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Neuroprotective Role of Prostaglandin PGE₂ EP2 Receptor in Hemin-mediated Toxicity

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Abstract

Heme (Fe²⁺ protoporphyrin IX) and hemin (Fe³⁺), the prosthetic group of hemoprotein, are cytotoxic due to their ability to contribute to the production of reactive oxygen species, increased intracellular calcium levels and stimulate glutamate mediated-excitotoxicity. Previous work by our group showed that blockade of the prostaglandin E₂ (PGE₂)-EP1 receptor reduced hemin-induced cytotoxicity in primary cortical neuronal cultures. However, the role of the prostaglandin E₂ (PGE₂)-EP2 receptor in hemin neurotoxicity remains unclear. Activation of the EP2 receptor in neurons results in increased cyclic AMP (cAMP) and protein kinase A signaling; therefore, we hypothesized that the activation of the EP2 receptor decreases hemin neurotoxicity. Using postnatal primary cortical neurons cultured from wildtype-control (WT) and EP2^{-/-} mice, we investigated the role of the EP2 receptor in hemin neurotoxicity by monitoring cell survival with the Calcein-AM live-cell and lactate dehydrogenase assays. MitoTracker staining was also performed to determine how mitochondria were affected by hemin. Hemin neurotoxicity in EP2^{-/-} neurons was 37.2 ± 17.0% greater compared to WT neurons. Of interest, cotreatment with the EP2 receptor agonist, butaprost (1 and 10 μM), significantly attenuated hemin neurotoxicity by 55.7 ± 21.1% and 60.1 ± 14.8%, respectively. To further investigate signaling mechanisms related to EP2 receptor mediating cytoprotection, neurons were cotreated with hemin and activators/inhibitors of both the cAMP-protein kinase A/exchange protein directly activated by cAMP (Epac) pathways. Forskolin, a cAMP activator, and 8-pCPT-cAMP, an Epac activator, both attenuated hemin neurotoxicity by 78.8 ± 22.2% and 58.4 ± 9.8%, respectively, as measured using the lactate dehydrogenase assay. Together, the results reveal that activation of the EP2 receptor is protective against hemin neurotoxicity in vitro and these findings suggest that neuroprotection occurs through the cAMP- Epac pathway in neuronal cultures. Therefore, activation of the EP2 receptor

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Conflict of interest statement

None declared.

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could be used to minimize neuronal damage following exposure to supraphysiological levels of hemin.

Keywords

Hemoglobin; Heme; protein kinase A; Inflammation; Neurons

1. Introduction

The toxicity of free heme has been clearly documented in several disease types. For example, in hemolytic anemia's such as sickle cell disease and thalassemia, release of heme from hemoglobin following lysis of red blood cells is known to cause stress and ultimately cell death (Kumar and Bandyopadhyay et al., 2005; Liu et al., 1988; Nagababu et al., 2008; Phumala et al., 2003; Walter et al., 2006). In a critical care situation such as hemorrhagic injury, neuronal cell death is caused by the lysis of red blood cells, which release hemoglobin and its breakdown product hemin (Chiu et al., 1996; Levy et al., 2002; Mohan et al., 2012; Regan et al., 1996; Wang et al., 2007; Wang et al., 2006). Physiological levels of free heme in the blood are maintained at low levels by the high binding affinity of protein such as serum albumin, hemopexin, and haptoglobin (Heide et al., 1964; Killander et al., 1964; Miller et al., 1996; Nagel and Gibson et al., 1971; Paoli et al., 1999; Shipulina et al., 2000). When internalized, free heme is catabolism by heme oxygenases, HO1 and HO2 isoforms (Loboda et al., 2008). In addition to HO1 and HO2 isoform regulated cellular damage, the oxidative state of iron (from Fe^{2+} to Fe^{3+} via the Fenton reaction) within heme can produce harmful superoxide free radicals in the brain that can lead to oxidative stress, initiate proteins and lipids oxidation, and damage to RNA and DNA leading to neuronal death (Smith et al., 1995; Valko et al., 2005).

Pathological conditions can increase the level of heme and iron. For example in acute conditions such as severe hemolytic crisis in sickle cell disease, heme levels can increase up to 20 μM or to greater than 200 μM in thalassemia (Phumala, Porasuphatana et al., 2003; Shaklai et al., 1985). Therefore, further studies on the cross talk between neuroinflammatory mediators such as prostaglandins and iron-containing hemoproteins or hemin are warranted.

