Neuroprotective Effect of Targeting Hepcidin against Ischemia-Induced Neuronal Damage

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Abstract

Ischemic stroke is one of the leading causes of death and disability in the US and worldwide. There has been very limited progress in developing effective therapeutic approaches for ischemic stroke, although numerous agents have been tested in animal models and in clinical trials. Among many issues, an incomplete understanding of the mechanism responsible for ischemia-induced neuronal injuries is a major limitation. Although iron plays important roles in mammalian metabolism and is essential for life, labile iron is highly toxic in the body. It has been clearly demonstrated that ischemia elevates labile iron levels in the brain. This elevation is a major damaging event in ischemic neurons and plays an important role in the evolution of a penumbra to infarct and subsequent worsening of the outcome after stroke. Additionally, post-ischemic reperfusion could cause a biochemical deterioration of ischemic brain tissue. It has been found that many vulnerable neurons undergo apoptosis during reperfusion. Research in the last 10 years has established that hepcidin is the central regulator of systemic iron homeostasis. It regulates the iron export function of the ferrous iron permease, ferroportin (FPN1), which is the only known iron exporter expressed by mammalian cells. Recently, we and others have revealed that hepcidin is widely expressed in rodent brains and that ischemia increases hepcidin levels in brain cells. This induction of hepcidin expression was associated with accumulation of intracellular labile iron. Furthermore, our data demonstrate that interfering with hepcidin binding to FPN1 did not rescue cells from cellular death following ischemia, so we postulate that targeting hepcidin directly could be an effective approach to protect against ischemia-induced neuronal death. Hypoxia-inducible factor 1 (HIF-1) is a master regulator in hypoxia. HIF-1 mediates angiogenesis, cell proliferation, and energy metabolism. Furthermore, upregulating HIF-1α, the regulatable subunit of HIF-1, provides neuroprotection in ischemic stroke. Heat shock protein 90 (HSP90), a molecular

chaperone that protects its client proteins from misfolding and degradation, binds with HIF-1α PAS domain and promotes the stabilization of HIF-1α. Recent reports show that reactive oxygen species (ROS) induce HSP90 cleavage and loss of its chaperoning function. Interestingly, our data demonstrate that down-regulation of hepcidin with shRNA approach reduced OGD/R-induced ROS and improved mitochondrial function. To understand the role of hepcidin in ischemia-induced neuronal death, we studied the effect of downregulation of hepcidin on HIF-1α and its major regulator, HSP90. We demonstrate that downregulation of hepcidin induced both HSP90 and HIF-1α expression, which subsequently protected neuronal cells from oxygen glucose deprivation/reperfusion (OGD/R) injury. Collectively, these findings suggest that hepcidin is a promising therapeutic target against ischemia/reperfusion injury.

This dissertation is dedicated to:

My grandfather, Awedh

&

All my family members

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ATP	Adenosine triphosphate
bHLH	Basic helix-loop-helix
BSA	Bovine albumin serum
CP	Ceruloplasmin
Cys326	Cysteine-326
DFO	Deferoxamine
DMT1	Divalent metal transporter 1
ELISA	Enzyme-linked immunosorbent assay
EPR	Electron paramagnetic resonance
ETC	Electron transport chain
Furs	Fursultiamine
HAMP	The gene encoding hepcidin
HIF-1α	Hypoxia inducible factor 1α
HSPs	Heat Shock Proteins
ICH	Intracerebral hemorrhage
IL	Interleukin
JAK	Janus Kinase
LPS	Lipopolysaccharide
MCAO	Middle cerebral artery occlusion
MRC	Maximal Respiratory Capacity
MS	Mass spectrometry
MTT	3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-tetrazolium bromide

NOS	Nitric oxide synthase
NOX	NADPH oxidase
ODD	Oxygen-dependent degradation domain
OGD	Oxygen-glucose deprivation
PAS	Per-aryl hydrocarbon nuclear translocator-sim
PBS	Phosphate buffered Saline
PHD	Prolyl hydroxylase domain
pVHL	Von Hippel Lindau tumor suppressor
r-tPA	Recombinant tissue plasminogen activator
ROS	Reactive oxygen species
STAT3	Signal transducer and activator of transcription 3
TAD	Transactivation domains
TBST	Tris-buffered Saline with Tween 20
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor-α
XO	Xanthine oxidase

Introduction

Iron Pathophysiology in Stroke

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Introduction

Stroke

Stroke is one of the leading causes of death and long-term severe disability worldwide. Stroke can be classified into ischemic or hemorrhagic forms according to its pathophysiological nature. The majority of all stroke cases (around 90%) are ischemic, which occur due to a sudden decrease or loss of blood supply to the brain by thrombi and/or emboli. Intracerebral and/or subarachnoid rapturing blood vessels cause hemorrhagic stroke, comprising about 10% of all strokes [1, 2]. Even though the incidence percentage of hemorrhagic stroke is lower than ischemic stroke, it cannot be ignored because this type is more dangerous compared to the ischemic form [3].

In the last ten years, research has focused on identifying effective therapeutic approaches for reducing death and disability related to stroke. Numerous agents have been tested in animal models and clinical trials, but there has been limited progress in developing effective therapeutic strategies for stroke [4-7]. An incomplete understanding of the mechanism responsible for stroke-induced neuronal injuries is a major issue underlying this limited progress. Disturbance of brain iron homeostasis contributes to neuronal injury following cerebral stroke [8-10].

Recently, there has been exciting progress in understanding iron metabolism in stroke. For example, hepcidin has recently been recognized as the central regulator of iron homeostasis and a bridge between inflammation and iron regulation [11, 12]. Many studies have shown that hepcidin enhances iron-dependent neuronal damage and subsequently has become a target for the development of novel therapeutics for iron toxicity in stroke [13, 14]. This review summarizes the pathophysiological role of iron in stroke as well as manipulation of iron-dependent pathways as a potential therapeutic approach to improve the outcome of stroke patients.

Brain iron regulation

Iron is essential for life as it is needed for many biological processes. In the brain, iron plays an important role in neurotransmission, myelination, cell division, and oxygen transport processes [15]. Brain cells maintain iron homeostasis by regulating iron import, storage, metabolism, and export [16, 17]. Many studies have demonstrated the role of transferrin, divalent metal transporter 1 (DMT1), ceruloplasmin (CP), ferritin, and ferroportin1 (FPN1) in the regulation of brain iron [18, 19]. Under physiological conditions, iron binds to transport proteins such as transferrin. Transferrin-bound iron is transported into brain cells via receptor-mediated endocytosis. Emerging evidence suggests that uptake of transferrin-bound iron into the brain cells by transferrin receptor-mediated endocytosis is the major known pathway of brain iron transport across the blood-brain barrier (BBB) [19]. This route of iron transport consists of several steps, including transferrin-iron binding, endocytosis of transferrin-bound iron, dissociation of iron from transferrin, and translocation of the iron inside brain cells. Unbound iron can gain access into brain cells through DMT1, but the majority of iron in brain cells is bound to storage proteins, such as ferritin. CP promotes iron release from intracellular stores. FPN1 is the first and solely known vertebrate iron exporter expressed in the brain. It has been shown that FPN1 mediates iron efflux from brain cells. Since iron import, storage, and export are tightly regulated, any alterations in these steps may disturb iron homeostasis in the brain resulting in iron neurotoxicity [19, 20]. Deficiency in FPN1 activity results in iron retention and further iron toxicity [21]. Increased free iron levels have been observed in ischemic and hemorrhagic brains; however, the exact mechanism behind it is not fully understood [8, 22].

Alterations of iron homeostasis in ischemic and hemorrhagic stroke

Iron plays a critical role in oxygen transport [15]. Under ischemic conditions, required levels of oxygen in the brain increase, resulting in high demand on iron transport and metabolism inside preserved brain regions [8]. Ferritin-bound iron is released in response to changes from ischemic insults, including acidosis and inflammatory mediators. Many studies have shown that the binding affinity of transferrin for iron decreases at low pH levels, resulting in dissociation of iron from transferrin [23]. Upon dissociation, unbound iron can easily gain access to the extracellular space where it is transported into neurons, resulting in increased intracellular iron levels [8, 24]. Recent observations have demonstrated that decreased iron efflux from neuronal cells contributes to putative alterations in iron homeostasis following ischemic stroke [25]. Furthermore, growing evidence indicates that iron accumulation in neurons following ischemia increases brain susceptibility to iron- induced damage [8, 23, 26]. In hemorrhagic stroke condition, many studies showed iron overload in the brain after intracerebral hemorrhage (ICH) [22, 27]. Red blood cell release and lysis have been considered the major factors mediating iron-induced brain damage after ICH [28]. Collagenase, which is commonly used to induce ICH, results in iron overload [29]. Moreover, iron overload may contribute to brain edema following ICH [10]. However, the potential mechanism, underlying iron-mediated neurotoxicity is unsatisfactorily understood.

Iron toxicity

Many studies have suggested that the severity of neuronal damage after ischemic stroke is proportional to the magnitude of brain iron accumulation [8]. For example, Patt *et al.* demonstrated that gerbils with low brain iron exhibited fewer neurological deficits compared to those with high brain iron levels [30]. Consistently, rats with iron overload exhibited about 60% greater infarct

volume than rats with lower iron load [31]. Iron accumulation in rat brains following ischemia [8, 32] induces lipid peroxidation and neuronal injury by catalyzing conversion of superoxide and hydrogen peroxide into highly reactive toxic hydroxyl radicals [33]. Increased formation of these free radicals results in oxidative stress and subsequent ischemic neuronal death [34]. In addition, it has been shown that experimental ICH causes brain injury via iron-induced oxidation and DNA damage [35, 36]. Results from animal studies indicated that Tirilazad mesylate, an inhibitor of iron-dependent lipid peroxidation, reduces neuronal injury in global and focal cerebral ischemia [37, 38]. Moreover, deferoxamine (DFO), a potent iron-chelating agent, reduces infract volume and neurological deficits in ischemic rats [39, 40] and human patients [41]. Rosenthal *et al.* have reported that DFO improves neurological status and reduces mortality rate in rats subjected to a 6.5 min-cardiac arrest [42]. DFO also improves neurologic function in collagenase-induced ICH model [29]. Together, these results support the notion that iron induces neurotoxicity following cerebral stroke.

Molecular mechanisms involved in iron toxicity

Ferroportin in ischemic and ICH stroke

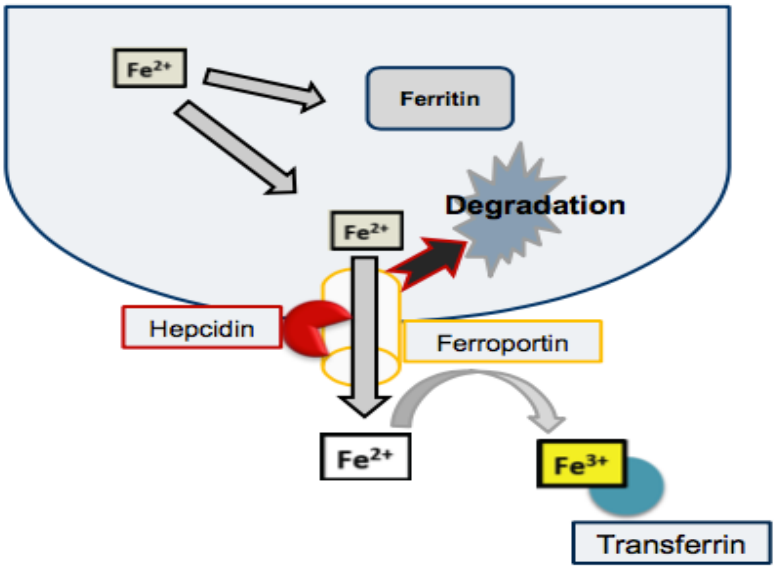
Ferroportin 1 (FPN1, encoded by SLC40A1) is a transmembrane protein, which contains 12 domains and is expressed in the duodenal enterocytes, placenta, macrophages, and brain [43-45]. In the brain, FPN1 is predominately expressed in neurons of the cerebral cortex, hippocampus, and cerebellum [45, 46] where it functions as the sole protein capable of exporting iron from cells [47]. In 2000, Donovan *et al.* found that a complete loss of ferroportin resulted in severe iron deficiency anemia and caused embryonic death in zebrafish and mammals [48]. Additionally, FPN1-mutated mice exhibited severe cellular iron accumulation [21, 49]. Emerging evidence shows that ischemic stroke disrupted the mechanisms that maintaining brain iron homeostasis and

further resulted in iron neurotoxicity [50]. Li *et al.* demonstrated that FPN1 expression was down-regulated in the cerebral cortex and hippocampus after cerebral ischemia. A decrease in FPN1 expression in an ischemic brain may result in iron overload and hepcidin synthesis [25]. Since brain endothelial cells express FPN1, this transporter plays an important role in ICH-induced iron overload. Many studies have showed that FPN1 significantly increased after ICH [51]. Despite the largely unknown neurophysiological role of FPN1 in the brain, FPN1 has been shown to act as a receptor for hepcidin in cultured neuronal cells [52].

Hepcidin

Hepcidin was first discovered in human blood and urine as a bactericidal peptide synthesized in the liver [53-55]. Since its discovery, many reports have demonstrated that hepcidin plays a critical role in systemic regulation of iron metabolism in mammals including human, rat, mouse, and pig [16, 56, 57]. The gene encoding hepcidin (HAMP) is expressed in a growing list of tissues, including liver, brain, lungs, intestines, and heart [58-62]. Hepcidin is a central regulator of iron homeostasis, but the exact mechanism of how hepcidin regulates iron acquisition has not been fully characterized. Nicolas *et al.* unambiguously recognized that inactivation of HAMP results in severe iron accumulation in the liver and pancreas [63]. Additionally, transgenic mice overexpressing HAMP exhibit a severe anemia [64]. Human patients with hepcidin mutations suffer from juvenile hemochromatosis [65]. Treating cultured cells expressing an FPN1-GFP with hepcidin results in translocation of FPN1-GFP from the cell surface into lysosomes where it is degraded [66]. Moreover, the injection of hepcidin into the cerebral ventricle suppresses FPN1 protein levels in different regions of the brain, such as the cerebral cortex, hippocampus, and striatum [14]. Xiong *et al.* reported that both brain and serum hepcidin was upregulated after ICH.

This increased expression of hepcidin reduces intracellular iron efflux from brain microvessel endothelial cells via regulating FPN1 [67]. Studies have shown that hepcidin binds to FPN1 and induces intracellular iron accumulation via interaction with FPN1 and causes its internalization and degradation in endolysosomes (**Scheme 1**) [66, 68]. It has been identified that residue Cys326 of FPN1 is required for hepcidin interaction and mutation of this residue induces FPN1 resistance to hepcidin binding [68]. Fernandes *et al.* proposed that a thiol subunit of the Cys326 residue initiates a disulfide bond with a cysteine residue in hepcidin [69, 70]. These findings indicate that increased iron accumulation and decreased iron efflux might be caused by the action of hepcidin. In physiological conditions, intercellular iron is mainly stored in ferritin and FPN1 mediates efflux of un-needed or excess iron. During many pathological conditions, including stroke, hepcidin is upregulated and binds to FPN1 and causes its internalization and degradation in endolysosomes. FPN1 degradation leads to iron accumulation in neurons and further results in neuronal death.

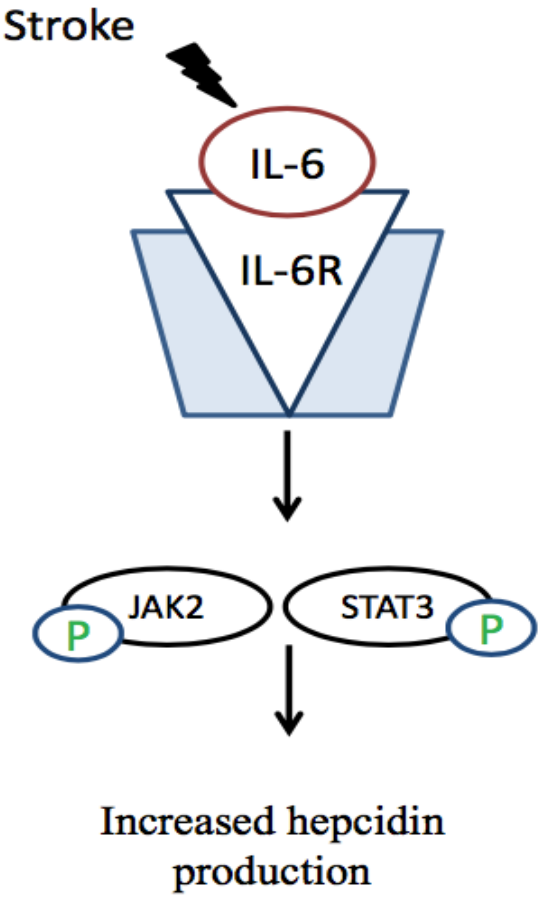


Scheme 1. Mechanism of hepcidin action. Hepcidin increases intracellular iron levels by causing the degradation of ferroportin, a cellular membrane protein that export iron to extracellular space.

Regulation of hepcidin-FPN1 pathway

Hepcidin expression is regulated and modulated at the transcriptional level by many factors, including iron status and inflammation [71]. For example, the inflammatory cytokine interleukin (IL)-6, a major inflammatory mediator, up-regulates hepcidin expression and reduces serum iron levels in humans and experimental animal models [72, 73]. Additional studies have suggested that hepcidin expression is up-regulated by inflammatory cytokines following infection and post-ischemic inflammatory conditions [43, 74]. The mechanisms underlying inflammation-induced hepcidin expression are complex and only partially understood. There is increasing evidence that inflammation positively regulates hepcidin expression, mainly through induction of signal transducer and activator of transcription3 (STAT3) pathway [75]. Upon inflammatory stimuli, interleukin-6 (IL-6) activates Janus Kinase (JAK), a kinase responsible for the STAT3 phosphorylation. Phosphorylated STAT3 (p-STAT3) translocates into the nucleus and binds its response element in the hepcidin promoter, resulting in the stimulation of hepcidin transcription (**Scheme 2**) [73]. At transcriptional levels, knockdown of STAT3 significantly down-regulates endogenous hepcidin mRNA expression, indicating that STAT3 is involved in the transcriptional control of hepcidin [75]. Recent studies show that administration of lipopolysaccharide (LPS), a key inflammatory mediator, increases hepcidin expression [76]. Furthermore, an in-vitro study demonstrated that iron-overloaded microglial cells exhibit increased production of proinflammatory cytokines, suggesting that hepcidin may provide a link between iron accumulation and inflammatory responses in the brain [74, 77]. Furthermore, iron disposition and IL-6 secretion are enhanced in mouse and rat brains following experimental ischemic stroke and ICH [14, 78]. This intracellular iron disposition results from reduced iron export in ischemic brain due to the down-regulated expression of FPN1 by hepcidin [79]. It has also been reported that ICH

models exhibited increased hepcidin and intercellular iron accumulation due to decreased iron efflux [67]. These findings indicate that hepcidin may promote a crucial association between inflammation and iron overload in brain tissues.

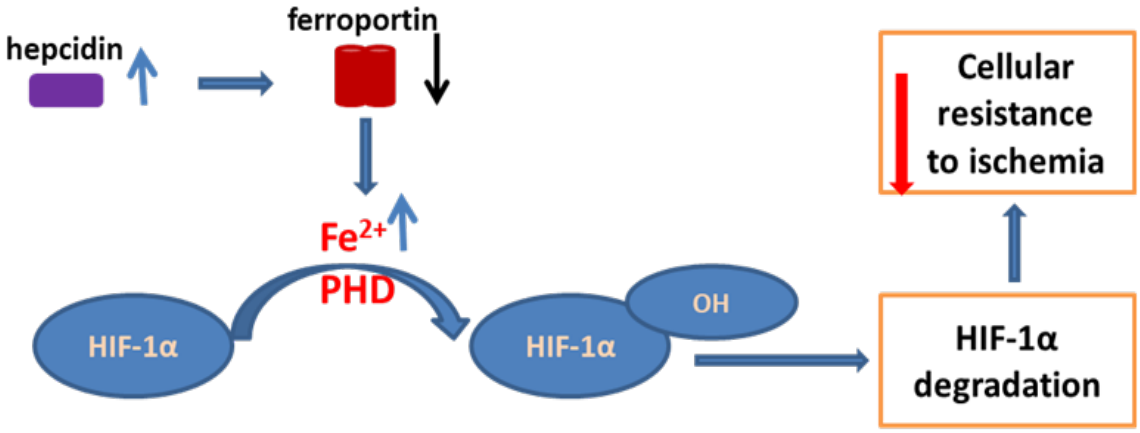


Scheme 2. **Inflammation-induced hepcidin transcription**. Inflammation plays a key role in upregulating hepcidin expression. Stroke initiates the production of inflammatory stimuli. Upon inflammatory stimuli, interleukin (IL)-6 activates Janus Kinase (JAK), a kinase responsible for the STAT3 phosphorylation. Phosphorylated STAT3 (p-STAT3) translocates into the nucleus and binds its response element in the hepcidin promoter, resulting in the stimulation of hepcidin transcription action.

