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Morphine-Mediated Cytoprotection against Hemin in SK-N-SH and A172 Cells

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Abstract

Study background: Heme and its catabolized form, hemin, are cytotoxic due to their ability to contribute to the production of reactive oxygen species, increase intracellular calcium levels and stimulate glutamate mediated-excitotoxicity. Previous work has shown that activation of the opioid receptors (i.e., mu, kappa and delta) is neuroprotective against ischemia-induced neuronal death and neurotoxicity induced by Aβ oligomers *in vitro*. However, the role of these opioid receptor in hemin toxicity remains unknown. Activation of the Mu-receptor results in decreased 3, 5'-cyclic adenosine monophosphate (cAMP) and adenylyl cyclase activity, which then results in reduced cAMP-dependent Ca^{2+} influx. Therefore, we hypothesized that the activation of the opioid receptors by morphine may decrease hemin toxicity.

Methods: The human neuroblastoma SK-N-SH and human astroglia A172 cells were treated with hemin (3.125, 6.25, 12.5, 25, 50 and 75 μM) for 18 h. In separate experiments, both cells types were pre- and co-treated with opioid receptor agonist morphine (10 μM) and hemin (75 μM) or naltrexone (10 μM) alone and pre- and co-treatment with hemin (75 μM) for 18 h. Cell viability was assessed using two assays, LDH and the number of live cells measured using the Calcein-AM assay.

Results: In both SK-N-SH and A172 cells, hemin-dose dependently induced significant cell death compared to the vehicle control. When pre-treated and co-treated with morphine, hemin toxicity in both cell types was significantly attenuated. The protection mediated by morphine from hemin was blocked by opioid antagonist, naltrexone.

Conclusion: Together, the results suggest that activation of the opioid receptors my morphine is protective against hemin toxicity *in vitro* and these findings suggest that cytoprotection may occur through the cAMP-AC pathway. Therefore, activation of for example the Mu-receptor could be used to minimize neuronal and glial damage following exposure to supraphysiological levels of hemin.

Keywords: Heme; Protein kinase A; Inflammation; Neurons; Morphine; Opioid receptor

Introduction

The toxicity of free heme has been documented in several disease types. For example, in hemolytic anemias such as sickle cell disease and thalassemia, release of heme from hemoglobin following lysis of red blood cells is known to cause cell death [1,2]. 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 [3-5]. Physiological levels of free heme in the blood are maintained at low levels (0.1 -1 μM) by the high binding affinity of proteins such as serum albumin, hemopexin and haptoglobin [6-8]. When internalized, free heme is catabolized by heme oxygenases (HO1 and HO2 isoforms) and therefore the amount of cellular damage free heme produces is limited by the stress-responsive HO1 isoform [9]. 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, initiation of lipid peroxidation and neuronal death.

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 (supraphysiological levels) in thalassemia [10]. Therefore, further studies on the cross talk between neuroinflammatory mediators and iron-containing hemoproteins such as hemin are warranted.

Morphine is a non-selective opioid receptor agonist and analgesic agent used in clinical practice to manage pain. Morphine and morphinans (class of compounds containing the basic morphine structure) agents have been shown to be neuroprotective in various models of inflammation and neurodegeneration. For example, morphine pretreatment of purkinje cells and hippocampal slices provided ischemic tolerance [11,12]. The same group also recently discovered that morphine-mediated neuroprotection was not only NMDA receptor mediated but also miR-134 dependent in primary cortical neuronal cultures [13]. Besides it critical role in anti-nociception, activation of the Mu-opioid receptor promotes cell proliferation and also affects neuronal differentiation both *in vitro* and *in vivo* [14-16]. Studies have shown that morphine protects neurons against microglia-mediated neuroinflammation and oxidative stress [17]. More recently, activation of the Mu- receptor attenuated β-amyloid peptide neurotoxicity through an mTOR-dependent mechanism [18]. For a more comprehensive review of morphinans mediated neuroprotection, Zhang et al. published a review in 2004 that discusses the utility of opioids in neurodegerative disease. The role of opioid

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receptor activation in the presence of supraphysiological levels of hemin remains to be studied. However, in the current study, we demonstrate and are the first to measure that opioid receptor activation by morphine (non-selective agonist) could protect neurons and astrocytes against hemin-induced toxicity. However, further experiments are needed with selective agonists and antagonist before we can conclude which subtype of opioid is more or less cytoprotective against hemin.