The proinflammatory biolipids, e.g., prostaglandins acting via their specific G-protein-coupled receptors, have been reported to have often-contradictory physiological functions in the central nervous system. The PGE_2 main recognized receptor types EP1, 2, 3, and 4, when activated, signal via different intracellular messaging systems. We and others have been postulating that activation of EP2 and EP4 receptors regulates adenylate cyclase and the generation of 3', 5'-cyclic adenosine monophosphate (cAMP), whereas the activation of EP1 and EP3 receptors regulates Ca^{2+} signaling (Carlson, 2003; Mohan et al., 2013). EP1 and EP2 receptors are expressed in cultured neurons and microglia as well as neurons of the cerebral cortex, striatum, and hippocampus (Mohan et al., 2013; Quan et al., 2013; Zhang et al., 1999). Also, activation of the EP2 receptor by PGE_2 is involved in long-term synaptic plasticity and cognitive function, as $\text{EP2}^{-/-}$ mice showed impaired hippocampal synaptogenesis (Chemtob et al., 1994; Yang et al., 2009). Our group has been focusing on studying the role of the EP receptors in relation to intracerebral hemorrhagic stroke and

hemin toxicity (Mohan et al., 2013; Singh et al., 2013). Previous data from our group and others have shown that following activation, different PGE₂ receptors can contribute or protect against N-methyl-D-aspartate (NMDA) neurotoxicity and ischemic stroke (Ahmad et al., 2005; Ahmad et al., 2008; Ahmad et al., 2006b; Milatovic et al., 2011; Miyagishi et al., 2013; Takadera et al., 2004). For example, in a mouse model of focal cerebral ischemia, we have shown that pretreatment with an EP2 receptor selective agonist, ONO-AE1-259-01 (1.0 and 2.0 nmol), was able to significantly decrease neurological deficits. In the same study, deletion of EP2 receptors aggravated ischemic brain damage (Ahmad et al., 2010). Activation of the EP2 receptors with butaprost protected neurons from amyloid β -peptide neurotoxicity in vitro (Echeverria et al., 2005). However, the role of the EP2 receptor in the presence of hemin remains unknown; this study aims to expand our understanding and provide new insight for EP2 receptor-mediated neuroprotection in the presence of hemin. We hypothesized that hemin-mediated cytotoxicity is blocked by the activation of EP2 receptors.

Several studies suggest that the mechanism by which PGE₂ affords neuroprotection is through EP2 or EP4 receptors, as they both increase cAMP, followed by a protein kinase A (PKA)-dependent pathway (Araki et al., 2000; Echeverria et al., 2005; McCullough et al., 2004). EP2 receptor-agonists (e.g. butaprost) are good pharmacological tools to study downstream intracellular signaling as they have similar potentiating effects as that measured by PGE₂ alone (Choi et al., 2001). The use of EP2 receptor agonists is also justified by the very short half-life of PGE₂. PGE₂ cannot be therapeutically used specifically against the EP2 receptor as the half-life of PGE₂ is less than 1 min. following intravenous injection and approximately 30 sec. in the circulatory system (Fitzpatrick et al., 1980; Kimball et al., 1980). Therefore, we propose that the activation of the EP2 receptor by butaprost could be used to minimize neuronal damage following exposure to supraphysiological (greater than 50 μ M) levels of hemin.

In addition to cAMP-PKA-dependent signaling, G-protein phosphorylation can also result in the activation of PKA-independent pathways (Ster et al., 2007). For example, the exchange proteins directly activated by cAMP (Epac1 and 2) is one such pathway that has received the most attention (Kawasaki et al., 1998; Roberts et al., 2014). Epac1 and 2 function as guanine nucleotide exchange factors for the G-protein Rap. Due to the abundance of G protein-coupled receptors that mediate cAMP signaling, Epac proteins may contribute to target biological functions such as regulation of neuronal differentiation, neuron outgrowth, and axonal generation (Christensen et al., 2003; Kiermayer et al., 2005; Murray et al., 2008; Shi et al., 2013). In the central nervous system, cAMP-Epac signaling is involved in learning and memory, neuronal excitability, brain oxidative stress, and neuronal death (Hara et al., 2012; Ouyang et al., 2008; Ster et al., 2007; Suzuki et al., 2010). This is the first study to measure the effect of the EP2 receptor in hemin-mediated neurotoxicity and introduce the role of Epac in EP2 receptor-mediated neuroprotection.