Iron-mediated hypoxia inducible factor 1α (HIF-1α) degradation

HIF-1α has been suggested to be a critical regulator of neurological outcomes following ischemic stroke due to its function as a central role in regulating neuronal adaptive responses to hypoxia. HIF-1 regulates genes that promote energy metabolism, angiogenesis, and erythropoiesis, all of which are critical in promoting cell survival in hypoxia. HIF-1α is a heterodimer, consisting of a constitutively expressed β subunit and a regulated α subunit. HIF-1α is preferentially degraded under normoxic conditions while under hypoxic conditions, it is stabilized, dimerizes with the β subunit, and subsequently induces adaptive gene expression and promotes cell survival. Under normoxic conditions, HIF-1α is degraded through the ubiquitin–proteasome system after recognized by the von Hippel Lindau tumor suppressor (pVHL), a component of the E3 ubiquitin ligase. The recognition by pVHL requires the action of a family of HIF prolyl hydroxylase domain (PHD) that hydroxylates proline residues on HIF-1α in the presence of oxygen. Ferrous ion is a cofactor of the enzyme PHD. Increase in free iron promotes HIF-1α degradation through the 26S pathway by elevating its hydroxylation and thus compromises cellular resistance to ischemic injury (**Scheme 3**). Conversely, reducing iron levels would be lower PHD activity and increase HIF-1α stability/accumulation. In fact, Hishikawa *et al.* have reported that DFO significantly attenuated basilar artery vasospasm and reduction of brainstem blood flow in a rat model of hemorrhagic stroke by increase in HIF-1α protein level and HIF-1α activity [80]. Similarly, in ischemic stroke DFO induced HIF-1 DNA binding and transcription of erythropoietin *in vivo* and ischemic tolerance [81, 82]. More recently, Sorond *et al.* reported that DFO infusion improved cerebrovascular function in older individuals, possibly by increased the activity of HIF-1α-regulated pathways [83]. Although it is arguable if the protective effect of DFO can be all attributed to HIF-1α activity [84], iron reduction could be an effective pharmacological approach

to activate HIF-1α and to enhance cerebrovascular function acute and chronic ischemic conditions. Hepcidin and FPN1 may provide additional targets for increasing HIF-1α levels



Scheme 3. New targets for increasing HIF-1α activity. Iron reduction could be an effective pharmacological approach to activate HIF-1α and to enhance cerebrovascular function acute and chronic ischemic conditions. Hepcidin and ferroportin may provide additional targets for increasing HIF-1α levels.

Post-ischemic and ICH inflammation regulates neuronal damage

Neuro-inflammation following ischemia plays a crucial role in neuronal injury [85]. The inflammatory response may promote cerebral swelling, which is often lethal [86]. Microglial cells, which are the resident macrophages of the brain, modulate neuronal damage by releasing several proinflammatory mediators such as cytokines and chemokines in response to post-ischemic inflammation [87, 88]. It has been reported that the number of activated microglia significantly increases in rat brains subjected to 2 hr-ischemia [89]. Many cytokines, including ILs and tumor necrosis factor-α (TNF-α), are up-regulated in ischemic brains [88, 90, 91]. Additionally, elevated cytokine levels following ischemia exaggerate the infarct severity and induce further neuronal death [88]. Intraventricular injection of recombinant IL-1β worsens the infract severity in ischemic mouse brains when compared with controls [91]. However, IL-1β deficient mice exhibit less neuronal damage in comparison with wild-type [92]. In addition to cytokines, chemokine levels

are elevated in ischemic animal models while their deficiency rescues neurons from ischemic injury [93, 94]. Consistently, mice lacking chemokine receptors exhibit less brain injury after ischemia [95]. Similar to ischemic stroke, microglial activation, cytokines and chemokines release have been reported in ICH [96]. These findings suggest that inflammation may contribute to neuronal injury and expand the brain lesion upon ischemia and ICH. Recent studies have reported that inflammation mediates iron accumulation in CNS cells [74]. As mentioned previously, increased iron deposition has been linked to neuronal damage following ischemia and ICH [8, 67]. These findings indicate that inflammation may drive iron accumulation in cerebral stroke. However, the hypothesis that hepcidin acts as a link between inflammation and iron neurotoxicity requires further investigation.

Effect of anesthesia on iron regulation in stroke

Postoperative cognitive dysfunction (POCD) refers to a decline in cognition after major surgery and anesthesia. Age is one major factor for cognitive complications following surgery [97]. Based on a prospective longitudinal study Monk *et al.* found that stroke is another major independent factor that increases incidence of prolonged POCD [98]. The authors argued that the increased incidence of prolonged POCD may be related to the concept of cognitive reserve because the patients may have lost critical neural mass with their stroke, leading to a decrease in their cognitive reserve and an increased susceptibility to POCD. Alternatively, the latest clinical evidence establishes a positive relationship between general anesthesia and development of POCD in the elderly [99-103]. It has been suggested that anesthetics may promote inflammation, increase oxidative stress, reduce neurotrophin expression, and cholinergic dysfunction, which contribute to POCD. We speculate that iron accumulation might be one contributing factor to anesthesia-mediated POCD due to the following factors. 1) Iron overload has direct links to key components

of POCD symptoms such as in Alzheimer's diseases [104, 105]. 2) It seems that oxidative stress contributes to the progression of POCD, and iron overload can cause oxidative stress. 3) It has been reported that anesthetics could increase the production of inflammation mediators, which can upregulate hepcidin expression, causing iron overload. 4) Preconditioning is to stimulate the endogenous protection mechanism with a mild stress. Sevoflurane and isoflurane preconditioning ameliorates inflammation, cerebral lipid peroxidation, and histologic injury in a rat model of focal cerebral ischemia [106], implying that the prolonged anesthetic exposure could cause adverse effects such as inflammation and lipid peroxidation. Future studies are needed to explore the potential role of iron metabolism in anesthetics-mediated brain dysfunction such as POCD.

Iron as a treatment target

In the last ten years, our understanding of the pathogenesis of stroke-induced brain injury has advanced. However, therapeutic options for cerebral stroke are still very limited. Current approved therapies rely mainly on the use of thrombolytic treatment in acute ischemic cases [5]. Recombinant tissue plasminogen activator (r-tPA) is the only thrombolytic drug that has been approved for clinical use to reduce neurological injury and improve the functional recovery of patients with cerebral ischemia [107]. The time window for effective treatment with r-tPA is limited to three hours after symptom onset. Because the large majority of patients with ischemic stroke are not aware of initial symptoms, many do not seek care within the three-hour time frame. Consequently, due to its short therapeutic window and potential hemorrhagic risk, the number of patients that benefit from this treatment is limited [5]. In term of ICH, several reports have shown that majority of ICH-patients who received anticoagulant and antiplatelet treatments had experienced the incidence of severe anticoagulant-associated ICH [108]. In addition, emerging

evidence demonstrated that antithrombotic drugs such as warfarin increased the risk of ICH by seven folds and is associated disability and death [109, 110]. This major challenge increases the demand for safe and effective therapeutic approaches to improve the outcome of this pathological condition. This section discusses some prospective therapeutic strategies that can protect the brain from the negative consequences of cerebral stroke. The negative consequences of stroke that could be beneficial therapeutic targets, including post-stroke inflammation and brain iron accumulation [8, 90, 96].

Anti-inflammatory treatment approach

As mentioned previously, post-ischemic inflammation is a large effector in the pathology of cerebral stroke [86, 90, 96]. Therefore, targeting inflammatory pathways can be a protective therapeutic strategy to reduce or limit brain injury after the stroke [5]. Many anti-inflammatory molecules have been tested as neuroprotective agents in acute ischemic stroke. Although most of these agents have failed to restore neurological functions in patients with ischemic stroke, blocking post-ischemic inflammation may improve pro-regenerative effects following ischemia. IL-10 and transforming growth factor beta (TGF-β) are major anti-inflammatory mediators in ischemic and ICH brain injury [86, 111, 112]]. Many studies have been conducted to determine the beneficial effect of these anti-inflammatory mediators in ischemic stroke treatment. Ooboshi *et al.* reported that overexpression of IL-10 protects neurons against focal and global brain ischemia. Their results also found that IL-10-treated ischemic brains exhibit less brain infarction and immune cell infiltrations in comparison with controls [113]. Recently, Cekanaviciute *et al.* demonstrated that TGF-β inhibits subacute neuroinflammation and preserves brain function in the peri-infarct cortex after ischemic stroke [114]. Clinically, minocycline is one of the most anti-inflammatory drugs that have been tested in treatment of human acute ischemic stroke and ICH [5, 115]. It has been

found that minocycline improves the prognosis of patients or animals with stroke compared with placebo treatment [116, 117]. Although the exact mechanism as well as the onset effect of these anti-inflammatory molecules in ischemia treatment remain to be clarified, controlling post-ischemic inflammatory pathway shows a promising potential target to treat ischemic stroke [86]. The next section discusses whether iron-chelating agents could be a neuroprotective tool against inflammation-induced iron neurotoxicity in ischemic stroke.

Iron-chelating therapy

Considerable evidence suggests that iron-mediated neurotoxicity plays a critical role in the pathogenesis of ischemic and ICH stroke [8, 10]. Additionally, it has been found that iron accumulation in the brain is induced by post-ischemic and ICH inflammation [14, 116]. Based on these notions, many studies have examined whether decreasing iron overload in the brain during ischemia decreases neurological defects and improves the outcome of ischemic stroke. Iron chelators bind excess iron in a stable complex to prevent iron accumulation and maintain a safe iron status [118]. In fact, DFO reduces ischemic and ICH stroke damage in rodent models [119, 120]. Additionally, DFO significantly decreases cortical infarct volume and improves neurologic deficits following ischemia in rat brains [40]. Furthermore, these neuroprotective effects of DFO are mediated by suppression of iron-induced free radical formation in animal models after ischemic stroke [41]. As discussed previously in this review, iron accumulation, FPN1 reduction and hepcidin up-regulation are all induced during ischemia-induced neuroinflammation [14, 74]. DFO dramatically blocks increased iron levels and reduces FPN1 down-regulation in LPS-treated neurons[121]. These findings suggest that DFO may also attenuate neuroinflammation-induced iron accumulation in neurons upon stroke via regulation of hepcidin pathway.

Targeting hepcidin pathway in ischemic and ICH stroke

Hepcidin, a negative regulator of the sole cellular iron exporter FPN1, plays a crucial role in intracellular iron accumulation [66, 68]. Therefore, therapeutic approaches that decrease hepcidin synthesis and/or increase FPN1 activity seem to be potential candidates to treat patient with iron overload disorders [122]. Recent studies have showed that hepcidin siRNA treatment induces the FPN1 expression and rescues iron accumulation in cerebral cortex, hippocampus, and corpus striatum of ischemic mouse brains [14]. In addition, hepcidin knockout mice ICH models exhibit reduced brain iron accumulation compared with wild type mice [67].These findings suggest that iron overload following ischemia and ICH is negatively correlated with increased FPN1 levels in hepcidin knockout models. Additionally, alteration of hepcidin-FPN1 interaction with a thiol-reactive compound fursultiamine prevents FPN1 internalization and degradation via sequestering the Cys326 residue of FPN1, a necessary residue for hepcidin binding to FPN1 [122]. Consequently, fursultiamine reduces intracellular iron accumulation via enhanced cellular iron export despite the presence of hepcidin [123]. As discussed previously, activation of IL6/STAT3 pathway induces hepcidin transcription [14]. Therefore, targeting IL6/STAT3 pathway with anti-IL6 antibodies can down-regulate hepcidin expression. Siltuximab and tocilizumab, anti-IL6 antibody drugs, are in clinical use to reduce hepcidin effects and improve cellular iron export in patients with anemia [122]. Recent studies have shown that anti-IL6 antibody significantly decreases the expression of IL-6 protein in the brain of ischemic animal models [124]. Furthermore, anti-IL6 antibody rescues the blood-brain barrier from ischemic injury in the fetal sheep model [125]. In addition, urocortin, anti-inflammatory neuropeptide, suppresses inflammatory cytokine production (e.g. IL-6 and TNF-α) and reduces neurological deficits

following ICH [126]. Taken together, targeting hepcidin and/or corresponding pathways could be an effective therapeutic approach to protect the brain from stroke injury.

Conclusion and future perspectives

Inflammation and iron accumulation play a crucial role in neuronal injuries caused by ischemic stroke. Hepcidin binds FPN1 and reduces its activity in excreting excess iron from the brain cells. The major factors that positively regulate hepcidin include iron status and inflammation. Modifying iron metabolism and post-stroke inflammation can reduce brain injury in stroke and the hepcidin pathway seems to be a promising molecular target for stroke therapy.

Increased hepcidin expression has emerged as a causative factor in several common iron disorders such as cerebral stroke. Targeting hepcidin pathways and/or interfering hepcidin-FPN1 interaction have become promising approaches for development of novel therapeutics for iron disorders. However, multiple potential opportunities for cross communication among these pathways can cause off-target effects such as an increased risk of infection. For example, IL-6 receptor-targeted therapeutics impair host defense. Moreover, interfering hepcidin-FPN1 interaction by fursultiamine antagonizes hepcidin effects rather than reducing its expression. Therefore, lowering hepcidin expression directly in cerebral stroke where it is elevated may have less possible off-target effects in comparison with other therapeutic approaches mentioned previously. In addition, the direct targeting of hepcidin in stroke models would help us to understand the specific role of hepcidin in stroke-induced brain injury. The major challenge of systemic administration of anti-hepcidin is that some negative effects need to be taken into account. Deficiency of systemic hepcidin results in increased serum iron, dietary iron hyperabsorption, and iron-dependent oxidative stress. To overcome this challenge, anti-hepcidin

could be delivered into the stroke-injured brain by using microinjection or nanoparticles as drug delivery systems. In the following, some experimental approaches are proposed in order to refine our understanding of the mechanistic role of hepcidin in cerebral stroke.

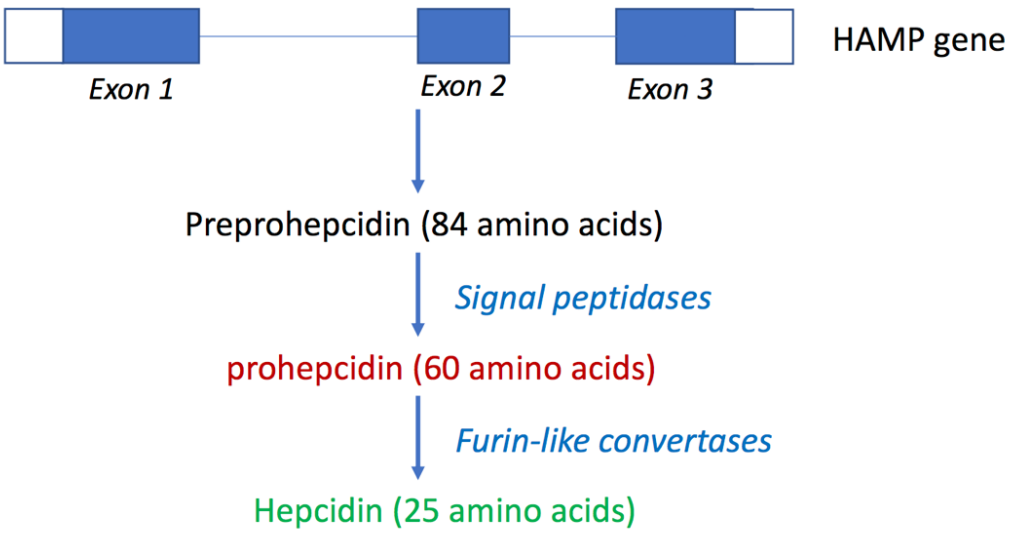
In summary, these future directions may improve our understating of the roles of hepcidin in iron-mediated neurotoxicity following cerebral stroke. Based on the results, novel therapeutic interventions might be developed to protect the brain from stroke injury.

Literature Review

Hepcidin Manipulation and Ischemia-Related Signaling Pathways

Hepcidin: Synthesis and activity

Hepcidin is encoded by the hepcidin antimicrobial peptide (HAMP) gene and synthesized primarily by the liver as an 84 amino acid preprohepcidin. In addition to the hepatocytes, macrophages and adipocytes produce hepcidin in a small quantity [127, 128]. Through enzymatic cleavage at N-terminal sequence, preprohepcidin is cleaved by signal peptidases into 60 amino acid prohepcidin that is amino-terminally processed to the mature 25 amino acid active peptide via furin-like convertases (**Scheme 4**) [55, 129-131]. NMR and Mass spectrometry studies demonstrated that the structure of hepcidin-25 contains eight cysteines connected by four disulfide bonds. Hepcidin-25 has shown antimicrobial and iron-regulatory activities [132]. Kulaksiz et al reported that hepcidin can be cleared by cellular co-degradation in lysosomes with FPN1 and by renal excretion. Due to low molecular weight of hepcidin, unbound form can pass the glomerular filtration and excrete in urine [133]. Recent studies have shown that hepcidin is directly and negatively correlated with creatinine and glomerular filtration rate, respectively. It has been demonstrated that hepcidin levels increases in patients with chronic kidney disease. This leads to increase serum iron and ferritin in these patients. [132, 134].



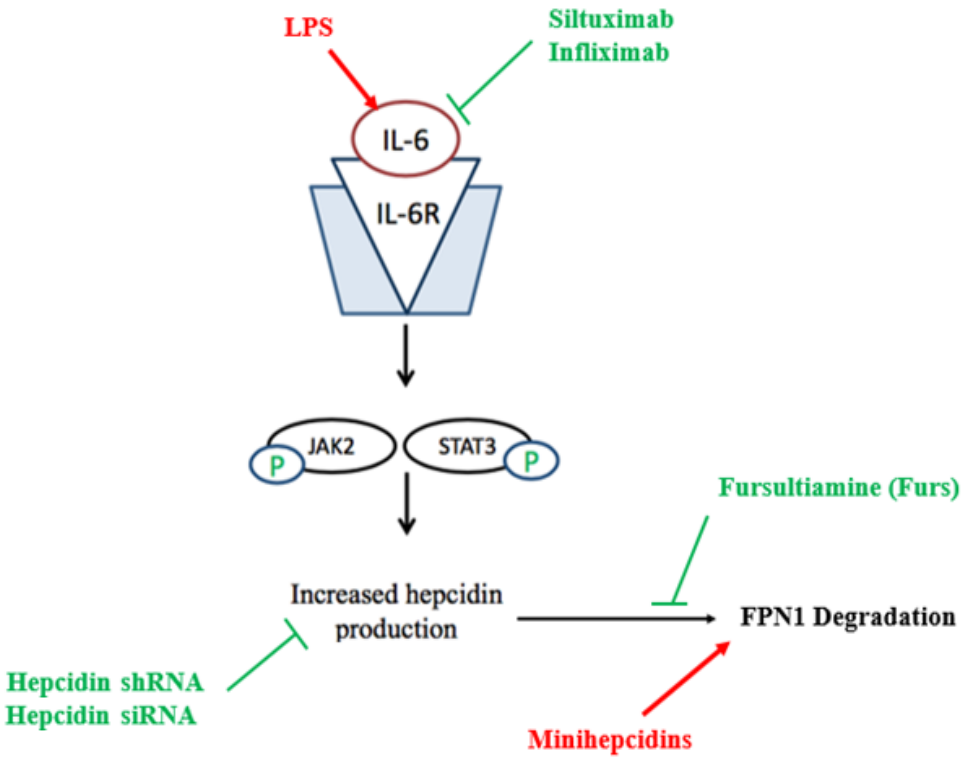
Scheme 4. Overview of hepcidin synthesis. Hepcidin is produced from preprohepcidin. Preprohepcidin is cleaved to prohepcidin via signal peptidases. Furin-like convertases cleaves prohepcidin to the mature bioactive form of hepcidin (hepcidin 25).

Analytical methods for hepcidin quantification

Many techniques have been used to measure or assess hepcidin-25. These approaches include mass spectrometry (MS), enzyme-linked immunosorbent assay (ELISA), immunocytochemistry, and western blotting analysis. MS has been used to distinguish between hepcidin isoforms. Although there are some concerns about the ELISA selectivity, it was sensitive enough to measure hepcidin-25 in serum and urine [133, 135]. Because hepcidin-25 has a compact structure with 4 disulfide bonds, western blotting analysis was not an ideal and successful approach to analyze hepcidin [135, 136]. In addition, immunohistochemistry, immunocytochemistry, and real-time PCR have been commonly used to evaluate hepcidin levels in various tissues [14, 137, 138].

Hepcidin manipulation

Since its discovery, many research groups have pursued to understand how hepcidin synthesis is regulated. Both positive and negative regulators have been investigated. Hepcidin positive regulators or agonists have been used to induce hepcidin transcription or action. Activating hepcidin upstream signaling pathways such as inflammation induces hepcidin transcription. Several peptides such as minihepcidins have been synthesized to mimic biological actions of endogenous hepcidin. Hepcidin negative regulators or antagonists inhibit hepcidin production through blocking the signaling pathways that induce hepcidin transcription. In addition, modulating hepcidin gene expression with antisense oligonucleotides is commonly used to specifically target hepcidin. Moreover, blocking hepcidin binding site at its receptor, FPN1, has been used to prevent the action of hepcidin (**Scheme 5**)[139].



