated with AL-8810 also had significantly reduced tape-removal times compared with mice in the vehicle-treated group at 48 hours (Fig. 1C). Compared with vehicle, AL-8810 had no effect on body temperature, heart rate, mean blood pressure, glucose, or blood gases (pH, PaCO₂, and PaO₂) after 1 hour of treatment (n = 5 per group, Table 1). Similar results were observed in FP^{-/-} mice. Genetic deletion of the FP receptor significantly reduced the cortical infarct volume at 48 hours after MCAO and enhanced the behavioral recovery after ischemia (Fig. 2). These data indicate that FP inhibition reduces anatomical and functional deficits after stroke.

FP receptor inhibition reduces neuronal damage and ROS formation following OGD

To study the effect of FP receptor in an in vitro model of ischemia, mouse hippocampal organotypic slice cultures were exposed to OGD. Cell death in the CA1 region was measured by PI labeling (Fig. 3). Slices were treated with 0.1, 1, or 10 µM AL-8810 after OGD, and PI fluorescence intensity was measured 24 hours later. Our data show that 10 µM AL-8810 reduced OGD-induced cell death compared with that of the vehicle-treated group (*P*<0.01; Fig. 3). Cell death after OGD was also significantly lower in slices from FP^{-/-}

mice than in those from WT mice ($P<0.001$; Fig. 4). We further addressed the selectivity of the antagonist in FP^{-/-} slice cultures. OGD-induced cell death did not differ between FP^{-/-} slice cultures treated with 10 μ M AL-8810 and those treated with vehicle (Fig. 4).

To determine which cell type(s) OGD affects, we studied neuronal and astrocyte cultures individually. OGD significantly increased cell death in neuronal cell cultures ($P<0.001$, Fig. 5A), but not in astrocyte cell cultures (data not shown). Treatment with 10 μ M AL-8810 significantly reduced neuronal cell death compared with vehicle treatment after OGD-induced injury ($P<0.05$, Fig. 5A). To show the living or dead neuronal cells, the culture neurons were double stained with the Hoechst 33342 and PI after OGD (Fig. 5B). The numbers of PI positive neurons were decreased by treatment with AL-8810 (Fig. 5B). PGF_{2 α} production was significantly increased after OGD in cultured neurons ($P<0.05$, $P<0.01$, Fig. 5C), but not in cultured astrocytes (data not shown). In addition, OGD-induced PGF_{2 α} production was significantly reduced by an NMDA receptor inhibitor (10 μ M MK-801 in cultured neurons), but not by an AMPA receptor inhibitor (10 μ M NBQX) ($P<0.05$, Fig. 5D). Examined together, these results indicate that the FP receptor contributes to acute ischemia-induced neuronal death.

After stroke, increased glutamate release, intracellular Ca²⁺ accumulation, and formation of ROS lead to oxidative stress and cell death. Therefore, we examined ROS formation and oxidative injury after OGD. During OGD, we treated the tissue slices with an ROS indicator, dihydroethidium. Dihydroethidium intensity data indicate that 10 μ M AL-8810 significantly reduced OGD-induced ROS formation in hippocampal slice cultures ($P<0.001$; Fig. 6A). Slices from FP^{-/-} mice also had reduced OGD-induced ROS formation compared with those from WT mice. OGD-induced ROS formation was unchanged in AL-8810-treated FP^{-/-} slices compared with the vehicle-treated FP^{-/-} group (Fig. 6).

FP receptor mechanism in stroke-related Ca²⁺ signaling

Next, we investigated the FP-associated Ca²⁺ response in cortical neuronal cell cultures. When cultured neurons were exposed to FP receptor agonist fluprostenol (Cayman Chemical) for 1 minute, [Ca²⁺]_i increased in a concentration-dependent manner (Fig. 7A, B). Pretreatment with 10 μ M of the inhibitor AL-8810 blocked this response ($P<0.01$; Fig. 7C, D). We studied the relationship between the FP receptor and the ryanodine receptor. When cells were perfused with 50 μ M ryanodine, a ryanodine receptor antagonist, the FP agonist-induced Ca²⁺ response was almost entirely blocked ($P<0.001$; Fig. 7C, D). In addition, we treated cultured neurons with 1 μ M fluprostenol and measured cell death after 24 hours. Fluprostenol increased the neuronal cell death ($P<0.01$) and AL-8810 reduced it significantly ($P=0.003$; $P<0.01$; Fig. 7E). Finally, to study OGD-induced Ca²⁺ increase and the effect of FP inhibition, we compared the Ca²⁺ level before and after OGD. OGD-induced Ca²⁺ increase was significantly reduced by treatment with AL-8810 ($P<0.01$; Fig. 7F).