Materials and Methods

Cell cultures

The human neuroblastoma cell line (SK-N-SH, ATCC # HTB-11; American Type Culture Collection, Manassas, VA) was used to measure changes in LDH. SK-N-SH cells were maintained in Royal Park Memorial Institute-1640 growth media (RPMI), supplemented with 10% fetal bovine serum, 50 U/mL penicillin and 0.05 mg/mL streptomycin and 10 μM retinoic acid. SK-N-SH cells were grown as a monolayer in a humidified incubator at 37°C, 5% CO_2 and 95% air in T75 culture flasks. The human astrocytoma cell line (A172, ATCC #CRL-1620; American Type Culture Collection, Manassas, VA, USA, donated by Dr. Randall Davis, Oklahoma State University, School of Medicine) was used to model a key component of neuroinflammation. Stock cultures of human astrocytoma cells were maintained in Dulbec-Co's modified Eagle's medium (DMEM) supplemented with, 10% fetal bovine serum, 50 U/ml penicillin and 0.05 mg/ml streptomycin. Culture medium was changed every 48-72 h. For both SK-N-SH and A172 cell-lines, experimental cultures were seeded at a cell density of $1 \times 10^4/cm^2$/well using 96 well plates to provide 80-90% confluence and all experiments were initiated in fresh medium. All cultures were maintained in a humidified incubator at 37°C, 5% CO_2 and 95% air and all experiments were conducted between passages 12 and 20.

Treatments

Forty eight hours old cultures were treated with vehicle (cell culture media contain 0.05% 0.1 M NaOH minus fetal bovine serum), hemin (Frontier Scientific, Logan, UT), morphine (Sigma, St. Louis, MO) and naltrexone (Sigma, St. Louis, MO) in cell culture media minus fetal bovine serum. Neurons and astroglia cells were treated with hemin (3.125, 6.25, 12.5, 25, 50 and 75 μM) for 18 h. In separate experiments, both cells types were treated with opioid receptor agonist morphine (10 μM) or naltrexone (10 μM) alone and co-treatment with hemin (75 μM) in growth media respective to each cell-line for 18 h.

Assays

Cell viability was also assessed using the CytoScan™-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. 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 by Mohan et al.

Microscopy

Micrographs of all cells were acquired using a 20X differential interference contrast (DIC) objective on a VWR® inverted fluorescence microscope monochrome digital camera. Calcein-AM-positive neurons were captured at 482/520 nm with a 20X objective using the same VWR® inverted fluorescence microscope.

Statistical analysis

All data are expressed as mean ± standard error of the means (SEM) and were analyzed using Two-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.

Results

Neurons are more susceptible to hemin toxicity

In SK-N-SH neuronal and A172 astrocytic cultures, hemin dose-dependently caused significant toxicity as measured using the LDH assay (LDH/LDH_{max} [%]). Hemin at 12.5 (41.9 ± 23.1%), 25 (94.6 ± 21.1%), and 50 μM (103.7 ± 18.6%) significantly ($p<0.05$) increased the amount of LDH released compare to vehicle (11.1 ± 2.9%; Figure 1A) in SK-N-SH cells. Hemin at 12.5 (28.0 ± 3.4%), 25 (36.3 ± 6.4%), and 50 μM (47.3 ± 6.4%) also significantly ($p<0.05$) increased the amount of LDH released by A172 cells compared to the vehicle (11.1 ± 2.9%; Figure 1A). Microphotographs of SK-N-SH and A172 cells following hemin treatment showed a change in morphology (increased blebbing/swelling pertaining to apoptosis) (Figure 1B, i-iv).