2. Materials and methods

2.1. Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee of University of Florida. All mice were maintained and housed in the university's vivarium under controlled conditions ($23^{\circ}\text{C} \pm 2^{\circ}\text{C}$; 12-h light/dark cycle), with access to food and water ad libitum.

2.2. Primary neuronal cell cultures

Postnatal mouse cortical neuronal cultures were performed as previously reported (Mohan et al., 2013). Briefly, neurons were isolated from 0- to 2-day-old C57BL6 male and female wildtype (WT) and EP2^{-/-} pups and cultured in serum-free Neurobasal medium and plated onto 24-well plates at a density of 5×10^5 cells/well supplemented with GlutaMax and B27 (Invitrogen, Carlsbad, CA). Fifty percent of the media was exchanged with fresh medium containing B27 (Life Technologies, Grand Island, NY) every 4 days. For direct comparison between WT and EP2^{-/-} neurons, "sister cultures" were used to increase the reliability of our data.

2.3 Cell survival assays

Seven- to 10-day-old neurons from WT and EP2^{-/-} pups were treated with vehicle (Neurobasal/B27 minus antioxidant-supplemented medium containing 0.05% 0.1M NaOH or 0.01% dimethyl sulfoxide), hemin (Frontier Scientific, Logan, UT), EP2 receptor-selective agonist, butaprost (Cayman Chemicals, Ann Arbor, MI), Forskolin (Sigma, St. Louis, MO), 8- pCPT-cAMP sodium salt (8CPT; Tocris Bioscience, Minneapolis, MN), Brefeldin A (BFA; Sigma), 6-Bnz-cAMP sodium salt (Bnz-cAMP), and H-89 (Sigma) in Neurobasal/B27 minus antioxidant-supplemented medium. Neurons were treated with hemin (vehicle, 12.5, 25, 50, 75, and 100 μM) for 18 h. In separate experiments, neurons were cotreated with EP2 receptor-selective agonist butaprost (1 and 10 μM) and vehicle or hemin (75 μM) for 18 h. To assess the number of live cells, the Calcein AM (Life Technologies) assay was performed as instructed in the manufacturer's protocol and as previously published (Mohan et al., 2013). Cell viability was also assessed using the CytoScanTM-Fluoro assay (G-Biosciences, St. Louis, MO). This is a fluorometric assay for estimating cell cytotoxicity based on the release of lactate dehydrogenase (LDH) from cells with damaged membranes. The low LDH activity (background) in wells subjected to medium exchange only was subtracted from all values to yield the signal specific for the neurotoxic insult mediated by hemin as described by (Koh et al., 1987). In addition to LDH and Calcein AM assays, an assay to measure the health of mitochondria was also performed. We used the MitoTracker Red CMXRos (500 nm) dye (Life Technologies), which is a membrane potential-dependent dye that labels mitochondria within live cells (Chazotte, 2011). Micrographs of neurons were acquired using a 20 $\times$ differential interference contrast (DIC) objective on a Leica DMI6000 B monochrome digital camera and captured using the MetaMorph software (Molecular Devices LLC, Sunnyvale, CA). Calcein AM-positive and MitoTracker-stained neurons were captured at 482/520 nm and 580/600 nm, respectively, with a 20 $\times$ objective using the same Leica DMI6000 B microscope. Any additional bright

field images were captured on an EVOS FL cell imaging system using a 20× objective (Life Technologies, Grand Island, NY, USA).

2.4. Statistical analysis

All data are expressed as mean $\pm$ standard error of the means (SEM) and were analyzed using one-way ANOVA (LDH and Calcein-AM assay) with Bonferroni's multiple comparison tests. Statistical difference between two groups was analyzed by two-tailed unpaired Student's *t*-test. Statistical differences were considered significant if $p < 0.05$. All data were analyzed by GraphPad Prism 6.0 software.