Scheme 5. Hepcidin manipulation via positive and negative regulators. LPS induces hepcidin transcription through activation inflammation mediators, such as IL-6. Minihepcidin mimics hepcidin action and induces FPN1 degradation. Anti-inflammatory cytokines (e.g. Siltuximab and infliximab), hepcidin shRNA, siRNA, and Fursultiamine inhibit hepcidin transcription or prevent hepcidin action [139].

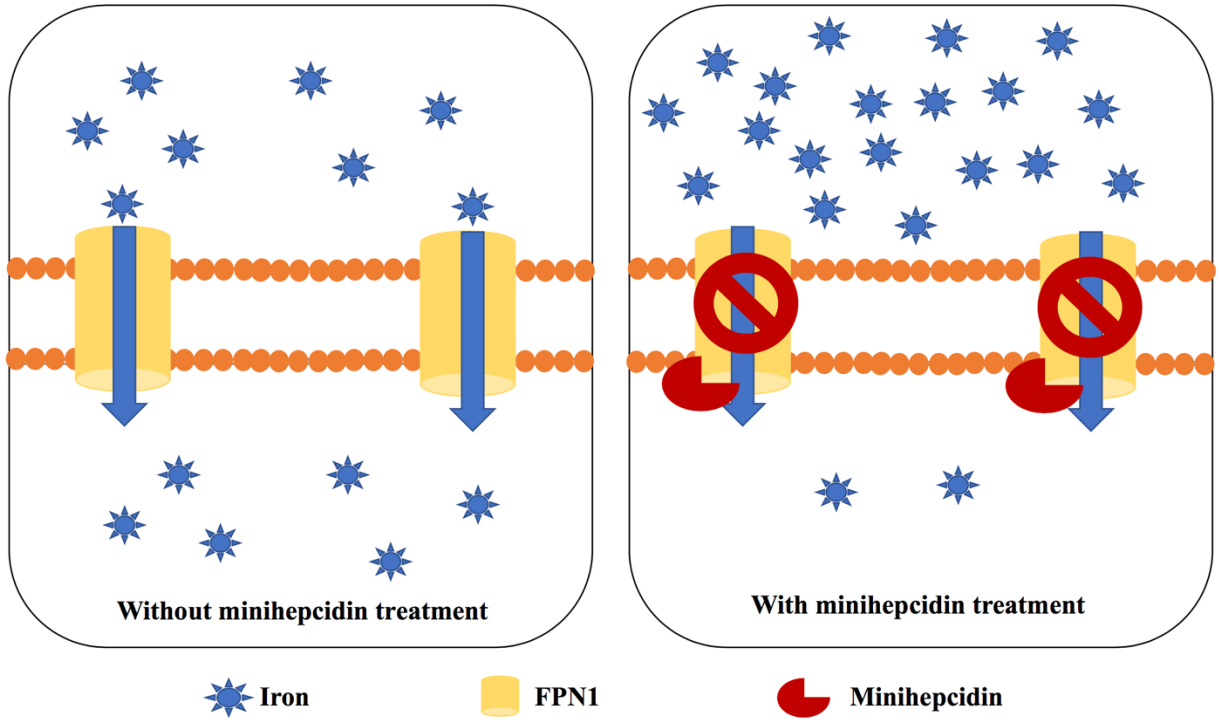
Positive regulation

Lipopolysaccharide

Lipopolysaccharide (LPS) induces hepcidin transcription due to increase in IL-6 levels. IL-6 activates its receptor that leads to JAK2/STAT3 activation. Activated STAT3 binds to response element in the hepcidin promotor and then stimulates the hepcidin production [75, 140].

Hepcidin mimics: Minihepcidins

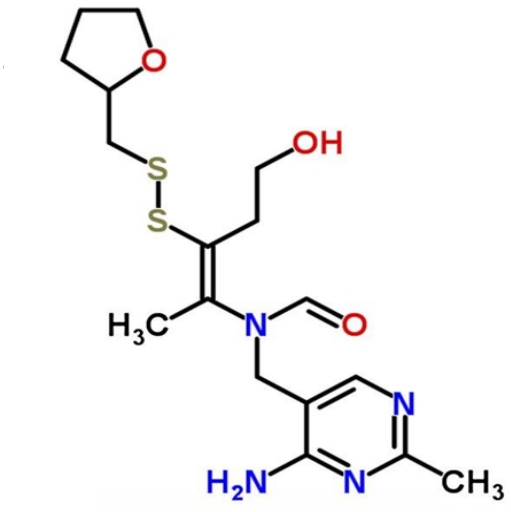
Natural hepcidin is not suitable for therapeutic use because it has a short half-life (several minutes). The complicated disulfide bond connectivity and the high cost make the synthesis of bioactive peptide in its normal form difficult. Due to this reason, Scientists have successfully synthesized hepcidin mimics, which were rationally designed based on the hepcidin structure. The common candidates for these hepcidin mimics are Minihepcidins [141]. Minihepcidins are small peptides mimic biological activity of the hepcidin (**scheme 6**). According to mutagenesis and truncations studies, the N-terminal fragment of hepcidin (9 amino acids) is critical for hepcidin's activity, as demonstrated by the induction of FPN1 degradation. According to a cellular bioassay study that tested varying lengths of minihepcidins on FPN1-GFP degradation, 9 N-terminal residues minihepcidin is more active than shorter ones[142]. Minihepcidins have long half-life and similar potency when compared to the natural hepcidin [142, 143].



Scheme 6. Minihepcidin action. Minihepcidin, synthesized polypeptide, mimics the action of natural hepcidin. It binds to FPN1 and induces its internalization and degradation, and thus inhibiting iron efflux [144].

Negative regulation

Short interfering and hairpin RNAs (siRNA and shRNA), commonly used for hepcidin gene silencing, have been shown to potentially induce hepcidin mRNA reduction. In addition, blocking the upstream pathways that are involved in hepcidin transcription, such as the IL-6/STAT3 pathway, decreases hepcidin production [141]. Recently, high-throughput screening of small hepcidin antagonists identified a Food and Drug Administration (FDA) approved thiamine derivative, Fursultiamine (Furs), that can antagonize the hepcidin effect on FPN1 (**Scheme 7**). Furs directly binds to hepcidin binding site (Cysteine 326 thiol residue) in FPN1 and prevents hepcidin-induced FPN1 internalization and degradation in lysosomes [123].



Scheme 7. Fursultiamine structure. A thiamine tetrahydrofurfuryl disulfide derivative has been identified as a unique and potent hepcidin antagonist. This drug irreversibly binds to Cysteine 326 thiol reside in FPN1[123].

Hepcidin in ischemic stroke

Hepcidin is widely expressed in murine brain and it has been shown that it plays an important role in regulating brain iron homeostasis through downregulating FPN1, the only well-known iron exporter [61, 145]. Our laboratory and collaborators have reported that FPN1 protein expression was downregulated in ischemic brains of mice and its downregulation was associated with an increased hepcidin mRNA level [14]. Hepcidin is upregulated in ischemic stroke, which further leads to accumulation of iron in brain tissues. It has been known that iron is involved in oxidative stress via acting as cofactor in ROS signaling pathways. A study showed that excessive superoxide induces release of iron from ferritin. In addition, generating highly reactive hydroxyl radicals from the Fenton reaction is facilitated by iron [33]. However, the exact role of hepcidin in

ischemic stroke has not been fully understood, therefore, more research needs to be done to gain potential information about the role of hepcidin in ischemic stroke.

ROS and Oxidative stress in ischemic stroke

Reactive oxygen species (ROS) are reactive oxygen-containing molecules that can damage macromolecules such as proteins and lipids. Although ROS play an important role in physiological processes including inflammatory response and vascular tone regulation, highly production of ROS can cause irreversible alternations or destruction, which lead to apoptosis and necrosis to the tissues or cells. Several studies have shown that ROS production increases in ischemic conditions and contributes to ischemic damage. Upon ischemia, many ROS molecules such as superoxide, peroxide, and hydroxyl radical were detected [146, 147]. In addition, reperfusion has been known to cause the second phase of ischemia/reperfusion injury and ROS formation is considered a major player in this phase [148-150]. There are many sources of ROS in ischemic stroke including the mitochondrial electron transport chain, NADPH oxidase (NOX), and xanthine oxidase (XO) [147, 151-153].

Following ischemic stroke, the limited supply of oxygen and glucose to the infract area leads to energy loss, resulting in subsequent membrane depolarization. This membranes depolarization increases intracellular calcium levels and induces glutamate release, leading to activate glutamate receptors [154, 155]. Activation of glutamate receptors contributes to excessive increased calcium, which causes proteolysis, lipolysis, increased nitric oxide synthase (NOS) activity, and mitochondrial damage. It has been found that increased NOS activity and mitochondrial damage induce caspase activation, inflammatory mediators release, and oxidative damage. Molecular oxygen reduction to H2O is considered a major pathway underlying ROS

generation. Dioxygen molecule reduces to produce superoxide anion radical (O^{-}_{2}) and hydrogen peroxide (H_2O_2). H_2O_2 can involve in the Fenton reaction in presence of Fe^{2+} transition metal. The Fenton reaction generates hydroxyl radical, which is the most toxic radical that causes oxidative damage and cellular death [155].

Emerging evidence shows excessive ROS formation causes severe damage during ischemia and cannot be balanced by endogenous antioxidant system. Cerebral reperfusion following ischemia-induced ROS production results in cytotoxicity via lipid peroxidation, DNA damage, and protein oxidation. In addition, this cellular injury impacts cell functions including cell transport, energy production, and ion balance [156].

Redox Reactions in Ischemic Stroke

Oxidation and reduction(redox) reactions occurs when electrons are transferred between two molecular species via chemical reactions that are facilitated by enzymes. The balance between oxidized molecules (lose electrons) and reduced molecules (gain electrons) in the cellular environment is known as the redox status. These reactions play a critical role in physiological and pathological conditions of biological organs. Redox reactions are involved in cell cycle regulation, cell growth, apoptosis, genes expression, and aging [157, 158].

At physiological conditions, endogenous antioxidants protect cells from the harmful effects of ROS via counteracting the production of ROS. However, excessive production of ROS or deficiency of antioxidants lead to alterations of redox status which initiate oxidative stress. Glutathione and thioredoxin systems are the predominant players that are involved in maintaining redox balance in the brain tissues. These systems act as disulfide reductase systems that detoxify the increase in ROS levels. The thioredoxin system consists of thioredoxin (Trx), thioredoxin

reductase (TrxR), and NADPH. The glutathione system consists of glutathione (GSH), glutathione reductase (GR), and NADPH.

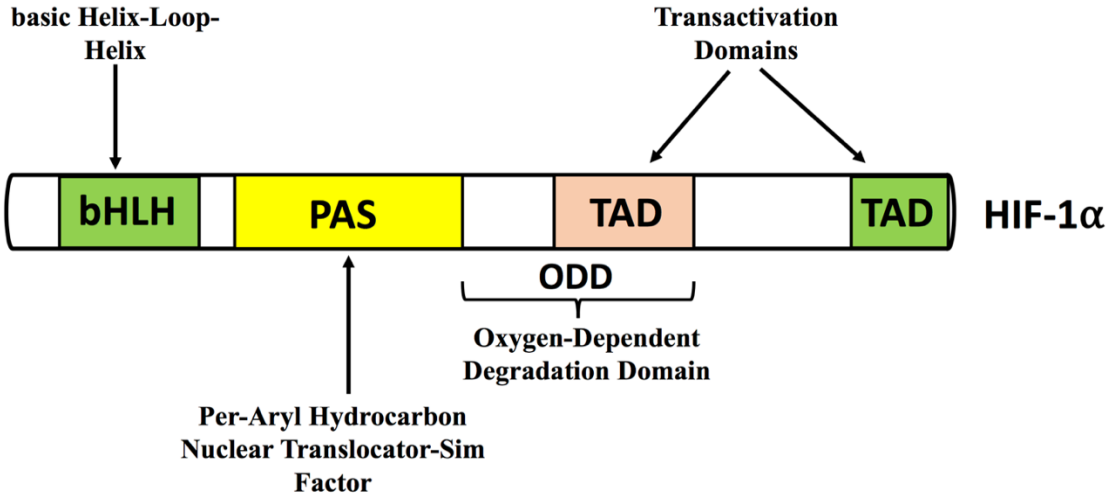
Trx and GSH antioxidants are expressed in neuronal and glial cells in the brain to control redox signaling. It has been shown that these antioxidant systems work together to fight against oxidant injuries. Interestingly, metabolites of one system are consumed by another system. For instance, NADPH is converted into $NADP^+$ via Trx redox cycling, and further this $NADP^+$ can be consumed by GSH redox cycling. The Trx and GSH are considered as H_2O_2 scavengers. Antioxidant enzymes such as superoxide dismutase play a critical role in conversion of superoxide radical to H_2O_2 which can be removed by redox systems. Briefly, the NADPH/ $NADP^+$ act as electron donor in the Trx and GSH systems which can scavenge H_2O_2 via glutathione peroxidase (GPX) and peroxiredoxin (Prx) enzymes. GPX reduces H_2O_2 into H_2O, in which reduced GSH is catalyzed to glutathione disulfide (GSSG). GR reduces GSSG to GSH, in which $NADP^+$ is generated from NADPH consumption [159].

Several studies showed that SOD protected ischemic brain following transient cerebral ischemia, which subsequently reduced or delayed the ischemia-induced neuronal death. In SOD-deficient mice, the mortality rate was increased following ischemic reperfusion conditions [160-162]. Recent studies have suggested that massive increased ROS levels induce neuronal GSH depletion and oxidative stress in ischemic brains and restoration of GSH can rescue neurons from oxidative stress [163]. The protective effect of Trx following cerebral ischemia has been well investigated. Administration of Trx reduces ischemic insult in animal models[164]. Taken together, redox-regulating activity attenuates ischemia-induced oxidative stress.

HIF-1α in Ischemic Stroke

HIF-1 is a heterodimeric transcription factor which is an important regulator in hypoxia. Structurally, HIF-1α has many motifs including basic helix-loop-helix(bHLH), per-aryl hydrocarbon nuclear translocator-sim factor (PAS), two transactivation domains (TAD), and oxygen-dependent degradation domain (ODD) (**Scheme 8**). bHLH and PAS domains play a critical role in creating a functional interface for protein-protein dimerization and DNA binding. The N-terminal TAD domain overlaps with ODD and together they are target for HIF-1α degradation. The C-terminal TAD contributes to HIF-1α activation via interacting with cysteine/histidine-rich region of the coactivator proteins in a hypoxic condition[165-167]. Numerous studies have showed that the role of HIF-1α in ischemic stroke. It is involved in glucose metabolism, angiogenesis, erythropoiesis, and cell survival. Previously, our lab and collaborators showed that HIF-1α expression was increased in different ischemic brain regions, including cerebral cortex, hippocampus, and corpus striatum. This activation of HIF-1α occurred in the early ischemia to promote its neuroprotection via inducing new blood vessels generation and providing new oxygen supply in the ischemic areas [14]. Emerging evidence has revealed that HIF-1α is rapidly degraded in reperfusion via the ubiquitin-dependent proteasomal process. Although the exact mechanism of HIF-1α degradation is not fully understood, extensive studies have demonstrated that activation of HIF-1α hydroxylation in normoixa via oxygen-dependent prolylhydroxlases (PDH) is the key mechanism underlying HIF-1α degradation. Hydroxylated HIF-1α can be easily recognized by von Hippel-Lindau tumor suppressor (pVHL) and further undergoing degradation by proteasomal system [168]. Genetic inhibition of HIF-1α with siRNA significantly exaggerated cell injury induced by ischemic conditions, suggesting the protective effects of HIF-1α against ischemic injury [169]. Emerging evidence showed that Heat Shock

Proteins (HSPs) particularly HSP90 can regulate HIF-1α stability and degradation. The next section will briefly discuss the role of HSP90 on HIF-1α regulation [170, 171].



Scheme 8. HIF-1α protein structure. HIF-1α protein contains different domains that regulate its stability. bHLH-PAS domains are necessary for heterodimer formation between HIF-1α and other proteins and for DNA binding. The N-terminal TAD domain overlaps with ODD and are a target for PHD-induced HIF-1α degradation. The C-terminal TAD domain locates at the C-terminus is important for transcriptional activation[166, 167].

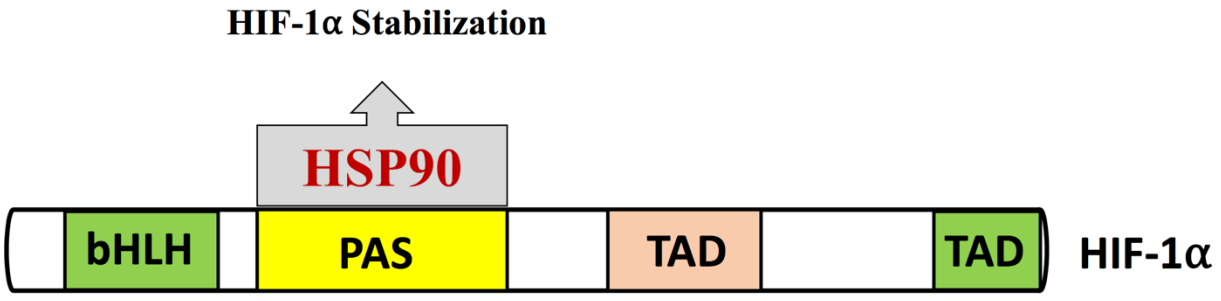
Heat Shock Proteins (HSPs)

Heat shock proteins (HSPs) are molecular chaperones that play an important role in the folding of newly synthesized proteins. They are induced under stress conditions and contribute to cytoprotection by stabilizing misfolded proteins and preventing their improper aggregation, allowing cells to repair their damaged proteins. Many studies have revealed that HSPs have a critical role in numerous pathological diseases, including neurodegenerative diseases and cancer. Based on their molecular weight, HSPs are classified into families, including HSP100, HSP90, HSP70, HSP60, and other small heat shock proteins. HSP90 is one of the most abundant HSP members in the eukaryotic cells. HSP90 interacts with its client proteins in different conformations. Interestingly, the role of HSP90 in regulation of numerous cellular proteins

becomes a promising therapeutic target for different pathological conditions. One of these proteins is HIF-1[170-172].

HSP90 - HIF-1α Interaction

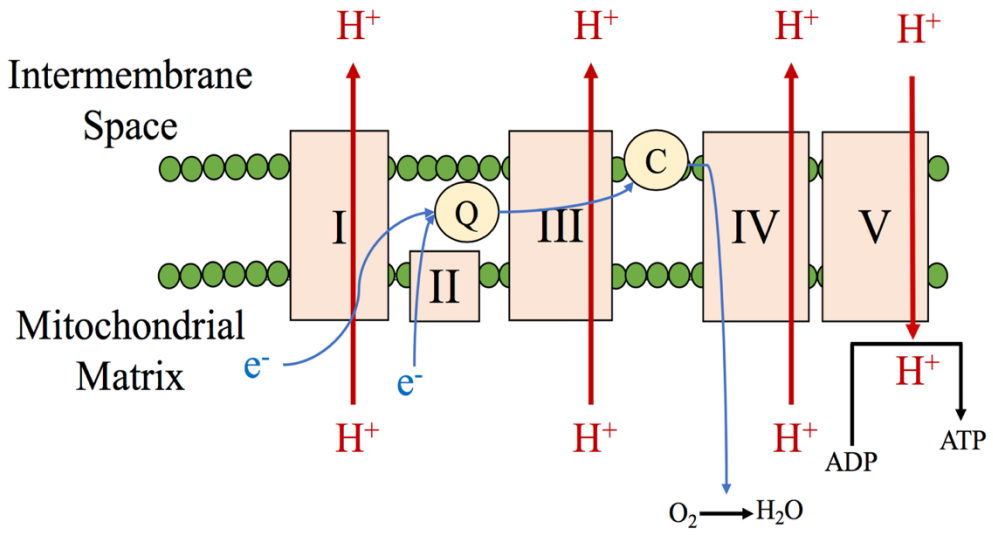
It has been known that HSP90 interacts with HIF-1α and induces its stability. However, the exact mechanism of interaction has not been fully understood. An in vitro study revealed that HSP90 binds to the PAS domain of HIF-1α [173]. Extensive studies have shown that pharmacological inhibition of HSP90 with Geldanamycin (GA) or 17-allylaminogeldanamycin suppresses HSP90 activity and induces HIF-1α degradation. In addition, inhibition of HSP90 promotes anti-angiogenic activity and reduces cell viability via inhibiting HIF-1α stabilization. Receptor of activated protein C kinase 1 (RACK1) induces HIF-1α degradation via competing with HSP90 for binding to the PAS domain of HIF-1α domain [174, 175]. HSP90 mutation decreases its binding affinity to HIF-1α [174, 176]. Therefore, HSP90 bound to PAS domain of HIF-1α plays a critical role in preventing ubiquitination and proteasomal degradation of HIF-1α [174, 177, 178]. The following scheme shows a representation of HSP90- HIF-1α interaction (**Scheme 9**).



Scheme 9. HSP90- HIF-1α interaction. HSP90 interacts with the HIF-1α PAS domain and induces HIF-1α stabilization [178].