Morphine-mediated attenuation of hemin toxicity is opioid receptor selective

To investigate the role of opioid receptors, neurons and astrocytes were pre-treated and co-treated with morphine and hemin in a separate cohort of experiments. In neurons, compared to vehicle treated cells, hemin (75 μM) treatment alone caused significant toxicity (74.0 ± 9.8%) as measured using the LDH assay (Figure 2A, $p<0.01$). When pre-treated (15 mins) and co-treated with morphine (10 μM), hemin-induced toxicity was significantly reduced (17.4 ± 6.5%) compared to hemin treatment alone (74.0 ± 9.8%) (Figure 2A, $p<0.01$). To measure the effect and role of the opioid receptors, cells were pre-treated with opioid receptor selective antagonist, naltrexone (10 μM) prior to morphine and hemin co-treatment. Following blockade of opioid receptors with the non-selective naltrexone, the cytoprotective effect of morphine against hemin was significantly blocked (46.1 ± 3.8%) indicating a opioid receptor mediated mechanism (Figure 2A, $p<0.001$). Following hemin treatment, microphotographs show that hemin caused a large number of neurons to undergo apoptosis and this decrease in the number of neurons was attenuated in the presence of morphine (Figure 2B, i-iii). Using the Calcein-AM assay in neurons, hemin treatment alone also caused a significant decrease (14.1 ± 5.4%) in the number of live-cells compared to the vehicle treatment alone (100.0 ± 0.4%) (Figure 2C, $p<0.001$). Pre-treatment and co-treatment with morphine (10 μM) significantly attenuated hemin-induced toxicity as measured using the Calcein-AM assay, increasing the number of live-cells (39.5 ± 5.3%) compared to the hemin treatment alone (14.1 ± 5.4%) (Figure 2C, $p<0.001$). Similar to results measured using the LDH assay, pre-treatment with naltrexone prior to morphine and hemin co-treatment significantly blocked any morphine-mediated neuroprotection against hemin and the percentage of live-cells were significantly less in SK-N-SH cells (14.1 ± 6.7%) compared to hemin plus morphine treated cells (Figure 2C, $p<0.001$). Following hemin treatment, microphotographs show a decrease in the number of Calcein-AM-positive neurons compared to vehicle treated cells and when co-treated with morphine, the number of live-cells increased compared to the hemin treatment alone (Figure 2D, i-iii).