3. Results

3.1. Knockout of the EP2 receptor renders neurons more susceptible to hemin neurotoxicity

In postnatal primary neuronal cultures from WT pups, hemin caused significant neurotoxicity as measured using the Calcein AM assay in a dose-dependent manner. Hemin treatment at 50 ($61.4 \pm 1.4\%$), 75 ($52.0 \pm 2.3\%$), and 100 μM ($39.1 \pm 3.8\%$) significantly decreased the number of live cells compared to vehicle ($96.8 \pm 2.4\%$; Fig. 1A); the 100% was established in conditions in which the cells did not receive any treatment (control). In addition, hemin treatment caused a change in the morphology (increased blebbing/swelling pertaining to apoptosis) at higher concentration of the WT neurons compared to vehicle-treated neurons. The microphotographs depict a decrease in the number of live neurons following an increasing concentration of hemin as represented by Calcein AM positive staining (Fig 1A, i–vi). To investigate the role of the EP2 receptor, hemin neurotoxicity in cells derived from EP2^{-/-} mice was compared to WT neurons. Hemin-induced neurotoxicity was significantly greater in EP2^{-/-} neurons ($51.5 \pm 2.5\%$) compared to WT neurons ($31.8 \pm 1.9\%$) as measured using the LDH assay (Fig. 1B, $p < 0.05$). Following hemin treatment, microphotographs show a decrease in the number of number of Calcein-AM-positive neurons from EP2^{-/-} mice (Fig. 1B, i–iii).

3.2. Butaprost, as a selective EP2 agonist, protects neuronal cultures from hemin

Using MitoTracker Red CMXRos, a red-fluorescent dye that stains mitochondria in live cells and whose accumulation is dependent upon the membrane potential of mitochondria, mitochondria status was assessed following hemin or butaprost cotreatment. The disruption of the mitochondria transmembrane potential is one of the earliest intracellular events and such disruption occurs after induction of cell death via mitochondria loss of homeostasis properties. We hypothesize that hemin-induced neuronal death may dissipate the mitochondrial membrane potential in conjunction with increased activation of the EP2 receptor via increased PGE₂ autocrine signaling. Following hemin treatment in WT neurons, mitochondrial staining significantly decreased. Hemin alone (75 μM ; $67.0 \pm 1.7\%$) significantly decreased the staining of mitochondria compared to vehicle ($94.2 \pm 2.1\%$). Butaprost (10 μM ; $82.8 \pm 3.5\%$) significantly increased the percentage of stained neuronal mitochondria compared to hemin alone (Fig. 2A, $p < 0.05$). Micrographs of MitoTracker-stained cells depict changes in mitochondria staining patterns between vehicle and hemin treatment alone and when cotreated with butaprost (Fig. 2A, i–iii). In separate experiments,

compared with hemin treatment alone ($32.77 \pm 4.33\%$), cotreatment with butaprost (1 and 10 μM ; $52.33 \pm 6.22\%$ and $70.9 \pm 4.11\%$) significantly improved the percentage of Calcein AM-positive cells. (Fig. 2B, $p < 0.01$). Bright-field and Calcein AM-positive fluorescence micrographs depict changes in the number of live neurons following treatment with vehicle and hemin alone or when cotreated with butaprost (10 μM ; Fig. 2B, i–iii). Cell viability was not significantly different between cultures treated with butaprost alone and those treated with vehicle (data not shown). Using the LDH assay, compared to hemin (75 μM ; $26.0 \pm 2.4\%$) alone, cotreatment with butaprost (1 and 10 μM ; $16.4 \pm 2.33\%$ and $14.77 \pm 1.6\%$) significantly decreased cytotoxicity measured using the LDH assay (Fig. 2C, $p < 0.01$).

To further elucidate the signaling mechanisms responsible for EP2 receptor mediated neuroprotection against hemin, we decided to conduct further experiments that focused on the alternative cAMP pathway signaling protein, Epac.

3.3. cAMP-Epac activation protects neuronal cultures against hemin-induced toxicity