Mitochondrial Bioenergetics

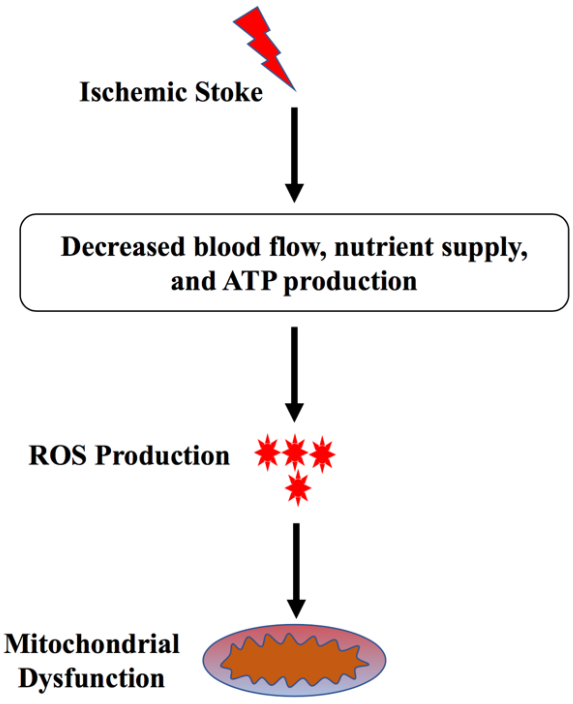
Mitochondria are widely distributed intracellular organelles that maintain cellular homeostasis via ATP synthesis-induced energy generation [179]. Morphologically, mitochondria primarily have two membranes, the outer and the inner membrane. The outer membrane consists of a phospholipid bilayer that is permeable to small molecules (*up to 10 kDa*), such as ions, water, and adenosine di- and triphosphate (ADP & ATP). The inner membrane of mitochondria is considered a reactive center of energy metabolism. Most importantly, this inner reactive mitochondrial membrane contains intramembrane channels and electron transport chain (ETC). The intramembrane channels play an important role in regulation of the molecules transport through the membrane. While ETC regulates and maintains the ATP synthetase via oxidative phosphorylation that takes place in the mitochondrial inner membrane through specialized multi-enzyme complexes (complexes I-V). These complexes are well characterized and include nicotinamide adenine dinucleotide (NADH) ubiquinone oxidoreductase (complex I), succinate dehydrogenase (complex II), cytochrome c reductase (complex III), cytochrome c oxidase (complex IV), and ATP synthase (complex V). Briefly, the mitochondrial respiration begins when complex I oxidizes NADH, which is generated from Krebs cycle, and uses electrons to reduce ubiquinone to ubiquinol. Ubiquinol receives electrons from complex II and then transfers these electrons to Coenzyme Q (Q), which further passes the electrons to complex III. Complex III passes electrons to complex IV where the electrons are used to reduce O_2 into H_2O. Hydrogen ions (protons) can be moved into the mitochondrial matrix via the free energy from the electron transfer. In complex V, the protons gradient that is generated from complexes couple electron transport with proton pumping is utilized by ATP synthase to generate ATP through ADP phosphorylation [179-183] **(Scheme 10)**.



Scheme 10. The complexes of the Electron Transport Chain (ETC). Electrons generated from substrates reduction enter complex I or II and are then transferred to Coenzyme Q (Q). The electrons are passed from Q to complex III which are further transferred to cytochrome c (C) and then to complex IV where the electrons are used to reduce O_2 into H_2O. Protons (H^+) re-enter to mitochondrial matrix via complex V. The protons gradient through matrix can be used by ATP synthase to generate ATP from ADP [183].

Mitochondrial Dysfunction in Ischemic Stroke

Ischemic stroke is characterized by lack of oxygen and nutrient supply (e.g. glucose). Following cerebral ischemia, ROS production is increased from many sources including the main source which is mitochondria. When the endogenous antioxidant system cannot rebalance the ROS levels, excessive oxidative stress induces superoxide anion (O_2^-) production, which is considered one of the highly reactive metabolites. O_2^- reacts with nitric oxide (NO) to produce peroxynitrite anion, which is able to be protonated to highly cytotoxic peroxynitrous acid. In addition, peroxynitrite is considered as an effective precursor to hydroxyl radical, which reacts with biological molecules, such as DNA, lipids, and proteins causing toxic alternations of these molecules. The consequences of biological molecules alternations include protein misfolding, protein aggregation, and mitochondrial dysfunction [162, 180, 184] **(Scheme 11)**.



Scheme 11. Ischemia-induced mitochondrial dysfunction. Ischemic stroke results in lack of blood, oxygen, and nutrient supply to an infarct region of the brain. Excessive ROS production induces mitochondrial fragmentation and dysfunction, and cell death[180].

Rationale and Significance

Stroke is a leading cause of death and disability in the United States and worldwide. Each year, 780,000 new and recurrent strokes occur in the United States, resulting in major social and economic consequences. The majority of all stroke cases (85%) are ischemic. There has been very limited progress in developing effective therapeutic approaches for ischemic stroke, although numerous agents have been tested in animal models and in clinical trials. Among many other issues, an incomplete understanding of the mechanism responsible for ischemia-induced neuronal injury is a major limitation [1].

Although iron plays important roles in mammalian metabolism and is essential for life, labile iron is highly toxic in the body. It has been clearly demonstrated that ischemia elevates labile iron levels in the brain. This elevation is a major damaging event in ischemic neurons and plays an important role in the evolution of a penumbra to infarct and subsequent worsening of the outcome after stroke. Additionally, post-ischemic reperfusion could cause a biochemical deterioration of ischemic brain tissue. It has been found that many vulnerable neurons undergo apoptosis during reperfusion [8].

Hepcidin has been recognized as the central regulator of iron homeostasis by its interaction with FPN1, the sole known cellular iron exporter, which leads FPN1 internalization and degradation. Because it was discovered and is primarily produced in the liver, research on hepcidin over the last decade has predominantly been limited to hepatocytes and liver tissue. Recently, hepcidin was found to be widely expressed in the brain. The mRNA level of hepcidin increased in the brains of rats subjected to ischemia. Moreover, degradation of FPN1 resulted in declined iron release and ultimately iron accumulation in these regions of the brain. These findings indicate that hepcidin mediates iron neurotoxicity in the ischemic brain by diminishing iron export [14].

HIF-1α is a master regulator in hypoxia. HIF-1α mediates angiogenesis, cell proliferation, and energy metabolism. Furthermore, induction of HIF-1α provides neuroprotection in ischemic stroke [168]. The regulating mechanism of HIF-1α has not been fully understood in ischemic brain cells. HSP90, a molecular chaperone that protects its client proteins from misfolding and degradation, binds with HIF-1α PAS domain and promotes the stabilization of HIF-1α. Recent reports show ROS induce HSP90 cleavage and loss of its chaperoning function [173, 185]. Recently, we have obtained key data to demonstrate that hepcidin induces ROS in human neuroblastoma cell line (SH-SY5Y) that were subjected to 2 h ischemic conditions and 24 h reperfusion when compared with control. *We postulate that Hepcidin induces ischemia-induced cell death by modulating HSP90/ HIF-1α pathway.*

To test the hypothesis that hepcidin mediates neuronal death upon ischemia via inducing ROS-induced HSP90/ HIF-1α degradation, the shRNA technique was performed to inhibit hepcidin expression in SH-SY5Y cells. Minihepcidin, a small peptide that mimics biological activity of the endogenous hepcidin, was utilized to study the role of hepcidin in ischemic stroke. To confirm the mechanistic role of hepcidin in ischemic stroke. SH-SY5Y cells treated with minihepcidin or transfected with hepcidin shRNA were exposed to 2 h *in vitro* ischemic conditions (oxygen-glucose deprivation (OGD)) in a hypoxic chamber and then 24 h reperfusion. ROS levels were examined by the electron paramagnetic resonance (EPR) spectroscopy. A standard protocol for western blotting and immunostaining was followed and primary antibodies against hepcidin, HSP90 and HIF-1α were used to examine the expression of these proteins. Neuronal death was evaluated by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) methods.

The data generated from these studies provided substantial key information regarding the mechanistic role of hepcidin in contributing to neuronal death following ischemic stroke. Based

on the results, novel therapeutic interventions might be developed to protect the brain from ischemic injury.

Materials and Reagents

Materials

The following table shows the core materials, reagents, and antibodies used in this dissertation.

Name	Company	Catalog Number
Poly-D-lysine hydrobromide	Sigma-Aldrich	P4832
Sodium Pyruvate	Gibco	10335
Glucose	Alfa Aesar	A15409
Glutamate	Sigma-Aldrich	176141
Fluorescein isothiocyanate–dextran	Sigma-Aldrich	FD40S
XF Assay Medium Modified DMEM	Seahorse Bioscience	102365-100
Oligomycin	Sigma-Aldrich	75351
Carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP)	Sigma-Aldrich	C2920
Rotenone	Sigma-Aldrich	R8875
Antimycin A	Sigma-Aldrich	A8674

CMH. hydrochloride	Enzo Life Science	ALX-430-117-M010
FPN1 Antibody	Novus Biologicals	NBP1-21502
HIF-1α Antibody	Novus Biologicals	NB100-131
Actin Antibody	Santa Cruz Biotechnology	sc-1616
Hepcidin-25	Abcam	ab75883
HSP90 antibody	Abcam	ab109248
HBSS	Worthington	P3C14111
Trypsin-EDTA	Gibco	25200-056
Protease inhibitor	ThermoFisher Scientific	1858566

Table 1: chemicals, reagents, and antibodies. A list of commercially available chemicals, buffers, solutions, reagents, and primary antibodies used in this dissertation.

Methods

Research Models

Primary Cortical Neurons

The University of Kansas Institutional Animal Care and Use Committee approved animal use. Primary neurons were prepared from the cortex of post-natal day 0 to 1 (P0-P1) brains that isolated from Sprague-Dawley rat (Charles River Laboratories). The cortical tissues were washed with HBSS (Hanks Balanced Salt Solution) and digested in 0.01% trypsin (Sigma-Aldrich) for 15-20 minutes and followed by gentle pipetting using a fire polished glass pipette. Cortical neurons were placed on cell culture flasks/plates or cover slips (Fisher Scientific) coated with Poly-L-Lysine in Dulbecco's Modified Eagle Medium (DMEM) and 10% Fetal Bovine Serum (FBS) at 37^{o}C in 5% CO_2 for 24 hr. Then, the neurons were maintained in the culture medium consisting of

Neurobasal media supplemented with 2% B27, 0.5 nM glutamine, and 1% penicillin/streptomycin. The medium was change 2-3 times a week and experiments were conducted after 10-14 days.

SH-SY5Y Cells

Human neuroblastoma cell line SH-SY5Y cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplement with 25% Opti-MEM reduced medium, 10% fetal bovine serum (FBS) and antibiotics (penicillin-streptomycin 1:100) at 37°C, at 5% CO_2. SH-SY5Y was transfected with shRNA plasmid using lipofectamine 2000 to downregulate protein of interest (POI). Cells were used for molecular and functional aspects at 90-100% of confluence.

In-Vitro Ischemic Model

Primary cortical neurons and SH-SY5Y cells were placed in hypoxic chamber with 1% O_2 and 5% CO_2 balanced with N_2 at 37°C and provided with glucose-free DMEM medium for 2 hr to represent Oxygen-Glucose Deprivation (OGD) condition, followed by 24 hr reperfusion, cells were incubated with 21% O2/ 5% CO2 at 37°C with complete medium for 24 hr. Control primary neurons and SH-SY5Y cells were only exposed to 21% O2/ 5% CO2 with complete culture medium.

Hepcidin Downregulation Model

Hepcidin shRNA lentiviral vector plasmid (sc-97892-SH) was used to knock down hepcidin gene in SH-SY5Y cells. Since this plasmid contains a puromycin resistance gene, puromycin was used for selection of cells stably expressing hepcidin shRNA. GFP scrambled shRNA was used as a control of the hepcidin knockdown model. Inhibition of hepcidin expression in SH-SY5Y cells was monitored by immunofluorescence approach.

Minihepcidin Treatment

Minihepcidins are synthetic peptides that mimic the biological activity of endogenous hepcidin. In this study we used minihepcidin ANP2-PR73 which was kindly provided by Dr. Piotr Ruchala from David Geffen School of Medicine at University of California, Los Angles. To test minihepcidin activity on our research model, FPN1 expression was determined in lysates from minihepcidin-treated cells. Briefly, SH-SY5Y cells were treated with 5, 10, and 20 μM minihepcidin ANP2-PR73 under normal conditions (37°C, at 5% CO_2). In addition, minihepcidin ANP2-PR73 effect upon OGD/R conditions were studied via treating SH-SY5Y cells with ANP2-PR73 during OGD/R conditions and further experiments, such as cell viability, immunoblot analysis, and EPR were conducted.

Immunoblot Analysis

Cells were washed with ice-cold 1X Phosphate buffered Saline (PBS) and lysed in 100-200 μL RIPA buffer containing protease inhibitor cocktail. The lysates were sonicated, and the protein concentration was determined using the plate reader. 10-20μg of protein samples were mixed with equal amount of loading buffer and boiled at 100°C for 10 min. Proteins were loaded into SDS-PAGE for electrophoresis separation for 1 hr. Separated proteins were transferred into PDVF membrane for 2 hr. Then, the membrane was blocked with 5% bovine albumin serum (BSA) in 1X Tris-buffered Saline with Tween 20 (TBST) for 1 hr. After blocking process, the membrane was incubated overnight at 4°C with a certain primary antibody depending on the aim of the experiment. Then, the membrane was washed three times with TBST, followed by secondary antibody incubation for 1 hr at room temperature (RT). The membrane was washed three times with TBST before incubated with ECL Western Blotting Detection Reagents. ChemiDoc™ XRS+

System (Bio-Rad, Hercules, CA) was used to detect the signals. ImageJ was used to quantify the integrated optical intensity (IOD) of each protein band. Actin protein expression was used as an internal control to normalize expression of the protein of interest. Primary antibodies that have been used were included in the **table1**.

Immunocytochemistry

Primary cortical neurons and SH-SY5Y cells-cultured on coverslip were washed twice with PBS and fixed using 4% paraformaldehyde in PBS pH 7.4 for 20 min at RT. Fixed samples were washed twice with ice-cold PBS and then incubated for 10 min at RT in PBS containing 0.3% Trition X-100 (PBST) for permeabilization. Samples were washed three times with PBS for 5 min each. Then, unspecific binding of antibodies was blocked with 1% BSA in PBST for 30 min. Samples were incubated with primary antibodies overnight at 4°C and followed by washing with PBS three times for 5 min each. Samples were incubated with fluorescent dye-labeled secondary antibodies for 1 hr at RT in the dark. DAPI was used to stain the nucleus before images were acquired on Leica microscope. Intensity were determined using ImageJ.

Cytotoxicity Assessment

Primary cortical neurons and SH-SY5Y cells survival was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Yellow MTT can be reduced to purple formazan by mitochondrial enzymatic reaction in the living cells. Cells were incubated with MTT for 4 hr and then the absorbance of the purple formazan was quantified by measuring the optical density (OD) at a wavelength of 570 nm.

Electron Paramagnetic Resonance (EPR)

Electron Paramagnetic Resonance (EPR) Spectroscopy (Bruker BioSpin) was used to determine superoxide levels in SH-SY5Y cells. Briefly, the control and OGD/R cells were incubated with 1mM cyclic hydroxylamine 1-hydroxy3-methoxycarbonyl-2,2,5,5-tetra-methylpyrrolidine (CMH), spin probe molecule, for 3 hr. The cells were washed with ice-cold PBS and samples were collected for EPR measurement. Samples were loaded into 50 µl glass capillary tubes and superoxide signal was detected by EPR spectrometer. Spectrometer settings were as follows: sweep width, 100 G; microwave frequency, 9.75 GHz; modulation amplitude, 1 G; conversion time,5.12 ms; time constant 5.12 ms; receiver gain, 2 × 102; number of scans, 30. Quantification of the EPR signal intensity was determined by comparing the intensity of the recorded middle-derivative EPR peak of each sample. The total protein of samples was determined to normalize the EPR data.

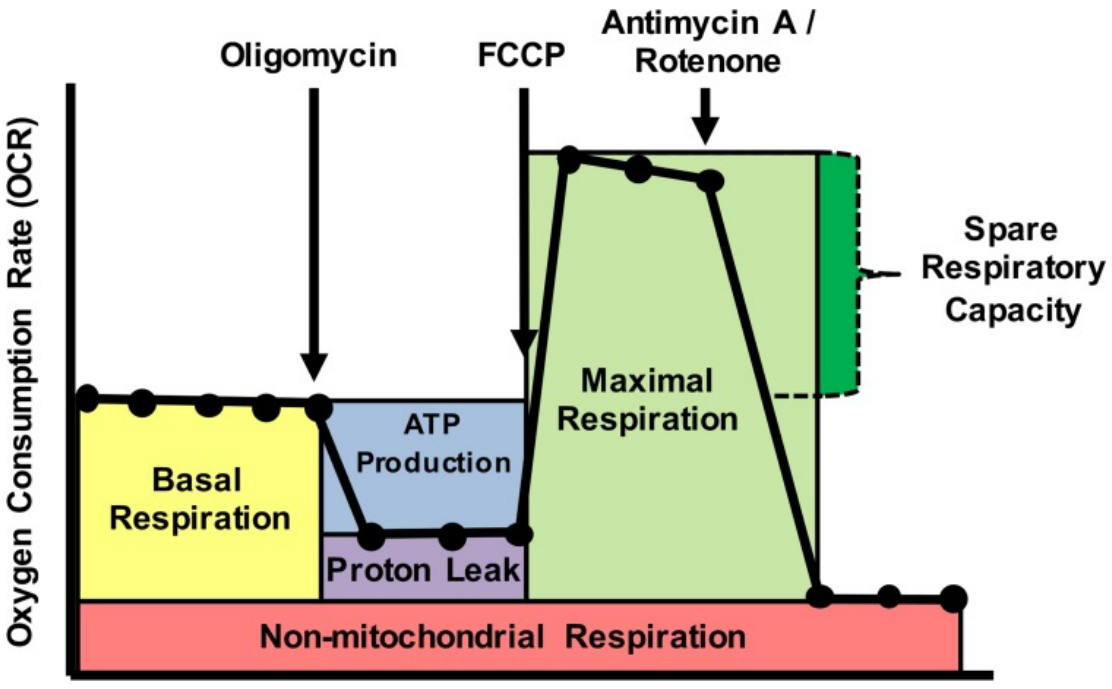
Mitochondrial Bioenergetics (mtBE) Assessment

SH-SY5Y cell mtBE was analyzed using a Seahorse XF96 Analyzer (Seahorse Biosciences, Billerica, MA). Briefly, Control and OGD/R cells were washed twice with pre-warmed XF assay medium containing 5.5 mM glucose and 1mM sodium pyruvate. Cells were incubated at 37°C without CO_2 for 1 hr before loading plate into the XF96 instrument. The XF Sensor Cartridge was loaded with 2µg/ml oligomycin (ATP synthase inhibitor), 2 µM carbonyl cyanide 4-trifluoromethoxy phenylhydrazone (FCCP; Protonophore), 1µM rotenone plus antimycin-A (mitochondrial complex I and III inhibitors) (**Table 2**). The cell plate then introduced into the Seahorse Analyzer using a 3-minute mix cycle to oxygenate the medium followed by a 4 min measurement of the Oxygen Consumption Rate (OCR). The total protein concentration of

samples was determined to normalize the mtBE data. The Maximal Respiratory Capacity (MRC) was evaluated using Wave Software.

Mitochondrial Bioenergetics (mtBE) Assessment: Inhibitors		
Compound	**Final concentration**	**Target**
Oligomycin	2 μg/ml	ATP Synthase
FCCP	2μM	Mitochondrial membrane
Rotenone	1μM	Complex I
Antimycin	1μM	Complex III

Table 2. The inhibitors that were used in mtBE assessment. It shows the compounds and its final concentrations and targets.



Scheme 12. Seahorse XF cell mito stress test profile. Modulators of mitochondrial respiration are added to cells to assess mitochondrial respiration, including Oligomycin, Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), Rotenone, and Antimycin [186].

Statistical analysis

Data were presented as means ± SEM from a minimum of three independent experiments. Unpaired Student *t*-test was applied for experiments containing two groups. One-way ANOVA was performed followed by post hoc Tukey's test for multiple comparisons. Differences of $p<0.05$ were considered statistically significant. Prism, ImageJ, and Excel were used for data analyses.

Results

Part 1: Role of hepcidin in ischemia-induced cell death

1.1. Hepcidin expression in primary cortical neurons and SH-SY5Y cells upon OGD/R conditions

Previously, our laboratory and collaborators have shown that the hepcidin mRNA level was increased in the ischemic side of the brain in a middle cerebral artery occlusion (MCAO) rat model compared with the non-ischemic side. In addition, the increased hepcidin mRNA level was associated with decreased FPN1 level in different mouse brain regions that affected by ischemia, including cerebral cortex, hippocampus, and corpus striatum [14]. To determine whether OGD/R conditions induce hepcidin protein expression in our in-vitro models, primary cortical neurons from rat pups (P0-P2) and SH-SY5Y cells were subjected to OGD conditions (1% $O_2$5% CO_2 balanced with N_2 at 37°C for 2 hr in glucose-free DMEM medium) followed by a 24 hr period of reperfusion (21% O2/ 5%CO2 with medium containing glucose). Hepcidin expression was analyzed by Immunocytochemistry. NeuN was used as a neuronal specific marker. As shown in **Figure 1.1.1**, the intensity of hepcidin immunostaining was significantly increased in the primary cortical neurons exposed to OGD/R conditions compared to those exposed only to normoxic conditions. Consistent with the observation in the primary cortical neurons, the OGD/R conditions significantly induced hepcidin expression in SH-SY5Y cells, compared to the SH-SY5Y cells were exposed only to normoxia (**Figure 1.1.2**).