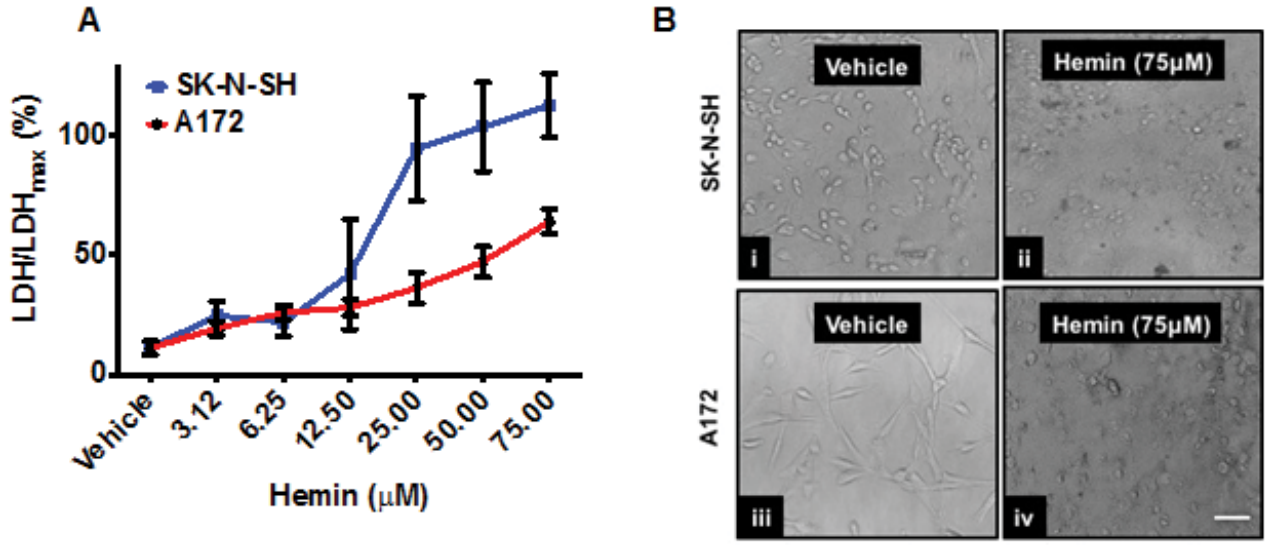

Figure 1: Hemin is toxic in both SK-N-SH neuronal and A172 astroglia cells. Hemin showed greater toxicity in SK-N-SH cells then A172 astrocytes as measured using the LDH assay (LDH/LDH$_{max}$ [%]). Hemin treatment for 18 h at 12.5 (41.9 ± 23.1%), 25 (94.6 ± 21.1%), and 50 μM (103.7 ± 18.6%) significantly increased the amount of LDH released compared to vehicle (11.1 ± 2.9%) in SK-N-SH cells. Hemin at 12.5, 25 and 50 μM significantly increased the amount of LDH released by A172 cells compared to vehicle. Microphotographs of SK-N-SH and A172 cells following hemin treatment showed a change in morphology (increased blebbing/swelling pertaining to apoptosis) (Figure 1B). Data are means ± S.E.M of duplicate measure from triplicate wells from n=6-9 per treatment group. *p<0.05, Two-way ANOVA with Bonferroni's multiple comparison tests vs. vehicle controls. Scale bar=20 μm.