Discussion

In our previous study, the FP receptor significantly reduced infarct volume in a transient MCAO mouse model (Saleem et al., 2009a). In this study, we showed that the inhibitor of the FP receptor significantly improved the outcome in mice after ischemia in terms of neurobehavioral function and infarct volume. In a previous study, OGD resulted in expression of apoptotic and necrotic cell death phenotypes, especially in neurons (Newcomb-Fernandez et al., 2001). We also demonstrated that FP receptor inhibition was protective against OGD-induced cell death and ROS formation in hippocampal slice cultures. Examined together, our results indicate that FP inhibition may be beneficial in protecting neurons from stroke- and neurotoxicity-induced insults.

Stroke is now ranked as the number 4 cause of death in the United States, desperately needing novel and effective treatments. Many pathophysiologic mechanisms are activated after stroke and brain injury (Moskowitz et al., 2010). These mechanisms are divided into glutamate-induced excitotoxicity, formation of ROS, and apoptotic-like cell death pathways (Besancon et al., 2008). Ca^{2+} dysregulation links many of these multifactorial signals (Berliocchi et al., 2005). Therefore, blockade of Ca^{2+} imbalance-related receptors, transporters, and intracellular signals reveals neuroprotective targets for stroke and neurodegeneration (Simard et al., 2006). After a stroke, loss of cerebral blood flow rapidly triggers energy deficits and neuronal depolarization that cause the release of large amounts of glutamate into the extracellular space. Over-activation of NMDA and AMPA receptors subsequently cause a generalized ionic imbalance within neurons, especially of Ca^{2+} . The Ca^{2+} overload is understood to induce a wide range of cell death enzymes, including ATPases, proteases, lipases, and DNases (Besancon et al., 2008). Furthermore, during stroke, the excess glutamate and hyperactivation of its receptors result in intracellular activation of phospholipid enzymes, phospholipid hydrolysis, and arachidonic acid release (Muralikrishna Adibhatla and Hatcher, 2006). Although blockade of glutamate receptors has been shown to be neuroprotective in preclinical studies, in clinical trials the results have been disappointing (Wahlgren and Ahmed, 2004). Studies have consistently shown that the NMDA-induced Ca^{2+} imbalance is related to a failure to remove Ca^{2+} from the cell (Bano and Nicotera, 2007; Kawano et al., 2006). After cerebral ischemia, the loss of Na^+/K^+ -ATPase activity in the plasma membrane can degrade the transmembrane Na^+ gradients necessary for $\text{Na}^+/\text{Ca}^{2+}$ exchange function and lead to Ca^{2+} accumulation.

Reports have suggested that prostaglandins perform diverse functions in neurodegenerative disorders through their G-protein-coupled receptors (Doré, 2006). The FP receptor mRNA is expressed in rat whole brain (Kitanaka et al., 1994), and it has been reported that the FP receptor is expressed in cortical cultures enriched in neurons (Carlson, 2003). Activation of the FP receptor initiates several events, including stimulation of the inositol 1,4,5-trisphosphate receptor-related Ca^{2+} signaling pathway and store-operated Ca^{2+} entry (Snetkov et al., 2006). One study demonstrated that FP receptor activation by $\text{PGF}_{2\alpha}$ in rodent cardiomyocytes increased the expression of early growth response factor-1 by activation of a Ras/Raf/mitogen-activated protein kinase signaling cascade (Xu et al., 2008). The amino acid sequence of the FP receptor has high sequence homology with that of the EP1 receptor, and the FP receptor shares the same branch of the phylogenetic tree with the EP1 and TP receptors. Interestingly, we have previously shown that the EP1 receptor in mice augments brain damage after excitotoxicity and focal cerebral ischemia (Ahmad et al., 2008). Although activation of the FP receptor initiates several events, including Ca^{2+} signaling, its role is not clear in stroke-related Ca^{2+} signaling. As previously described, activated EP1 can reduce $\text{Na}^+/\text{Ca}^{2+}$ exchange activity and cause dysregulation of Ca^{2+} homeostasis following ischemia (Kawano et al., 2006). Therefore, we also studied NMDA-induced Ca^{2+} response to determine the mechanism of FP receptor activity in NMDA neurotoxicity. NMDA-induced Ca^{2+} responses were not different between cultured neurons from WT mice and those from $\text{FP}^{-/-}$ mice (data not shown). However, we demonstrated that OGD increases $\text{PGF}_{2\alpha}$ production, that activation of the FP receptor induces a Ca^{2+} response in cultured neurons, and that this response is connected to the ryanodine receptor pathway. In addition, the treatment of FP receptor agonist in cultured neurons increases cell death and FP receptor blockade decreases OGD-induced Ca^{2+} response. These data indicate that the protective effect of FP receptor inhibition and its mechanism in stroke-related Ca^{2+} signaling differs from that of the EP1 receptor-related Ca^{2+} pathway. In addition, there is direct evidence of endoplasmic reticulum (ER) stress after brain ischemia/reperfusion (Hayashi et al., 2005). Disruption of ER Ca^{2+} homeostasis plays an important role in induction of ER stress. Therefore, the FP receptor may affect ER Ca^{2+} homeostasis via ryanodine receptor signaling with ER Ca^{2+} release, a possibility that requires further study.