To determine how butaprost affects hemin-induced neurotoxicity, we assessed the effect of EP2 receptor-mediated signaling pathways, cAMP-PKA, and Epac signaling. Neuronal cultures were treated with hemin (75 μM) or hemin cotreated with forskolin (10 μM), 8CPT (100 μM), BFA (10 μM), 6-Bnz-cAMP (50 μM), and H-89 (10 μM ; (Holz et al., 2008; Misra et al., 2005; Suzuki et al., 2010; Vliem et al., 2008; Zhou et al., 2013). The effect of these compounds, when cotreated with hemin, on neuronal survival was measured using LDH, Calcein AM assays, and MitoTracker staining. These concentrations were chosen as being selective for cAMP, Epac, and PKA and (Holz et al., 2008; Murray et al., 2008; Suzuki et al., 2010; Vliem et al., 2008; Zhou et al., 2013). Dose-response studies with Epac agonist 8CPT significantly reduced hemin neurotoxicity. Cotreatment with 8CPT (10, 50, and 100 μM) and hemin (75 μM) significantly decreased ($51.1 \pm 1.6\%$, $51.5 \pm 2.3\%$, and $42.1 \pm 2.8\%$, respectively) the levels of LDH compared to hemin alone ($79.6 \pm 3.4\%$; Fig. 3A). In separate experiments, WT neurons were cotreated with hemin (75 μM) and cAMP agonist, forskolin (10 μM) or Epac agonist, 8CPT (100 μM). Forskolin ($31.1 \pm 4.8\%$) and 8CPT ($36.5 \pm 1.1\%$) significantly decreased the levels of LDH compared to hemin ($65.0 \pm 5.8\%$) treatment alone (Fig. 3B). Similarly, forskolin and 8CPT significantly increased the number of Calcein AM-positive neurons ($76.0 \pm 1.3\%$ and $71.0 \pm 5.2\%$, respectively) and mitochondrial staining ($74.3 \pm 3.7\%$ and with 8CPT (10, 50, and 100 μM) and hemin (75 μM) significantly decreased ($51.1 \pm 1.6\%$, $51.5 \pm 2.3\%$, and $42.1 \pm 2.8\%$, respectively) the levels of LDH compared to hemin alone ($79.6 \pm 3.4\%$; Fig. 3A). In separate experiments, WT neurons were cotreated with hemin (75 μM) and cAMP agonist, forskolin (10 μM), or Epac agonist, 8CPT (100 μM). Forskolin ($31.1 \pm 4.8\%$) and 8CPT ($36.5 \pm 1.1\%$) significantly decreased the levels of LDH compared to hemin ($65.0 \pm 5.8\%$) treatment alone (Fig. 3B). Similarly, forskolin and 8CPT significantly increased the number of Calcein AM-positive neurons ($76.060 \pm 1.3\%$ and $71.0 \pm 5.22\%$, respectively) and mitochondrial staining ($74.3 \pm 3.7\%$ and $73.2 \pm 10.4\%$, respectively) compared to hemin (Calcein-AM; $42.5 \pm 2.6\%$; MitoTracker; $47.6 \pm 2.4\%$) treatment alone (Fig. 3, C and D). Cotreatment with the Epac antagonist BFA (10 μM) did not significantly change the viability of neurons compared to the hemin treatment alone. In addition to cAMP-Epac activation, the role of the cAMP-PKA pathway was studied. However, activation (with 6-Bnz-cAMP) and inhibition

(with H89) of PKA did not show any significant neuroprotection against hemin (Fig. 3, B–D).

4. Discussion

The main finding of this study is that PGE₂ EP2^{-/-} neurons are more susceptible to hemin neurotoxicity than WT neurons. And that activation of the EP2 receptor protected neurons from hemin neurotoxicity; we have documented a unique and significant protective ability of EP2 receptors against hemin-induced neurotoxicity.

Previous studies have revealed that the mouse brain and neuronal cultures express EP2 receptors (Choi et al., 2006) (www.brain-map.org). The group led by Rivest and colleagues has shown that EP2 receptors are expressed in astrocytes and neurons of the cerebral cortex, striatum, and hippocampus (Zhang et al., 1999). Using primary hypothalamic cultures, hemin (50 µM) treatment for 3 h resulted in the release of PGE₂ into the surrounding cellular milieu (Lee et al., 2001). In the same study, the increase in hemin-induced PGE₂ was inhibited by methylene blue, a soluble guanylate cyclase inhibitor, indicating that PGE₂ receptors were activated following hemin treatment (Lee et al., 2001). Hemin may cause toxicity through various mechanism; as a pro-oxidant, it can alter proteins on the surface of the membrane and can also aggregate which would affect the integrity of cellular membranes and therefore the expression and function of lipid and receptors expressed on the surface of cells (Higdon et al., 2012). On a similar note, our original experimental aim as to measure the expression of the EP2 receptor in vehicle control, hemin plus butaprost treated neuronal cultures. However preliminary experiments showed that 18 h of hemin treatment induced a significant decrease in RNA yield (increased degradation) that would have otherwise been used for qRT-PCR analysis.