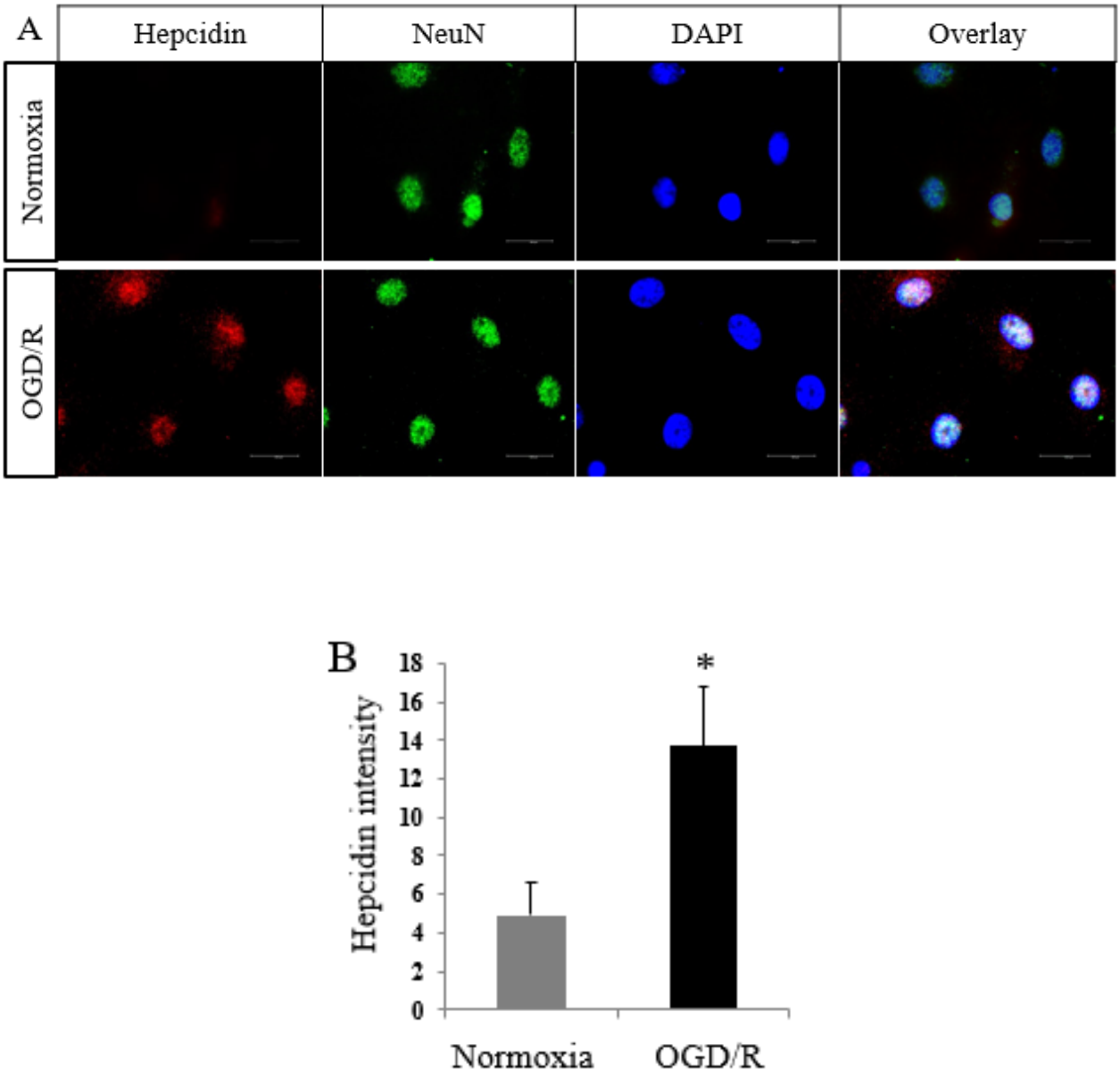


Figure 1.1.1| Hepcidin expression is induced in primary cortical neurons exposed to OGD/R conditions. The protein expression of hepcidin was analyzed by double immunostaining with the neuronal marker NeuN after primary cortical neurons subjected OGD/R conditions. (**A**) Double immunostaining of hepcidin (red) and NeuN (green). (**B**) Quantification of Hepcidin intensity in primary cortical neurons exposed to normoxia or OGD/R conditions. **p*<0.05 versus normoxia. (*n*=3). Scale bar, 20µm.

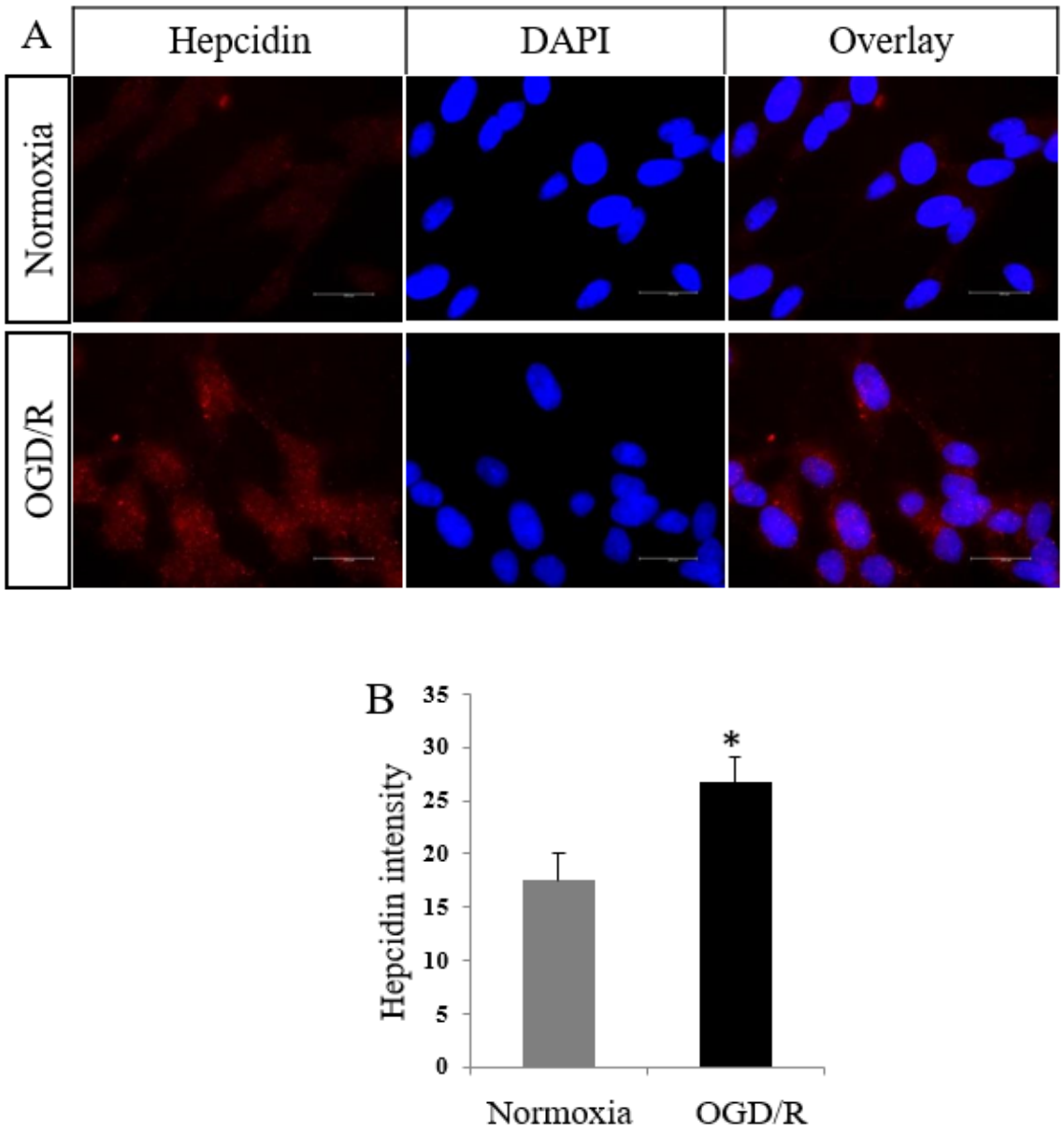


Figure 1.1.2| Hepcidin expression is induced in SH-SY5Y cells exposed to OGD/R conditions. The protein expression of hepcidin was analyzed in SH-SY5Y cells subjected to normoxia or OGD/R conditions. (**A**) Immunostaining of hepcidin (red) and nuclear marker DAPI (blue). (**B**) Quantification of Hepcidin intensity in SH-SY5Y cells exposed to normoxia or OGD/R conditions. *p<0.05 versus normoxia. (n=3). Scale bar, 20µm.

1.2. OGD/R conditions induce death in primary cortical neurons and SH-SY5Y cells

Numerous studies have investigated that ischemia/reperfusion induces cell dysfunction, injury, and death in different brain regions [184, 187, 188]. We assessed the death rate in primary cortical neurons from rat pups and SH-SY5Y cells subjected to OGD/R conditions. Our results showed that OGD/R conditions significantly induced death in primary cortical neurons and SH-SY5Y cells, compared to normoxic conditions. (**Figure 1.2.1**).

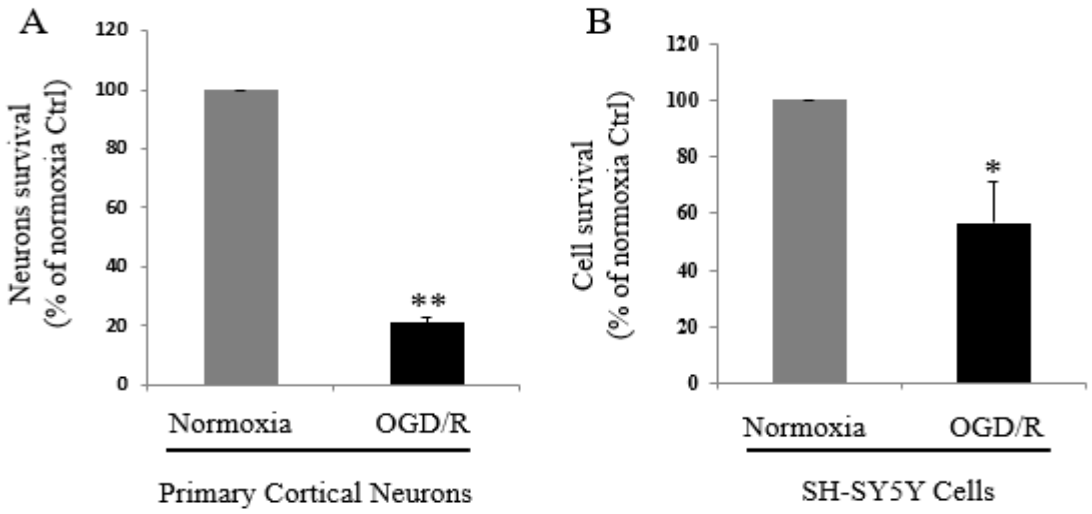
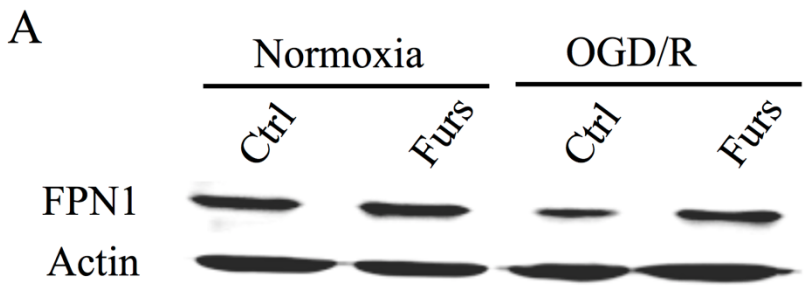


Figure 1.2| OGD/R conditions induce death in primary cortical neurons and SH-SY5Y cells. Neuronal and cellular death was assessed in primary cortical neurons and SH-SY5Y cells were subjected to normoxia or OGD/R conditions. (**A**) Quantification of neuronal death in primary cortical neurons exposed to normoxia or OGD/R conditions. **p<0.001 versus normoxia. (**B**) Quantification of cellular death in SH-SY5Y cells exposed to normoxia or OGD/R conditions. *p<0.05 versus normoxia (n=3).

1.3. Blocking hepcidin binding on FPN1 fails to prevent OGD/R-induced cell death in SH-SY5Y cells

FPN1, a sole well-known cellular iron exporter, is a receptor for hepcidin. Hepcidin binds to cysteine 326 on FPN1 and causes its internalization and degradation in lysosomes [52, 66]. Fursultiamine (Furs), a thiamine derivative compound, can block the binding site of hepcidin on FPN1 gene [123]. First, we determined whether FPN1 expression is downregulated following OGD/R conditions. As shown in **Figure 1.3.1**, FPN1 expression was downregulated in SH-SY5Y cells exposed to OGD/R conditions, compared to the normoxic conditions.

To investigate whether blocking cysteine 326 of FPN1 with Furs rescues hepcidin-induced FPN1 degradation, we treated SH-SY5Y cells with 20 μM Furs that were subjected to OGD/R conditions. Immunoblotting analysis showed that blocking hepcidin binding on FPN1 prevented hepcidin-induced FPN1 degradation in SH-SY5Y cells upon OGD/R conditions, compared to control SH-SY5Y cells (**Figure 1.3.1)**.



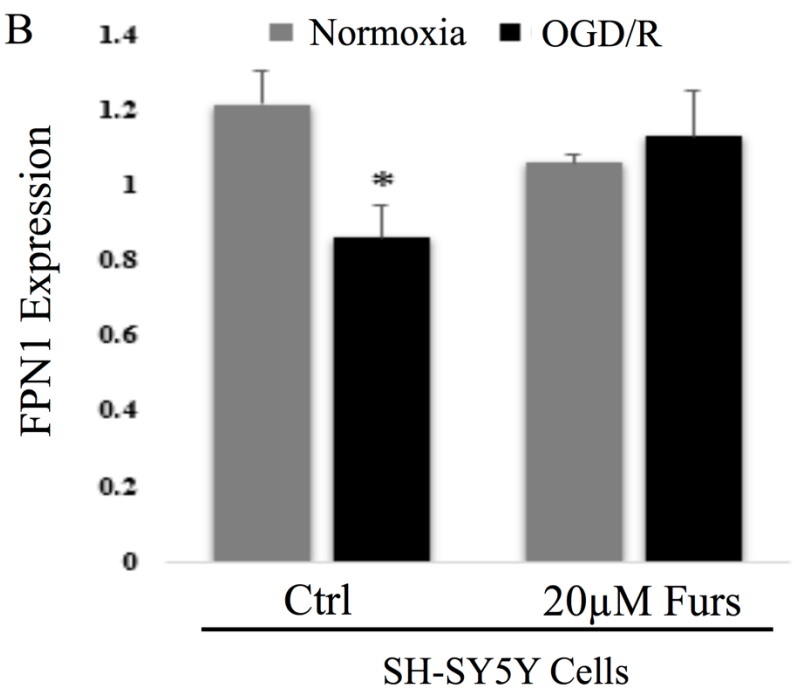


Figure 1.3.1| Blocking hepcidin binding on FPN1 prevents hepcidin-induced FPN1 degradation in SH-SY5Y cells following OGD/R conditions. (**A**) The protein level of ferroportin (FPN1) was analyzed in Furs-treated SH-SY5Y cells were subjected to normoxia or OGD/R conditions. (**B**) Quantification of FPN1 intensity in Furs-treated and control SH-SY5Y cells exposed to normoxia or OGD/R conditions. *p<0.05 versus normoxia. (n=3).

As previously mentioned, blocking hepcidin binding on FPN1 with Furs prevented FPN1 degradation. To investigate whether blocking hepcidin binding site on FPN1 rescues SH-SY5Y cells from cell death following OGD/R conditions, we treated SH-SY5Y cells t with 10 and 20 μM Furs were subjected to OGD/R conditions. MTT assay showed that Furs exacerbated death rate in SH-SY5Y cells after OGD/R exposure. As shown in **Figure 1.3.2**, cell death significantly increased in Furs-treated SH-SY5Y cells under OGD/R conditions, compared with control SH-SY5Y cells under the same conditions.

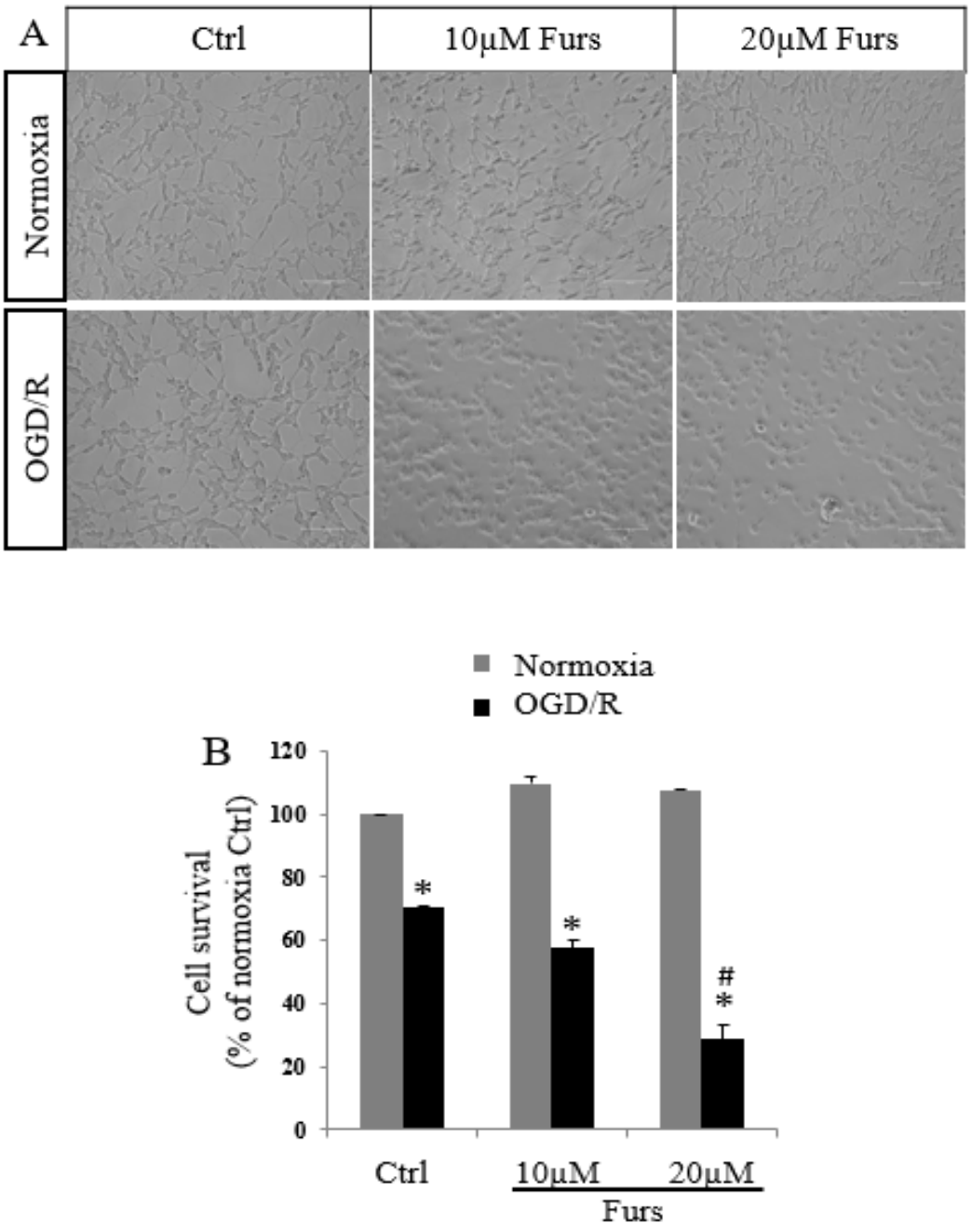


Figure 1.3.2| Interfering with hepcidin-FPN1 binding induces SH-SY5Y cell death following OGD/R conditions. (**A**) Microscopic images of Furs-treated SH-SY5Y cells subjected to normoxia or OGD/R conditions. (**B**) Quantification of cell death in Furs-treated SH-SY5Y cells exposed to normoxia or OGD/R conditions. *p<0.05 versus normoxia. $^{\#}p$<0.05 versus OGD/R Ctrl (n=3).

1.4. Knockdown of hepcidin reduces OGD/R-induced cell death

Hepcidin shRNA lentiviral vector plasmid was used to knockdown hepcidin gene in SH-SY5Y cells. Stable hepcidin shRNA-transfected SH-SY5Y cells were subjected to OGD/R conditions. As shown in **Figure 1.4.1**, OGD/R conditions failed to induce hepcidin expression in hepcidin knockdown SH-SY5Y cells, compared to scrambled shRNA SH-SY5Y cells exposed to the same conditions. This result validated our hepcidin knockdown model for further functional and molecular studies on hepcidin's rules in ischemic conditions.