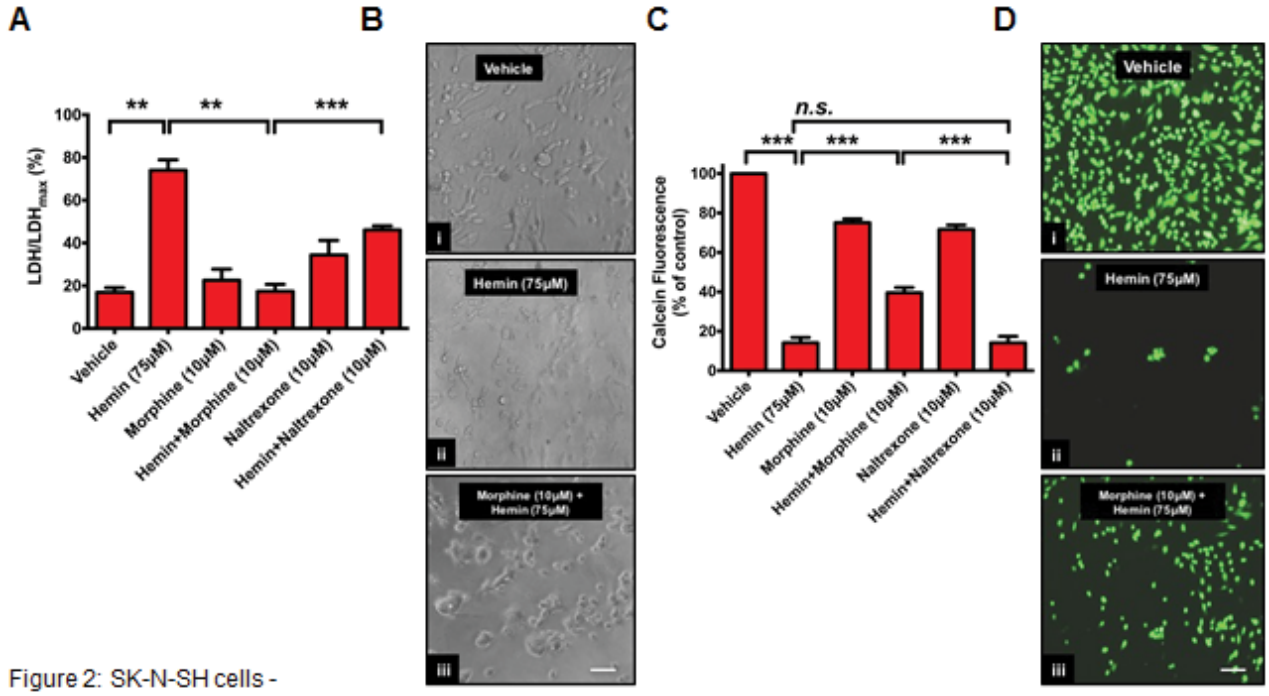

Figure 2: SK-N-SH cells -

Figure 2: Morphine-mediated attenuation of hemin toxicity in SK-N-SH cells. When pre-treated and co-treated with morphine (10 μM), hemin-induced toxicity was reduced compared to hemin treatment alone. To measure the role of the opioid receptors, cells were pre-treated with opioid selective antagonist, naltrexone (10 μM) prior to morphine and hemin co-treatment. Following blockade of the opioid receptors with naltrexone, the cytoprotective effect of morphine was significantly blocked (Figure 2A and 2C). Microphotographs of SK-N-SH cells following pre-treatment and co-treatment with morphine and hemin treatment showed an increase in the number of live-cells number (Figure 2B and 2D, i-iii). Data are means ± S.E.M of duplicate measure from triplicate wells from n=3-6 per treatment group. **p<0.01, ***p<0.001, Two-way ANOVA with Bonferroni's multiple comparison tests vs. vehicle or hemin or hemin plus morphine. Scale bar=20 μm.

In a separate cohort of experiments, A172 astrocytes were also treated with hemin and compared to vehicle treated cells. Hemin (75 μM) treatment alone caused significant toxicity (68.90 ± 3.4%) as measured using the LDH assay (Figure 3A, $p<0.01$). When pre-treated (15 mins) with morphine (10 μM), hemin-induced toxicity was significant reduced (40.1 ± 8.3%) compared to hemin treatment alone (68.9 ± 3.4%) (Figure 3A, $p<0.01$). Following pre-treatment with naltrexone (10 μM) the cytoprotective effect of morphine against hemin was significantly blocked (88.1 ± 5.2%) indicating a opioid receptor mediated mechanism (Figure 3A, $p<0.001$). Following hemin treatment, microphotographs show that hemin caused a large number of astrocytes to undergo apoptosis and this decrease in the number of astrocytes was attenuated in the presence of morphine (Figure 3B, i-iii).

Discussion

In the present study, we demonstrated that morphine attenuated hemin-induced toxicity. Morphine's ability to decrease hemin toxicity was shown to be dependent on the activation of opioid receptors expressed (non-selectivily) in neurons and astrocytes. We have documented a unique and significant protective ability of opioid receptor activation against hemin-induced toxicity.

Hemin may cause its toxicity through various ways; 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 also the expression and function of lipid and receptors expressed on the surface of cells [19]. Previous studies have suggested that opioid receptors are involved in protecting against neurodegeneration indicated in Alzheimer's disease, ischemia and excitotoxic brain injury [20-23].

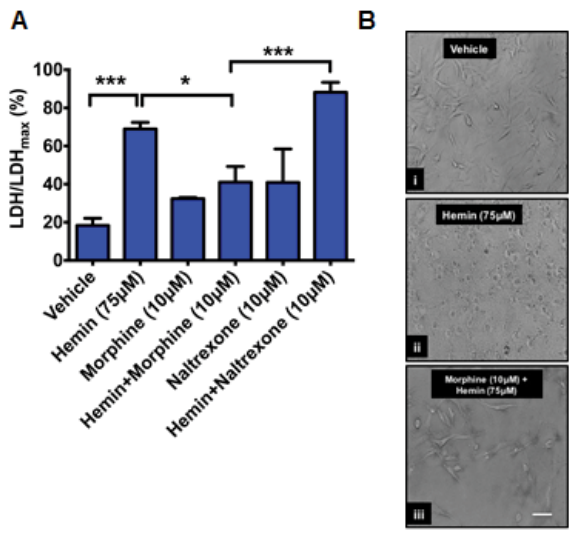

Figure 3: Morphine-mediated attenuation of hemin toxicity in A172 astroglia cells. When pre-treated and co-treated with morphine (10 μM), hemin-induced toxicity was reduced compared to hemin treatment alone. To measure the role of the opioid receptors, cells were pre-treated with opioid receptor antagonist, naltrexone (10 μM) prior to morphine and hemin co-treatment. Following blockade of the opioid receptor with naltrexone, the cytoprotective effect of morphine was significantly reduced (Figure 3A). Microphotographs of A172 cells following pre-treatment and co-treatment with morphine and hemin treatment showed an increase in the number of astroglia cells (Figure 3B, i-iii). Data are means ± SEM of duplicate measure from triplicate wells from n=3-6 per treatment group. *p<0.05 for hemin vs. hemin plus morphine. ***p<0.001 for hemin plus morphine vs., naltrexone pre-treated cells. Two-way ANOVA with Bonferroni's multiple comparison tests vs. vehicle or hemin or hemin plus morphine or hemin plus naltrexone. Scale bar=20 μm.