Previous studies have suggested that prostaglandin receptors are involved in mechanisms of neurodegeneration in cerebral ischemia and excitotoxic brain injury (Ahmad et al., 2006a; Doré et al., 2003; Mohan et al., 2012; Mohan et al., 2013; Serrano et al., 2011; Singh et al., 2013). When activated, EP2 receptor couples to G_{αs}-protein, resulting in increased cAMP signaling. cAMP signaling is involved in multiple functions of various cell types, therefore, local levels of cAMP are highly regulated via the actions of multiple feedback systems (Castro et al., 2014). The precise coordination of proteins that manufacture, degrade, and are activated by cAMP is critical to maintaining coherent downstream signaling events triggered by specific extracellular stimuli (Baillie, 2009). Phosphodiesterase enzymes are the main biological method by which cAMP become deactivated (dephosphorylated) has been found at different expression levels following ischemic insult, which may explain why contiguous cAMP gradients can be formed simultaneously following a single G protein-coupled receptor activation event (Mitome-Mishima et al., 2013). Others and our group have demonstrated that activation of EP2 receptors can be used as a neuroprotective target in paradigms of acute toxicity in vitro and in ischemic stroke in rodents (Ahmad et al., 2006b; Liu et al., 2005). In contrast, however, PGE₂ has been reported to promote NMDA-induced neurotoxicity in rat embryonic cortical mixed cultures via EP2 receptor-dependent elevated levels of cAMP (Takadera et al., 2006). Differences for these conflicting observations of the role of the EP2 receptor may be from differences in the rodent model used to culture

neurons, tissue culture protocols, the concentration of agonist used to elicit activation of the EP2 receptor, assays used to measure cytotoxicity, and the proportion of non-neuronal cell types. The role of non-neuronal cells such as microglia has been previously reported; microglia are less vulnerable to hemin toxicity due to increase expression of inducible nitric oxide synthase and heme oxygenase-1 (Cai et al., 2011). This microglia-mediated mechanism may be responsible for modulating the adaptive response of activated microglia to hemin toxicity and not the EP2 receptor (Cai et al., 2011). The degree to which a specific EP2 receptor agonist activates the downstream signaling cascade depends on the affinity and specificity of the drugs. Also, more relevant to this study, the classical paradigm of membrane-only cAMP signaling has been challenged by reports of sustained cAMP production following receptor internalization that may render a different cellular response (Calebiri et al., 2009). This novel signaling model may explain how a different agonist can activate a common receptor and trigger different cellular outcomes (Ferrandon et al., 2009).

Despite some of our earlier observations that suggested EP2 receptor-dependent neuroprotection could be through the generation of cAMP, the signaling cascade responsible for EP2 receptor-dependent neuroprotection remains incomplete. Using non- and specific cAMP-PKA and Epac activators and inhibitors, our results suggest that butaprost-mediated neuroprotection from hemin may have occurred through the cAMP-Epac pathway in neuronal cultures. However, activation of Epac was fully mimicked by forskolin treatment, suggesting the possible synergistic action of Epac and PKA on cAMP mediated neuroprotection. In addition to Epac/PKA synergy, our data also showed that treatment with the Epac antagonist did not significantly affect the number of live cells (Calcein AM assay) compared to hemin treatment alone. If Epac was the sole protein responsible for neuroprotection, then blockade of the Epac protein should have either exacerbated hemin-induced cell death or at least maintained the levels of live cells measured following hemin treatment alone (see Fig. 1C). We believe that the high, but not statistically significant reading from the Calcein AM assay following treatment with BFA, BnZcAMP, H89 and hemin were probably from live, non-neuronal cell types that are more resistant to hemin cytotoxicity. The robust nature of non-neuronal cells to hemin cytotoxicity may be mediated by the “activational nature” (e.g. activated microglia) of these cell types (Cai et al., 2011). Our results further substantiate the neuroprotective role of EP2 receptors in neuronal cultures, but further in vivo studies (post-treatment with butaprost) using a mouse model of intracerebral hemorrhage and the exact role of cAMP-Epac signaling in EP2 receptor-mediated neuroprotection is warranted.