Ischemia leads to the activation of ROS-generating enzymatic systems (Moskowitz et al., 2010). The increase in $[Ca^{2+}]_i$ promotes ROS formation by the protein kinase C pathway, nitric oxide pathway, and mitochondrial depolarization in ischemia (Brennan et al., 2009). We found that OGD-induced ROS formation was decreased by FP receptor depletion or FP receptor inhibitor. The results indicate that inhibiting the FP receptor can protect neurons in early periods of ischemia by reducing Ca^{2+} overload and ROS formation.

Conclusion

In this study, we used AL-8810 to evaluate the role of FP receptor in ischemic damage. AL-8810 reduced OGD-induced neuronal cell death in slice cultures from WT mice but had no effect on cell death or ROS production in slices from $FP^{-/-}$ mice, indicating that the drug is selective for FP. However, potent and selective FP antagonists should be developed for more pharmacologic studies and therapeutic applications. Our findings provide novel targets for intervention and treatment of cerebral ischemia and help to elucidate the therapeutic potential and role of the FP receptor in acute stroke.

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Abbreviations

Ca^{2+}	calcium
COX	cyclooxygenase
FP receptor	PGF _{2α} receptor
$FP^{-/-}$	FP receptor knockout
pMCAO	permanent middle cerebral artery occlusion
OGD	oxygen-glucose deprivation
WT	wildtype
HBSS	Hanks' buffered saline solution
LDH	lactate dehydrogenase
ER	endoplasmic reticulum.

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Highlights

- Infarct volumes and behavioral deficits were reduced in FP antagonist AL8810 mice
- Similar results in FP knockout mice after permanent ischemic stroke
- FP receptor blockade protects against oxygen glucose deprivation cell death in organotypic slices
- Neuronal death is less in cells treated with AL8810 not in cultured astrocytes
- FP receptor agonist increases intracellular calcium levels of cultured neurons

Fig. 1.

Effects of FP receptor inhibition on ischemic brain injury and neurologic dysfunction after pMCAO in mice. (A) Representative photographs of infarcted brain slices from vehicle- and AL-8810-treated mice. (B) Cortical infarct volume, shown as a percent of total cortical volume and corrected for swelling, was significantly smaller in AL-8810-treated mice than in vehicle-treated mice after 48 hours. (C) The tape removal time in the sticky tape test assessed 48 hours after ischemia was significantly lower in AL-8810-treated mice than in vehicle-treated mice, indicating reduced neurologic dysfunction. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 10-13$ mice).

Fig. 2. Effects of FP receptor deletion on ischemic brain injury and neurologic dysfunction after pMCAO. (A) Representative photographs of infarcted brain slices. (B) Cortical infarct volume was significantly smaller in FP^{-/-} mice than in wild-type (WT) mice after 48 hours. (C) The tape removal time was significantly lower in FP^{-/-} mice than in WT mice at 24 and 48 hours. * $P < 0.05$, ** $P < 0.01$ ($n = 10-12$ mice).

Fig. 3. Effects of FP receptor inhibition on OGD-induced cell death in cultured hippocampal slices. (A) Fluorescence images (4X magnification) show PI staining in hippocampal slices subjected to OGD for 1 hour. Images were taken before OGD (Base), 24 hours after OGD (OGD), and 24 hours after NMDA incubation (Maximal Death). (B) After OGD insult, the slice cultures were treated with AL-8810. The neuronal damage was significantly lower in 10 μ M AL-8810-treated slices than in vehicle-treated slices after OGD-induced injury. ** P <0.01 (n = 19–23 slices).