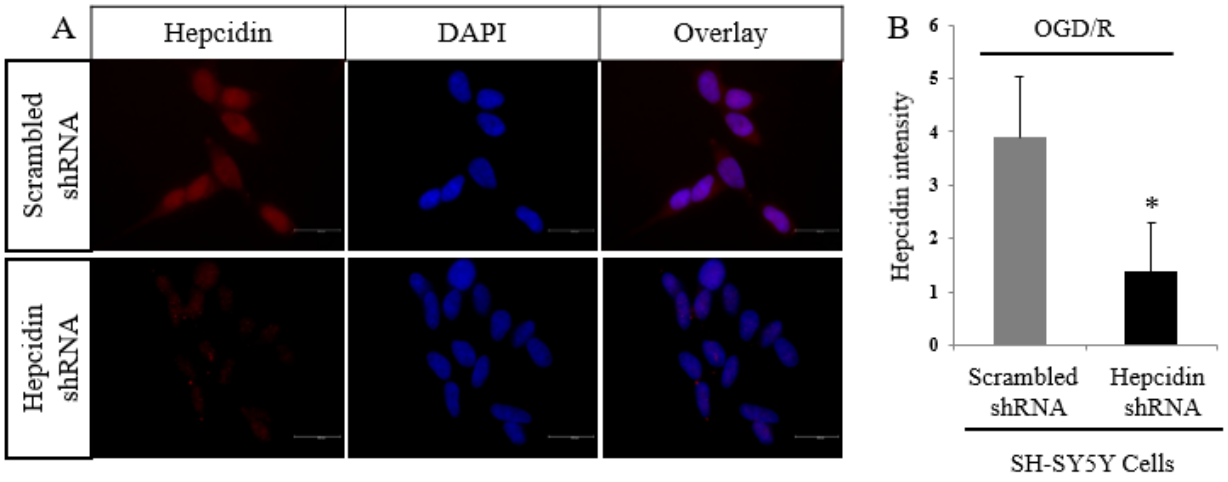


Figure 1.4.1| Hepcidin down-regulation in SH-SY5Y cells. The protein expression of hepcidin was analyzed in Scrambled or Hepcidin shRNA-SH-SY5Y cells subjected to OGD/R conditions. (**A**) Immunostaining of hepcidin (red) and nuclear marker DAPI (blue). (**B**) Quantification of Hepcidin intensity in Scrambled or Hepcidin shRNA-SH-SY5Y cells exposed to OGD/R conditions. *p<0.05 versus Scrambled SH-SY5Y cells. (n=3). Scale bar, 20μm.

Our previous results demonstrated that blocking hepcidin binding site exacerbated the death rate of SH-SY5Y cells following OGD/R conditions. To confirm this observation, we studied the effect of targeting hepcidin on cell death directly via shRNA approach. Hepcidin shRNA lentiviral vector

plasmid was used to knock down hepcidin gene in SH-SY5Y cells. Stable hepcidin shRNA transfected SH-SY5Y cells were subjected to OGD/R conditions. As shown in **Figure 1.4.2**, we found that the cell death in Hepcidin shRNA SH-SY5Y cells was lower than that in Scrambled shRNA SH-DY5Y cells following OGD/R conditions.

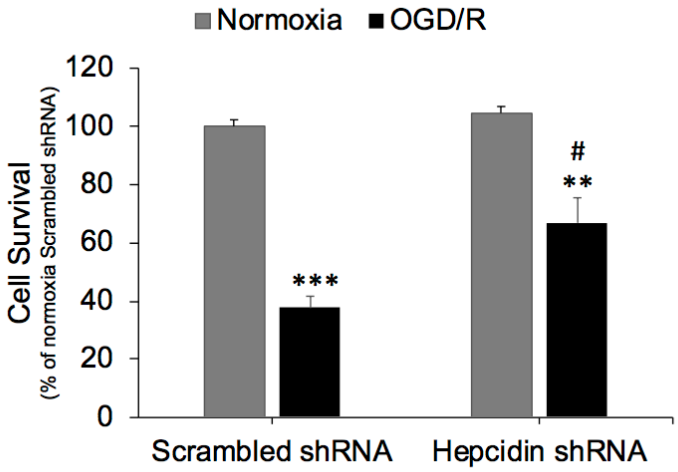


Figure 1.4.2| Hepcidin down-regulation reduces SH-SY5Y cell death following OGD/R conditions. Cell death was assessed in Scrambled or Hepcidin shRNA-SH-SY5Y cells subjected to normoxia or OGD/R conditions. ***p<0.0001 versus Scrambled shRNA cells under normoxia. **p<0.001 versus Hepcidin shRNA cells under normoxia $^{\#}p$<0.05 versus Scrambled shRNA SH-SY5Y cells under OGD/R (n=3).

1.5. Minihepcidin, ANP2-PR73, mimics the action of biological endogenous hepcidin

Our previous data in this study showed that hepcidin was upregulated and induced FPN1degradation, the sole known cellular iron exporter. FPN1 is considered as hepcidin receptor because endogenous hepcidin binds to residue Cys326 of FPN1. Due to the very short half-life of natural hepcidin, researchers have synthesized small peptides named minihepcidins which mimics

the biological activity of hepcidin[142]. Synthetic minihepcidin ANP2-PR73 was used in this study to investigate mechanistic roles of hepcidin in ischemic stroke. Our results showed that minihepcidin ANP2-PR73 induced FPN1 degradation in a concentration dependent manner under normal conditions. ANP2-PR73 at 10 and 20 μM significantly decreased FPN1 expression when compared with control (**Figure 1.5.1)**.

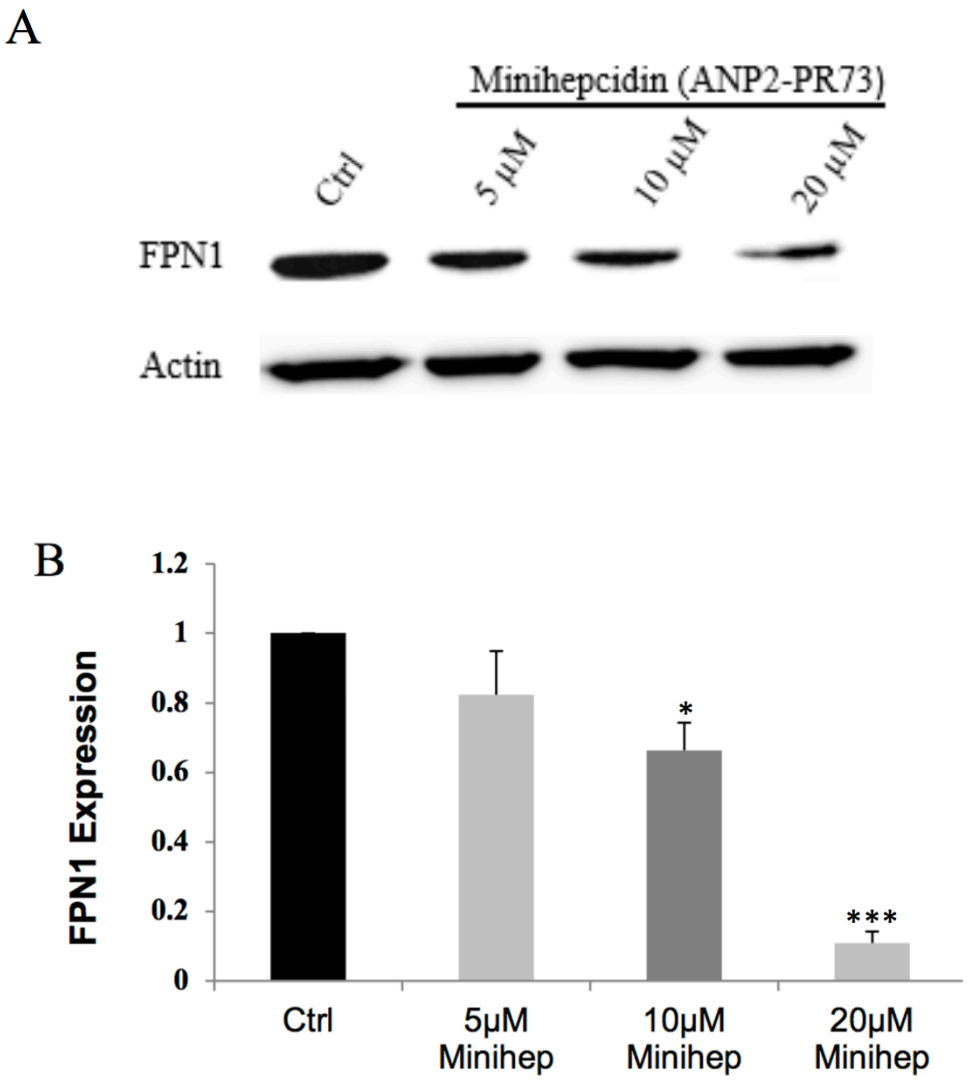


Figure 1.5.1| Minihepcidin ANP2-PR73 induces FPN1 degradation in SH-SY5Y cells under normal conditions. (**A**) The protein level of FPN1 was analyzed in ANP2-RP73-treated SH-SY5Y

cells were incubated at normal conditions. (**B**) Quantification of FPN1 intensity in ANP2-RP73-treated SH-SY5Y cells were incubated at normal conditions. *p<0.05, ***p<0.0001 versus control SH-SY5Y cells (Ctrl). (n=3).

To evaluate the effect of minihepcidin ANP2-PR73 on cell death, SH-SY5Y cells were treated with minihepcidin ANP2-PR73 for 12 hr under normoxia, and then cell survival was assessed. We found that 10 μM of ANP2-PR73 significantly induced cell death in SH-SY5Y under normal conditions (37°C, at 5% CO_2). Interestingly that iron-chelating agent, Deferoxamine (DFO) couldn't rescue cells from the death that induced by minihepcidin (**Figure 1.5.2)**.

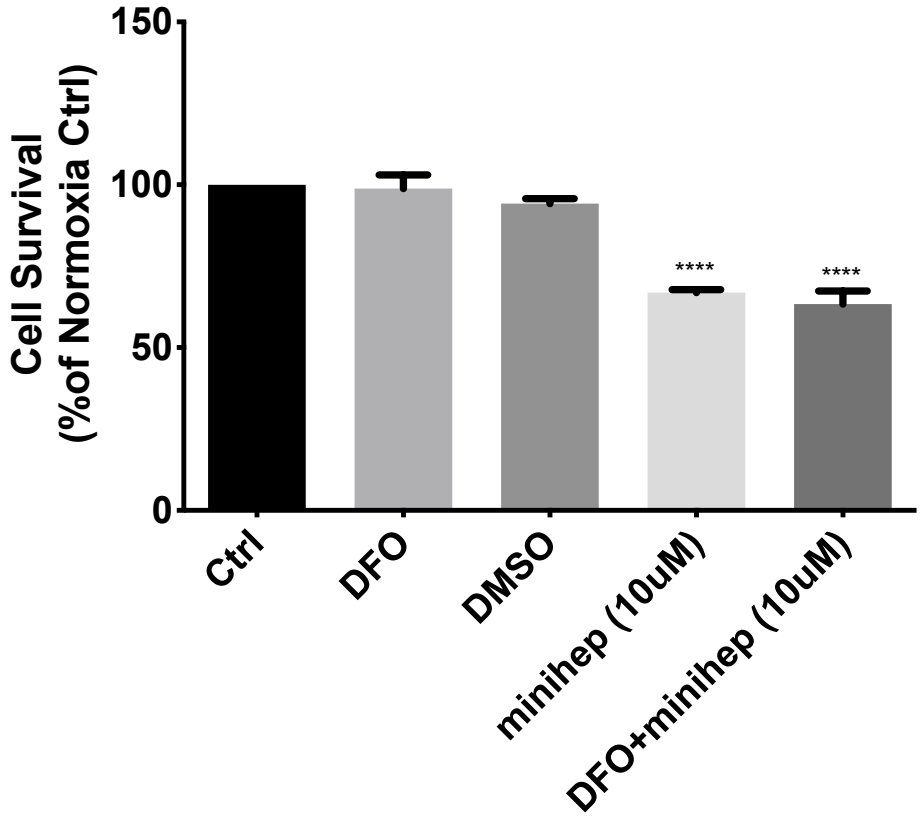


Figure 1.5.2| Minihepcidin induces cell death in SH-SY5Y cells under normoxia. The cell survival was evaluated in minihepcidin ANP2-PR73-treated and control SH-SY5Y cells under normal conditions using MTT assay. ****p<0.0001 versus control SH-SY5Y cells. (n=3).

1.6 Minihepcidin, ANP2-PR73, diminishes the protective effect of hepcidin knockdown on cell death upon OGD/R conditions

We confirmed in the previous section that minihepcidin, ANP2-PR73, induced cell death in SH-SY5Y cells at normal conditions. To investigate the effect of minihepcidin in cell death upon OGD/R conditions. Scrambled shRNA and Hepcidin shRNA SH-SY5Y cells were treated with 10 μM minihepcidin and exposed to OGD/R conditions. We found that minihepcidin diminished the protective effect of hepcidin knockdown on cell survival upon OGD/R condition. The cell death was significantly higher in minihepcidin treated-hepcidin shRNA cells than in control hepcidin shRNA cells. In addition, we didn't observe a significant effect of minihepcidin on cell death when compared with scrambled shRNA cells (**Figure 1.6.1)**.

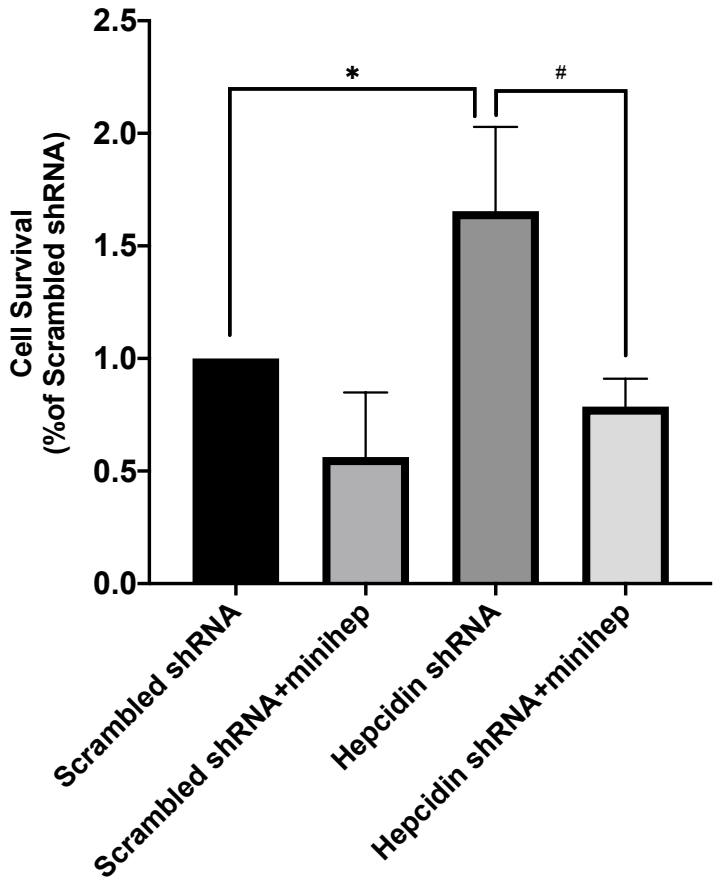


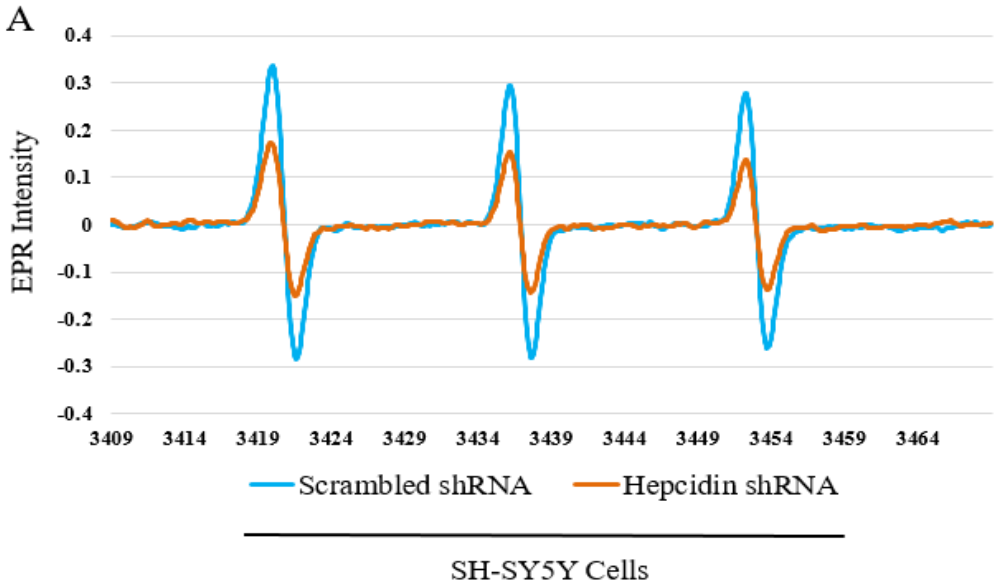
FIGURE 1.5.3| Minihepcidin diminishes the protective effect of hepcidin downregulation on OGD/R induced cell death. The cell death was evaluated after minihepcidin ANP2-PR73 treatment in Scrambled or Hepcidin shRNA-SH-SY5Y cells subjected to OGD/R conditions. *p<0.05 versus scrambled shRNA control. $^{\#}p$<0.05 versus hepcidin shRNA control. (n=3).

Part 2: Hepcidin induced ROS production and Mitochondrial dysfunction

2.1 Role of hepcidin in ROS production

2.1.1 Downregulating hepcidin reduces ROS production in SH-SY5Y cells following OGD/R conditions

Previous studies showed that lack of oxygen during ischemia induced anaerobic products of glycolysis, which further increased superoxide ($O_2^{\cdot -}$) conversion to highly toxic free radicals [156]. To study the role of hepcidin on ischemia-induced superoxide production, Hepcidin shRNA and Scrambled shRNA-transfected SH-SY5Y cells were subjected to OGD/R conditions. Cells were incubated with 1mM CMH for 3 hr and collected for EPR measurement. Samples were loaded into 50 µl glass capillary tubes and superoxide signal was detected by EPR spectrometer. As shown in **Figure 2.1.1**, we found that the EPR peak intensity of superoxide in hepcidin shRNA transfected SH-SY5Y cells was significantly lower than the peak intensity of superoxide in Scrambled shRNA transfected SH-SY5Y cells. These data demonstrated that knockdown of hepcidin in SH-SY5Y cells significantly reduced superoxide production upon OGD/R conditions.



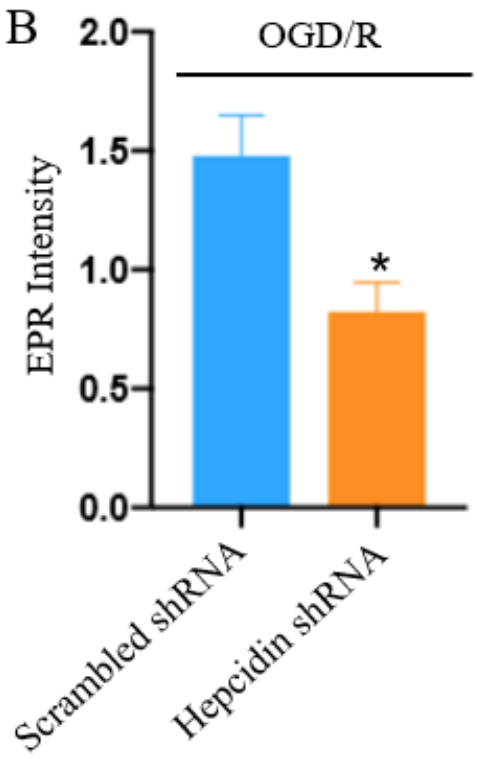
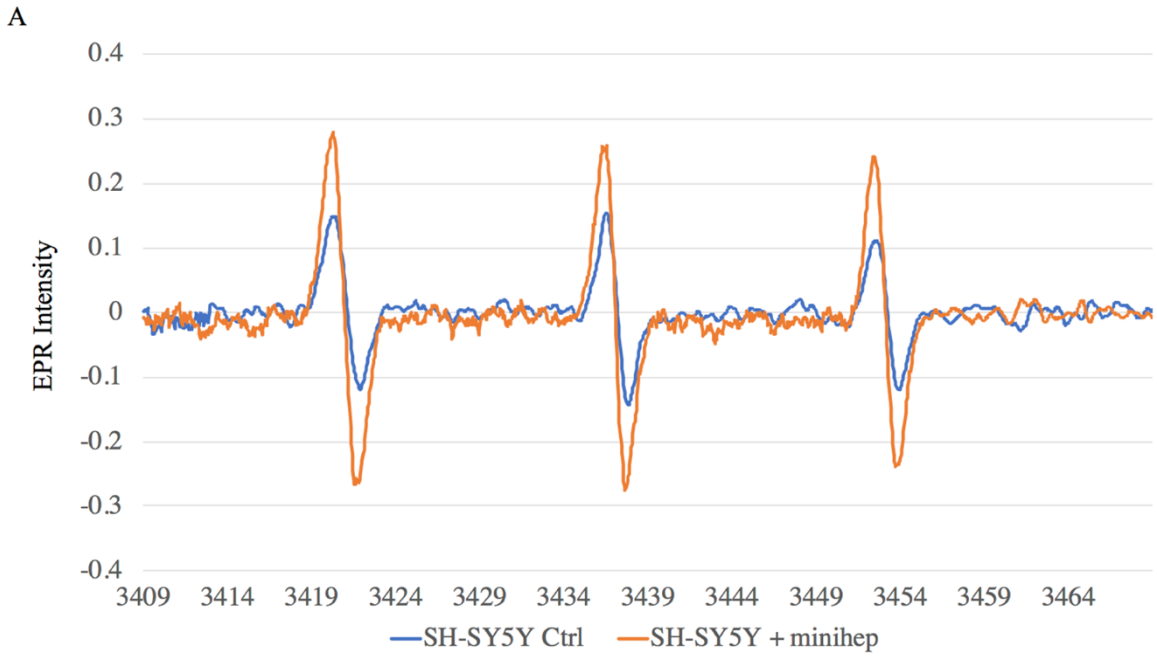


Figure 2.1.1| Hepcidin knockdown reduces ROS production in SH-SY5Y cells following OGD/R conditions. The ROS level was analyzed in Scrambled or Hepcidin shRNA-SH-SY5Y cells subjected OGD/R conditions. (**A**) EPR spectrum (blue) represents Scrambled shRNA and EPR spectrum (orange) represents Hepcidin shRNA SH-SY5Y cells (**B**) Quantification of EPR intensity in Scrambled or Hepcidin shRNA-SH-SY5Y cells exposed to OGD/R conditions. *p<0.05 versus Scrambled SH-SY5Y cells. (n=3).