To date, four different opioid receptor types mu (μ), delta (δ), kappa (κ), opioid receptor like-1 (ORL1) and their genes have been characterized at the cellular, molecular, and pharmacologic levels [24]. All four opioid receptors are seven-transmembrane spanning proteins that couple to inhibitory G proteins. After being activated by an agonist, such as the endogenous μ-opioid peptide endorphin, or exogenous agonists, such as morphine, the Gα and Gβγ subunits dissociate from one another and subsequently act on various intracellular effector pathways [25,26]. Following activation of opioid receptors, guanosine triphosphate (GTPase) modulates agonist binding to opioid receptors in membrane preparations from brain tissue. Agonist stimulation of opioid receptors inhibits cyclic adenosine monophosphate (cAMP) production [27,28]. It is widely accepted that all four opioid receptor types couple to pertussis-toxin-sensitive G proteins, including $G\alpha^{i}$, to cause inhibition of cAMP formation. Considering the consistent signaling between the subtypes of opioid receptors, studies have shown that the OPRM1 (mu) and OPRD1 (delta) receptor to be indicated in neuroprotection. Activation of OPRM1 or OPRD1 with selective opioid agonist prior (precondition) to oxygen-glucose deprivation (OGD) dose-dependently reduced neuronal death in rat hippocampal slice cultures. Also by the same group, pre-treatment with morphine reduced brain infarct volume and improved neurological functional outcomes in rats subjected to occlusion of the right middle cerebral artery [23]. Future experiments will be conducted using selective agonist against mu, delta, kappa opioid receptors to determine which opioid receptor subtype has greater cytoprotective role against hemin toxicity.

The cytoprotection provided by the presence of opioids before insult is called opioid preconditioning [29]. During the 2000-2006 period, seven studies showed that morphine preconditioning reduced neuronal cell death [11,20-23,30,31]. However, more recent studies have shown that opioid preconditioning may protect neurons by modulating neuroinflammation and inflammatory pathways [32-34]. These studies and the data presented here in our study with the use of astrocytes support the role of glial cells in opioid-mediated cytoprotection. Both microglia and astrocytes play an important role in the inflammatory response in the brain following stroke, brain infection and neurodegerative diseases. However, there is very little information regarding mechanisms that could be used to protect glial cells. Here, we have showed for the first time that incubation of A172 human astroglia cells with morphine prior to hemin treatment protects astrocytes and this morphine preconditioning effect was abolished by naltrexone, a non-selective opioid receptor antagonist. The exact signaling mechanisms involved in protecting astrocytes from hemin by morphine remain to be studied. Published studies have shown that morphine preconditioning can modulate TNFα, IFNγ and eNOS [32,34,35] and therefore may decrease neuroinflammation-mediated neuronal cell damage and death. Hemin is known to increase inflammatory-injury by activating TLR-4 [36]. However, this *in vitro* study suggests that hemin may also protect astrocytes by inhibiting NOS through the induction of HO-1 [37]. The difference between Sheng et al. study and our study is that our astrocytes were not activated; Sheng et al. used treated astrocytes before hemin exposure. Activated astrocytes are known to provide a natural, innate level of neuroprotection but if over-activated they can be key to the development of neurodegeneration [38].

In conclusion, we report that activation of the opioid receptors by morphine promotes cytoprotection against hemin toxicity in neurons

and astrocytes. Our data supports previous studies that highlight the importance of opioid preconditioning in cellular functions and health in disease. Supraphysiological hemin levels in the brain after intracerebral hemorrhage and how the stimulation of the opioid receptor pathway may affect outcomes remain unknown. However, we demonstrate that the activation of opioid receptors could be an effective therapeutic target for the early short-term treatment of hemin-mediated cytotoxicity.

Conflict of Interest Statement

Nothing to declare.

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