Fig. 4. Effects of FP receptor deletion on OGD-induced cell death in cultured hippocampal slices. (A) Fluorescence images show PI staining in hippocampal slices subjected to OGD. (B) After OGD, neuronal cell death in the CA1 region measured as PI intensity was significantly lower in hippocampal slices from FP^{-/-} mice than in those from WT mice. Cell death in cultured FP^{-/-} slices was not changed by 10 μ M AL-8810 compared with vehicle treatment. ** P <0.01, *** P <0.001 (n = 21–23 slices).

Fig. 5. OGD-induced cell death and PGF_{2α} production in cultured neurons. Cultured neurons and astrocytes were subjected to OGD for 1 hour; 24 hours later, the LDH assay was performed to assess cell death. (A) Cell death was significantly higher in neuronal cell cultures exposed to OGD than in those exposed to normal growing conditions (control). Neuronal cell death was significantly lower in cultures treated with 10 μ M AL-8810 than in vehicle-treated cultures. *** P <0.001 vs. control, # P <0.05 vs. vehicle-treated group. (B) Representative images of the Hoechst 33342 and PI double staining (a: phase contrast, b: sham, c: OGD + vehicle, d: OGD + 10 μ M AL8810, Bar = 50 μ m). Images display typical results from three independent experiments. (C) To study the effect of ischemia on PGF_{2α} production, cultured neurons and astrocytes were subjected to OGD for 1 hour. In cultured neurons, PGF_{2α} production was significantly increased at 2 hours and 6 hours after OGD. * P <0.05, ** P <0.01. (D) OGD-induced PGF_{2α} production was significantly decreased by 10 μ M MK-801, but not by 10 μ M NBQX. * P <0.05.

Fig. 6. Effects of FP receptor inhibition on OGD-induced ROS formation in hippocampal slices. (A) Dihydroethidium (DHE) fluorescence revealed that AL-8810 (10 μ M) significantly reduced OGD-induced ROS formation in hippocampal slice cultures. (B) OGD-induced ROS formation was significantly lower in hippocampal slices from FP^{-/-} mice than in those from WT mice. AL-8810 had no additional effect on slices from FP^{-/-} mice. *** P <0.001 (n = 20–22 slices).

Fig. 7.

Effects of FP receptor inhibition and ryanodine receptor inhibition on FP agonist-induced Ca^{2+} response in cultured neurons. (A) Ca^{2+} response was evoked by 1-minute exposure to 1, 10, 100, and 1000 nM fluprostenol--an FP receptor agonist. (B) Mean change in $\text{Ratio}_{340/380}$ ($n = 17-19$ cells). (C) Cultured neurons were superfused with 10 μM AL-8810 or 50 μM ryanodine solution for 10 minutes before treatment of 100 nM fluprostenol. Pretreatment with 10 μM AL-8810 almost completely inhibited the FP agonist-induced Ca^{2+} response. The fluprostenol-induced Ca^{2+} response was also inhibited by pretreatment with 50 μM ryanodine, a ryanodine receptor antagonist. (D) Mean change in $\text{Ratio}_{340/380}$; *** $P < 0.001$ ($n = 18-19$ cells). (E) Treatment with 1 μM fluprostenol increases the neuronal cell death and 10 μM AL-8810 significantly attenuated the induced neuronal cell death. ** $P < 0.01$ vs. control, # $P < 0.05$ vs. vehicle-treated group. (F) OGD-induced Ca^{2+} response was significantly reduced by 10 μM AL-8810 ** $P < 0.01$ ($n = 17-18$ cells).

Table 1

Physiologic parameters in vehicle and AL-8810-treated mice (n = 5).

Parameters	Vehicle	AL-8810
Rectal temperature, °C	36.8 ± 0.1	36.7 ± 0.1
Heart rate, beats/min	525 ± 20	529 ± 11
Mean blood pressure, mm Hg	86.2 ± 3.0	88.0 ± 3.5
PaCO ₂ , mm Hg	38.9 ± 1.3	39.7 ± 1.6
PaO ₂ , mm Hg	127 ± 8	125 ± 7
pH	7.34 ± 0.02	7.36 ± 0.02
Glucose, mg/dL	122 ± 9	126 ± 8