2.1.2 Minihepcidin ANP2-PR73 induces ROS production in SH-SY5Y cells under normal conditions

Our previous result showed that hepcidin knockdown significantly reduced ROS production in SH-SY5Y cell following OGD/R condition. To confirm the role of hepcidin on ROS production, we studied the effect of a minihepcidin, ANP2-PR73, on ROS production in SH-SY5Y cells under normoxia. SH-SY5Y cells were treated with 10 µM of ANP2-PR73 for 12 hr. Then, the cells were incubated with 1mM CMH for 3 hr and collected for EPR measurement. Samples were loaded into 50 µl glass capillary tubes and superoxide signal was detected by EPR spectrometer. The EPR measurements indicated that minihepcidin significantly induced ROS levels in SH-SY5Y under normal conditions (37°C, at 5% CO_2) (**Figure 2.1.2**).



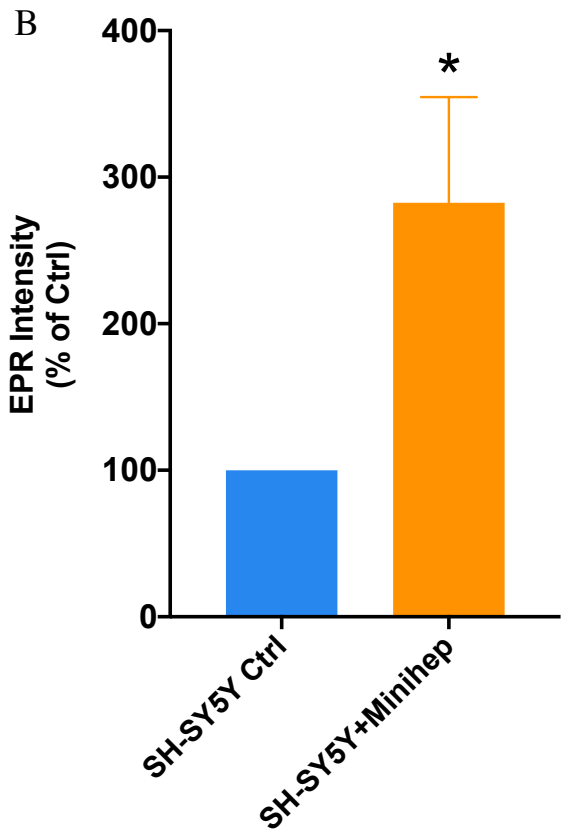


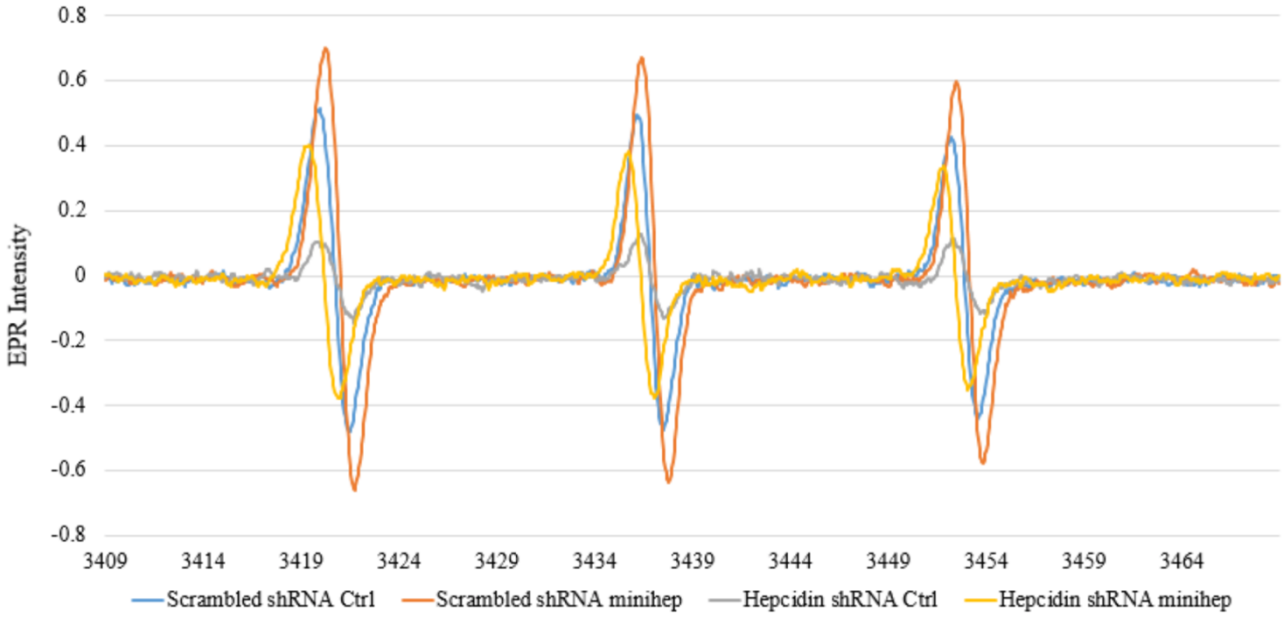
Figure 2.1.2| Minihepcidin induces ROS in SH-SY5Y cells under normoxia. The ROS levels were evaluated in minihepcidin ANP2-PR73-treated SH-SY5Y cells under normal conditions using EPR. EPR spectrum (blue) represents control SH-SY5Y cells and EPR spectrum (orange) represents minihepcidin ANP2-PR73- treated SH-SY5Y cells (**B**) Quantification of EPR intensity in minihepcidin ANP2-PR73-treated and control SH-SY5Y cells under normal conditions. *p<0.05 versus control SH-SY5Y cells (Ctrl). (n=3).

2.1.3 Minihepcidin, ANP2-PR73, diminishes the effect of hepcidin knockdown on ROS production following OGD/R conditions

To study whether minihepcidin ANP2-PR73 induces ROS production following OGD/R conditions, Scrambled shRNA and Hepcidin shRNA SH-SY5Y cells were treated with 10 μM

minihepcidin and exposed to OGD/R conditions. Cells were incubated with 1mM CMH for 3 hr and collected for EPR measurement. Samples were loaded into 50 µl glass capillary tubes and superoxide signal was detected by EPR spectrometer. We found that minihepcidin significantly induced ROS levels in both scrambled shRNA and Hepcidin shRNA cells that exposed to OGD/R conditions. Interestingly, minihepcidin diminished the effect of hepcidin knockdown on ROS production upon OGD/R condition. The ROS level was significantly higher in minihepcidin treated-hepcidin shRNA cells than hepcidin shRNA cell control (**Figure 2.1.3**).

A



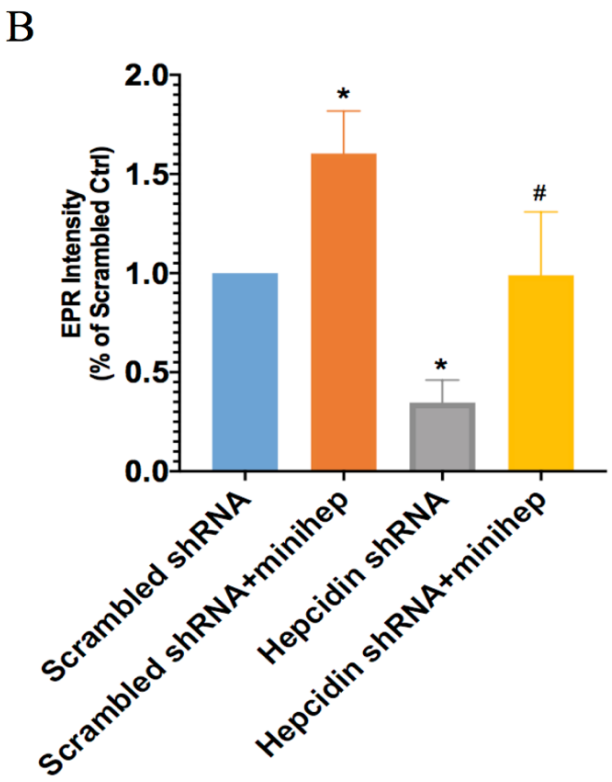
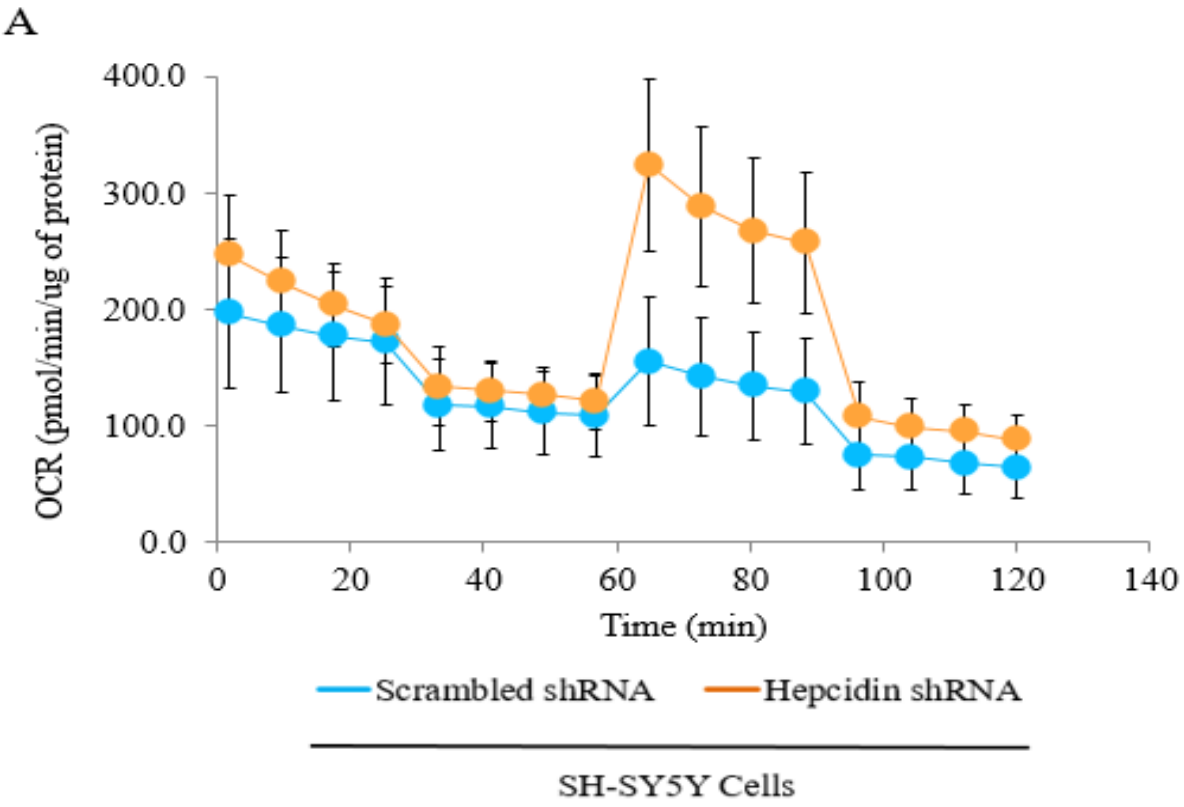


Figure 2.1.2| Minihepcidin diminishes the protective effect of hepcidin downregulation on OGD/R induced ROS production. ROS levels were evaluated in minihepcidin ANP2-PR73-treated Scrambled or Hepcidin shRNA-SH-SY5Y cells subjected to OGD/R conditions using EPR. EPR spectrum (blue) represents control Scrambled shRNA SH-SY5Y cells, EPR spectrum (orange) represents minihepcidin ANP2-PR73- treated Scrambled shRNA SH-SY5Y cells, EPR spectrum (grey) represents control Hepcidin shRNA SH-SY5Y cells, EPR spectrum (yellow) represents minihepcidin ANP2-PR73- treated Hepcidin shRNA SH-SY5Y cells *p<0.05 versus scrambled shRNA control. $^{\#}p$<0.05 versus hepcidin shRNA control. (n=3).

2.2 Role of hepcidin in Mitochondrial dysfunction

2.2.1 Hepcidin knockdown improves mtBE deficits in SH-SY5Y cells following OGD/R conditions

Ischemic stroke results in lack of blood, oxygen, and nutrient supply to an infarct region of the brain. Excessive ROS production induces mitochondrial fragmentation and dysfunction. Our previous result in this study demonstrated that hepcidin plays an important role in ROS production and knockdown of hepcidin reduces ROS production in SH-SY5Y cells following OGD/R conditions. To study whether hepcidin knockdown protects cells from ROS-induced mitochondrial dysfunction, Hepcidin shRNA and Scrambled shRNA-transfected SH-SY5Y cells were subjected to OGD conditions for 2 hr followed by a 24 hr period of reperfusion. Mitochondrial function was evaluated through real-time measurements of oxygen consumption using the Seahorse extracellular flux analyzer. As shown in **Figure 2.2.1**, we found that the mitochondrial MRC in hepcidin shRNA SH-SY5Y cells was significantly higher than Scrambled shRNA SH-SY5Y cells. These data demonstrated that knockdown of hepcidin in SH-SY5Y cells improves mitochondria function upon OGD/R conditions.


A
400.0
300.0
200.0
100.0
0.0
OCR (pmol/min/ug of protein)
0
20
40
60
80
100
120
140
Time (min)
Scrambled shRNA
Hepcidin shRNA
SH-SY5Y Cells

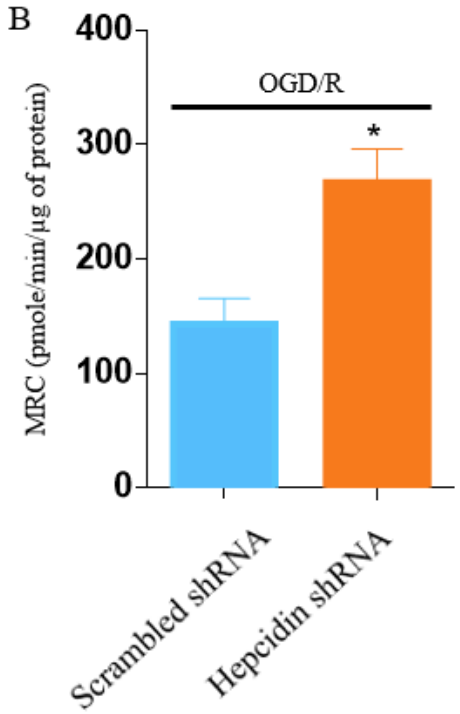

B
400
300
200
100
0
MRC (pmole/min/µg of protein)
OGD/R
*
Scrambled shRNA
Hepcidin shRNA

Figure 2.2.1| Hepcidin knockdown improves mtBE deficits in SH-SY5Y cells following ischemic conditions. Mitochondrial oxygen consumption was analyzed in Scrambled or Hepcidin shRNA-SH-SY5Y cells subjected to OGD/R conditions. (**A**) OCR levels (blue) represents Scrambled shRNA and OCR level (orange) represents Hepcidin shRNA SH-SY5Y cells (**B**) Quantification of MCR in Scrambled or Hepcidin shRNA-SH-SY5Y cells exposed to OGD/R conditions. *p<0.05 versus Scrambled SH-SY5Y cells. (n=3).

2.2.2 Minihepcidin, ANP2-PR73, diminishes the protective effect of hepcidin knockdown on mitochondrial dysfunction following OGD/R conditions

Our previous result in this study confirmed that hepcidin knockdown improves mitochondrial dysfunction in SH-SY5Y cells following OGD/R conditions. To study whether minihepcidin ANP2-PR73 induces mitochondrial dysfunction following OGD/R conditions, Scrambled shRNA and Hepcidin shRNA SH-SY5Y cells were treated with 10 μM minihepcidin and exposed to OGD/R conditions. Mitochondrial function was evaluated through real-time measurements of oxygen consumption using the Seahorse extracellular flux analyzer. We found that there was no significant difference between MRC in minihepcidin treated and control Scrambled shRNA SH-SY5Y cells. However, there was a significant difference between MRC in minihepcidin treated and control Hepcidin shRNA SH-SY5Y cells. These results indicated that miniehpcidin diminished the protective effect of hepcidin knockdown in mitochondrial dysfunction following OGD/R conditions (**Figure 2.2.2**).

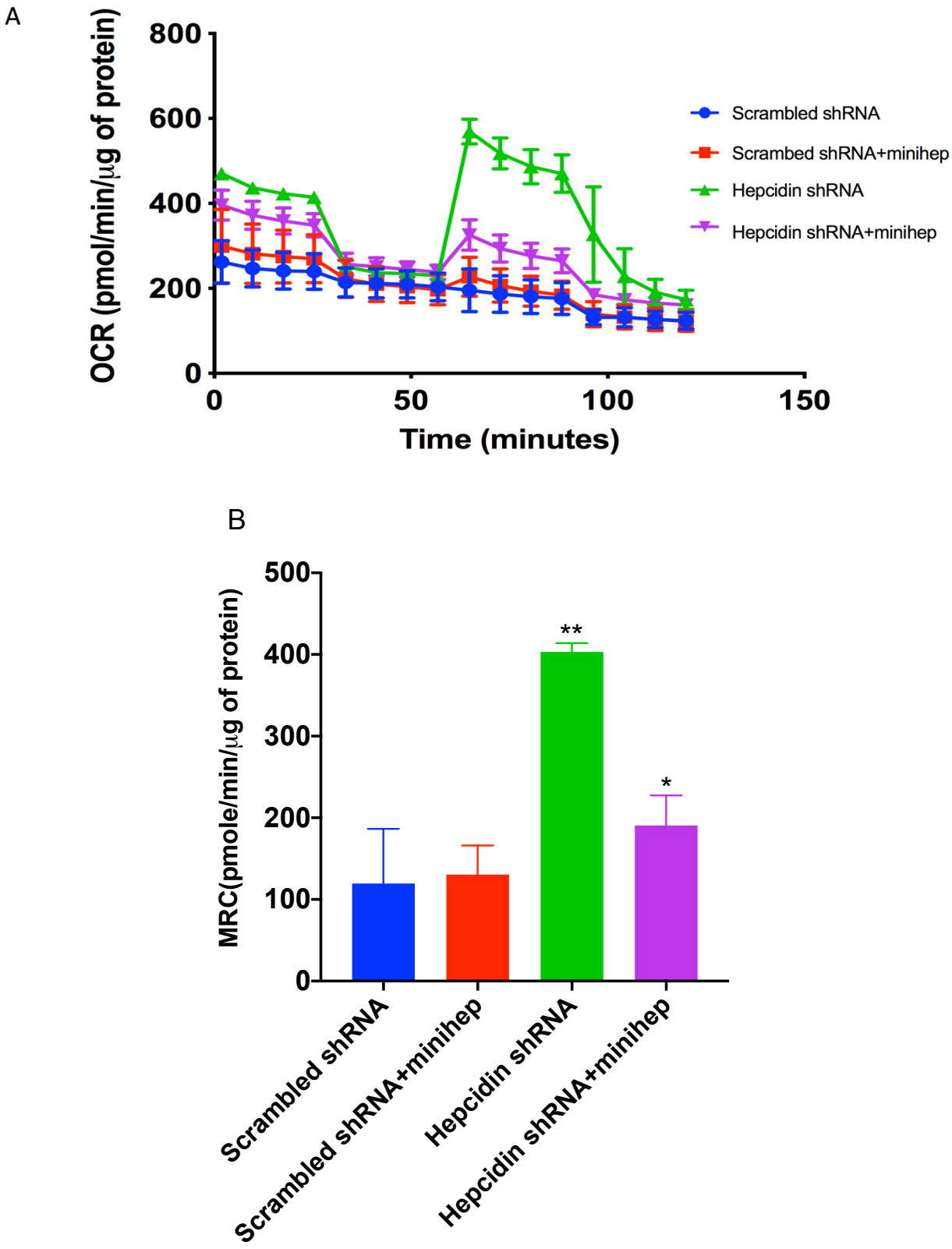


Figure 2.2.2| Minihepcidin diminishes the protective effect of hepcidin downregulation on OGD/R induced mitochondrial dysfunction. Mitochondrial oxygen consumption was analyzed

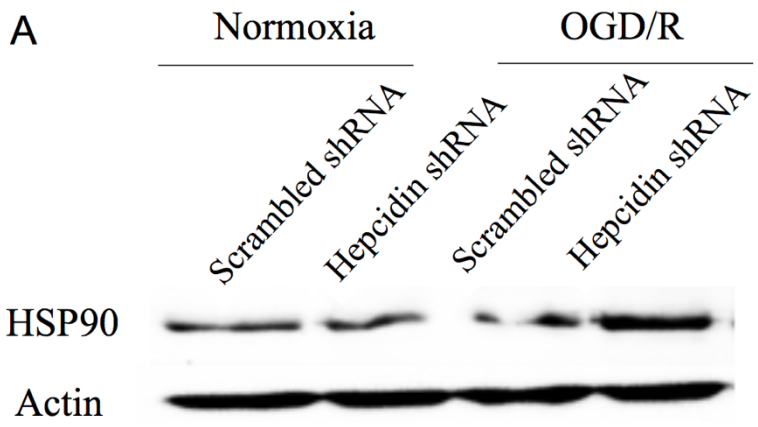
in minihepcidin ANP2-PR73-treated Scrambled or Hepcidin shRNA-SH-SY5Y cells subjected to OGD/R conditions. (**A**) OCR levels (blue) represents Scrambled shRNA and OCR level (orange) represents Hepcidin shRNA SH-SY5Y cells (**B**) Quantification of MCR in Scrambled or Hepcidin shRNA-SH-SY5Y cells exposed to OGD/R conditions. *p<0.05 versus Scrambled SH-SY5Y cells. (n=3).