In conclusion, we report that selective activation of the EP2 receptor by butaprost promotes neuroprotection against hemin cytotoxicity and our data supports previous studies that highlight the importance of the cAMP-Epac signaling cascade in cellular functions and health. Supraphysiological hemin levels in the brain after intracerebral hemorrhage and how the stimulation of the PGE₂-EP2 receptor pathway may affect outcomes remain unknown. However, we demonstrate that EP2 receptors could be an effective therapeutic target for the early short-term treatment of hemin-mediated neurotoxicity.

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Highlights

- Hemin-induced neurotoxicity was increased in EP2^{-/-} neurons
- Treatment with EP2 receptor agonist butaprost reduced hemin neurotoxicity
- Activation of the cAMP-Epac signaling pathway reduced hemin neurotoxicity

Fig. 1. Hemin-induced neurotoxicity is increased in EP2^{-/-} neurons. WT neurons were treated with hemin (12.5–100 μM) for 18 h. (A) Neuronal cell viability was measured by the Calcein AM assay (% of control) from WT neurons and (Ai–iv) phase-contrast images of Calcein AM-positive neurons were captured. (B) Neuronal cell viability was measured by LDH assay (LDH/LDH_{max} %) from WT and EP2^{-/-} neurons following hemin (75 μM) treatment and (Bi–iii) bright-field images of Calcein AM-positive cells. Data represents means $\pm$ SEM of duplicate measure from triplicate wells from $n = 4$ per genotype. Statistical analysis was carried out using a one-way ANOVA, with Bonferroni's multiple comparison tests. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. vehicle. # $p < 0.01$ vs. WT. Scale bar = 20 μm .

Fig. 2.

EP2 receptor agonist, butaprost, protects neurons from hemin. WT neurons were cotreated with hemin (75 μ M) and butaprost (1 and 10 μ M) for 18 h. (A) Changes in mitochondrial staining was measured using MitoTracker Red and the amount of absorption measured and (Ai-iii) shows images of MitoTracker Red-positive of neurons following treatments. (B) Change in Calcein AM fluorescence following treatment with vehicle, hemin (75 μ M) alone, or cotreatment with butaprost and (Bi-iii) shows images of Calcein AM-positive neurons following treatments. (C) Hemin- induced neurotoxicity was measured by LDH (LDH/LDHmax %). Data represents means $\pm$ SEM of duplicate measure from triplicate wells from $n = 8$ experiments. Statistical analysis was carried out using a one-way ANOVA, with Bonferroni's multiple comparison tests. [#] $p < 0.001$ vs. vehicle; ^{*} $p < 0.05$; ^{**} $p < 0.01$; ^{***} $p < 0.001$ vs. hemin. Scale bar = 20 μ m.

Fig. 3.

Activation of the cAMP-Epac protects neurons from hemin. (A) WT neurons were treated with vehicle, hemin (75 μM), and 8CPT (10, 50 and 100 μM) for 18 h and LDH levels were measured. (B–D) WT neurons pre- and cotreated with cAMP agonist, forskolin (FSK-10 μM), Epac agonist, 8CPT (100 μM), Epac antagonist, BFA (10 μM), PKA activator, BnZcAMP (50 μM), and PKA inhibitor, H89 (10 μM), and hemin (75 μM) for 18 h. (B) neuronal cell viability was measured by LDH assay (LDH/LDHmax %), (C) Calcein AM assay (% of Control) and (D) by using MitoTracker Red (% of Control) absorption assay from WT neurons. Data represents means ± SEM of duplicate measure from triplicate wells from n = 4 experiments. Statistical analysis was carried out using a one-way ANOVA, with Bonferroni's multiple comparison tests. #p < 0.001 vs. vehicle alone; *p < 0.05; **p < 0.01; ***p < 0.001 vs. hemin.

Fig. 4.

EP2 receptor modulates cAMP-Epac intracellular signaling in the presence of heme. Intracellular signaling pathways involved in the EP2 receptor-mediated neuroprotection in the presence of heme in neurons. Agonist = EP2-receptor agonist (butaprost); G_s = guanine nucleotide-binding protein, stimulatory (s)-type; AC = adenylyl cyclase; PI3K/MAPK/Akt = phosphoinositide 3-kinase/mitogen-activated protein kinase/protein kinase B signaling; FSK = cAMP agonist (forskolin).