Part 3: Role of hepcidin on HSP90 and HIF-1α expression following OGD/R conditions

3.1 Role of hepcidin on HSP90 following OGD/R conditions

3.1.1 Downregulating hepcidin increases HSP90 expression in SH-SY5Y cells upon OGD/R conditions

HSP90, a molecular chaperone, protects its client proteins from misfolding and degradation through enhancing their stability. One of these client proteins is HIF-1, which is a master regulator of cellular adaption to hypoxia[173]. Numerous studies have shown that inhibition of HSP90 leads to HIF-1α degradation [170, 173, 174]. To determine whether knockdown of hepcidin reduced cell death following OGD/R conditions via inducing HSP90 expression, Hepcidin shRNA and Scrambled shRNA transfected SH-SY5Y cells were subjected to OGD/R conditions. Immunoblotting showed that HSP90 level was significantly increased in hepcidin shRNA SH-SY5Y cells upon OGD/R conditions, compared to Scrambled shRNA SH-SY5Y cells exposed to the same conditions (**Figure 3.1.1)**.



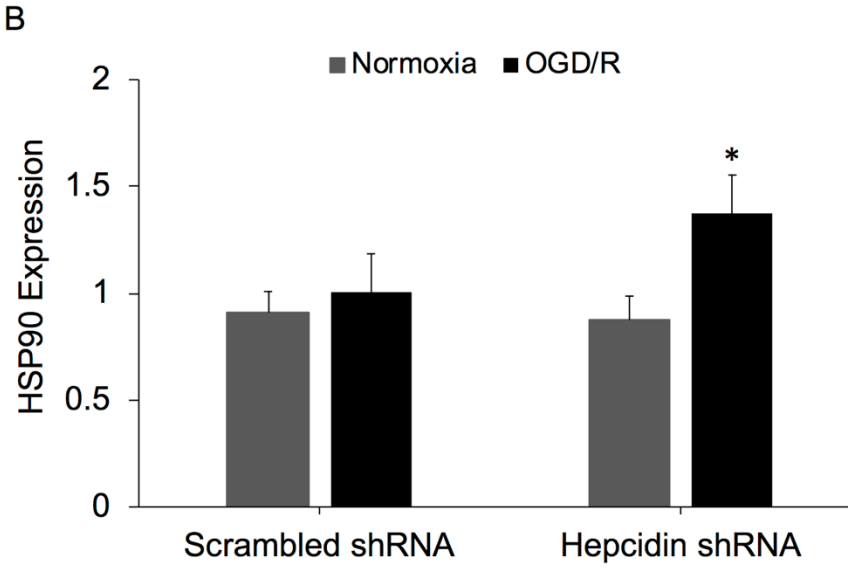


Figure 3.1.1| Hepcidin knockdown induces HSP90 expression in SH-SY5Y cells following OGD/R conditions. (**A**) The protein level of HSP90 was analyzed in Scrambled or Hepcidin shRNA-SH-SY5Y cells subjected to OGD/R conditions. (**B**) Quantification of HSP90 intensity in Scrambled or Hepcidin shRNA-SH-SY5Y cells exposed to OGD/R conditions. *p<0.05 versus Scrambled SH-SY5Y cells. (n=3).

3.1.2 Minihepcidin ANP2-PR73 diminishes the effect of hepcidin knockdown on HSP90 expression in SH-SY5Y cells upon OGD/R conditions

To study whether minihepcidin ANP2-PR73 reduces HSP90 expression in SH-SY5Y cells exposed to OGD/R conditions. Scrambled shRNA and hepcidin shRNA SH-SY5Y cells were treated with minihepcidin ANP2-PR73 for 12 hr and cell survival was evaluated. Interestingly, we found that 10 μM of ANP2-PR73 significantly reduced HSP90 expression in Hepcidin shRNA SH-SY5Y cells following OGD/R conditions compared to control Hepcidin shRNA SH-SY5Y

cells. However, minihepcidin didn't significantly reduce HSP90 expression in Scrambled shRNA SH-SY5Y cells compared to control Scrambled shRNA SH-SY5Y cells exposed to the same conditions (**Figure 3.1.2)**.

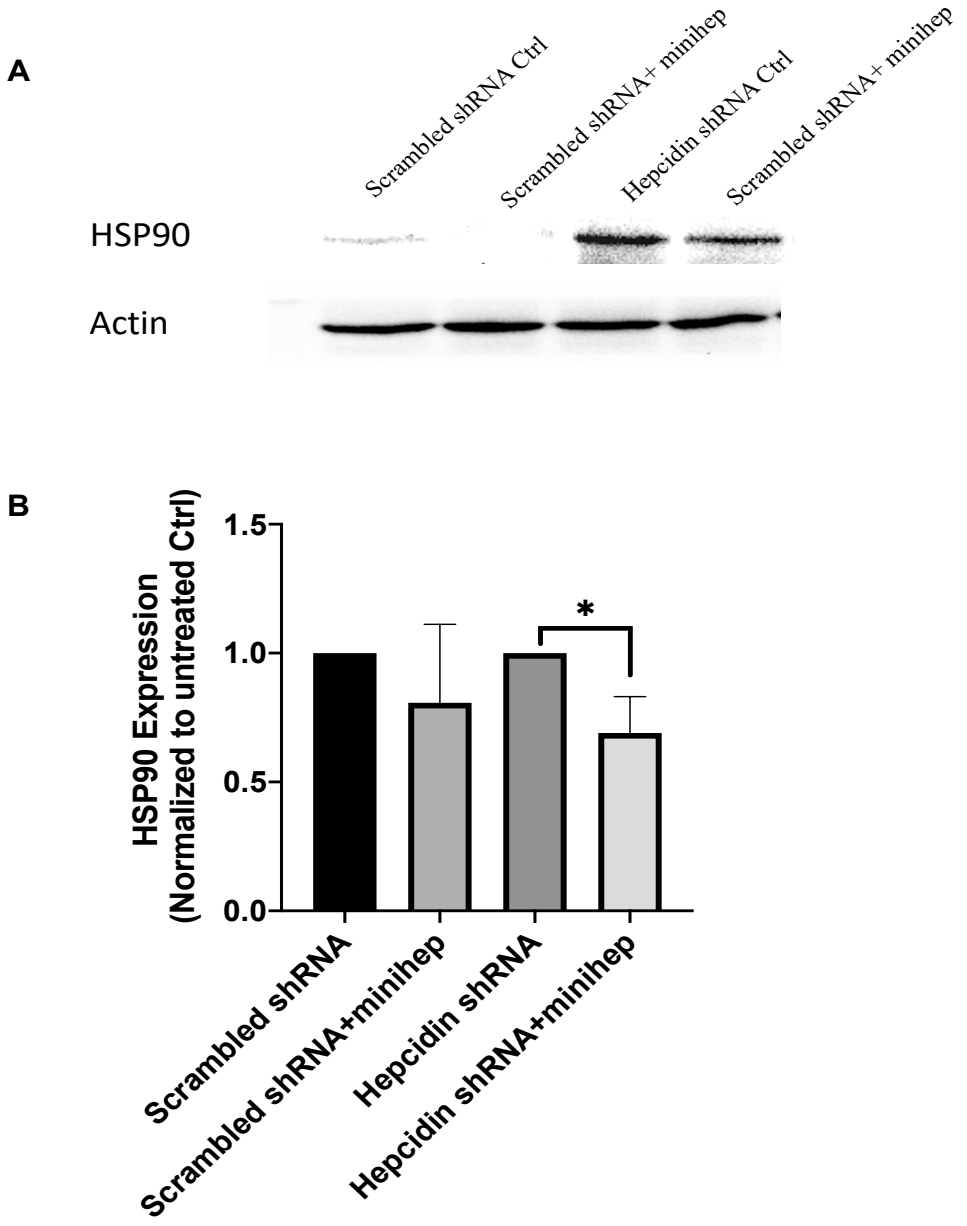


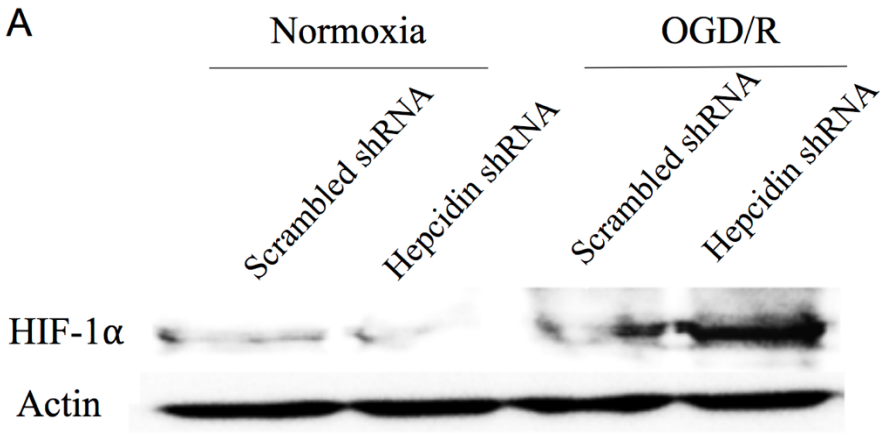
Figure 3.1.2| Minihepcidin diminishes the effect of hepcidin knockdown-induced HSP90 expression following OGD/R conditions. (**A**) The protein level of HSP90 was analyzed in minihepcidin ANP2-PR73- treated and control Scrambled or Hepcidin shRNA-SH-SY5Y cells

subjected to OGD/R conditions. (**B**) Quantification of HSP90 intensity in Scrambled or Hepcidin shRNA-SH-SY5Y cells exposed to OGD/R conditions. *p<0.05 versus controls hepcidin shRNA cells. (n=4).

3.2 Role of hepcidin on HIF-1α following OGD/R conditions

3.2.1 Hepcidin knockdown induces HIF-1α in SH-SY5Y cells upon OGD/R conditions

Emerging evidence showed that HIF-1 α promotes cell survival in ischemic tissues. HIF-1 α is stabilized by HSP90. To determine whether knockdown of hepcidin-induced HSP90 stabilizes HIF-1α upon OGD/R conditions via HSP90 upregulation, Stable hepcidin shRNA and Scrambled shRNA transfected SH-SY5Y cells were subjected to OGD conditions for 2 hr followed by a 24 hr period of reperfusion. Immunoblotting showed that HIF-1 α was significantly upregulated in hepcidin shRNA SH-SY5Y cells upon OGD/R conditions, compared to Scrambled shRNA SH-SY5Y cells exposed to the same conditions (**Figure 3.2.1)**.



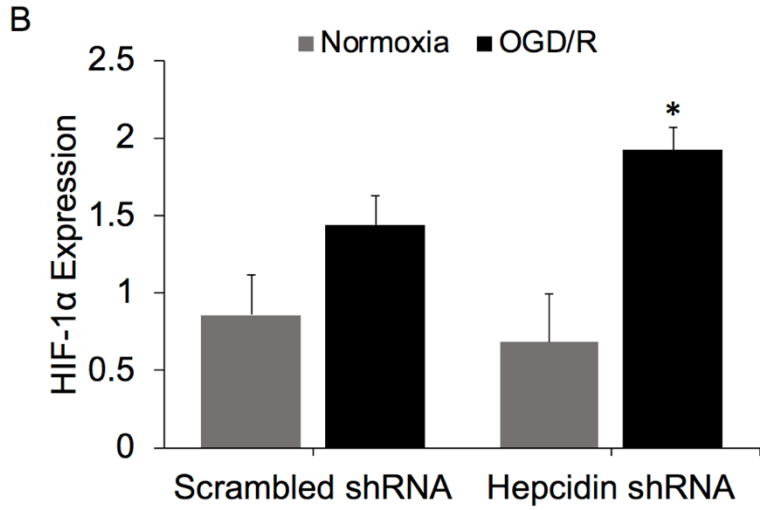


Figure 3.2.1| Hepcidin knockdown induces HIF-1α expression in SH-SY5Y cells following ischemic conditions. (**A**) The protein level of HIF-1α was analyzed in Scrambled or Hepcidin shRNA-SH-SY5Y cells subjected to OGD/R conditions. (**B**) Quantification of HIF intensity in Scrambled or Hepcidin shRNA-SH-SY5Y cells exposed to OGD/R conditions. *p<0.05 versus Scrambled SH-SY5Y cells. (n=3).

3.2.2 Minihepcidin ANP2-PR73 diminishes the effect of hepcidin knockdown on HIF-1α expression in SH-SY5Y cells upon OGD/R conditions

Our previous result in this study showed that hepcidin knockdown induces HSP90 and HIF-1α expression. In addition, we found that minihepcidin reduced HSP90 expression in cells that express Hepcidin shRNA. Numerous studies have shown that inhibition of HSP90 leads to HIF-1α degradation. To study whether minihepcidin ANP2-PR73 reduces HIF-1α expression in SH-SY5Y cells exposed to OGD/R conditions. Scrambled shRNA and hepcidin shRNA SH-SY5Y cells were treated with minihepcidin ANP2-PR73 for 12 hr. Interestingly, we found that 10 μM of ANP2-PR73 significantly reduced HIF-1α expression in both Scrambled and

Hepcidin shRNA SH-SY5Y cells following OGD/R conditions compared to control Scrambled or Hepcidin shRNA SH-SY5Y cells that exposed to the same conditions (**Figure 3.2.2)**.

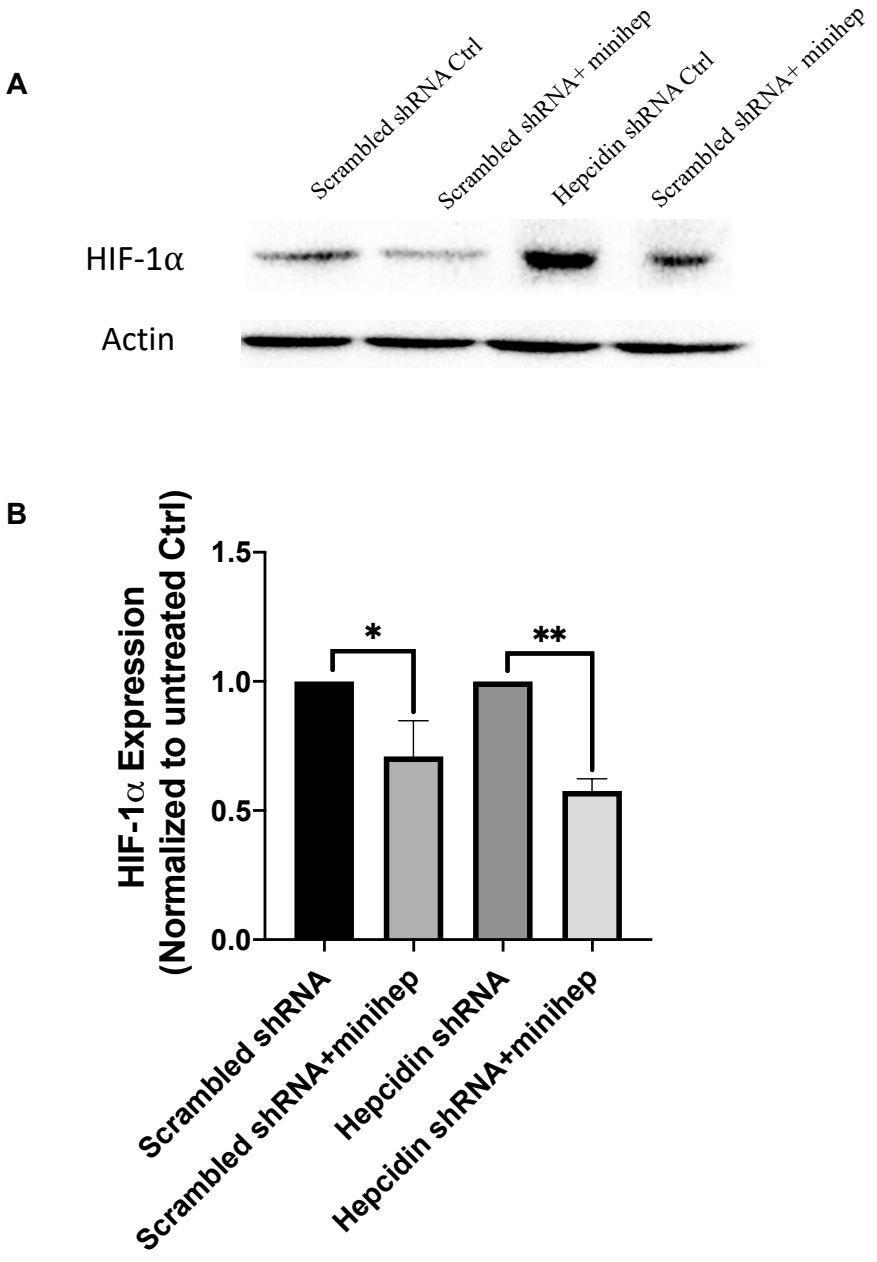


Figure 3.2.2| Minihepcidin diminishes the effect of hepcidin knockdown-induced HIF-1α expression following OGD/R conditions. (**A**) The protein level of HIF-1α was analyzed in minihepcidin ANP2-PR73-treated and control Scrambled or Hepcidin shRNA-SH-SY5Y cells

subjected to OGD/R conditions. (**B**) Quantification of HIF-1α intensity in minihepcidin-treated and control Scrambled or Hepcidin shRNA-SH-SY5Y cells exposed to OGD/R conditions. *p<0.05 versus control scrambled shRNA, **p<0.05 versus control hepcidin shRNA (n=3).

Discussion and Conclusion

This dissertation explored the role of hepcidin in ischemic conditions. First, it reported the effect of pharmacological interfering hepcidin-FPN1 binding with Furs. Second, it demonstrated the protective role of downregulating hepcidin in SH-SY5Y cells exposed to OGD/R conditions. In addition, it explored molecular mechanisms of hepcidin downregulation involved in cell survival upon OGD/R conditions. Finally, it showed how hepcidin mimetic molecules contributed to OGD/R-induced cell death.

Hepcidin is mainly synthesized in liver as a secreted hormone regulates iron homeostasis via degradation of FPN1, the sole known cellular iron exporter [55, 189]. Recently, it was shown that hepcidin is expressed in brain tissues [61]. Even though the mechanistic role of hepcidin in brain tissues has not been fully elucidated, previous study from our collaborative work revealed that hepcidin is upregulated upon brain pathological conditions that associated with inflammation such as ischemic stroke [14]. The level of the iron storage protein, ferritin was increased in the cerebral cortex, hippocampus, and corpus striatum in the ischemic brain. This increase in ferritin levels was associated with decrease in FPN1 levels in these brain regions of ischemic rat brains leading to accumulation of intracellular iron. Most importantly, the increased ferritin and decreased FPN1 levels in the ischemic brains were associated with increase of mRNA levels and immunohistochemistry staining of hepcidin compared with controls [14, 145]. As mentioned previously in the introduction that inflammation is considered a positive regulator of hepcidin transcription via activation of STAT3 signaling pathway via IL-6 [73, 74]. It has been found that the mRNA and protein levels of IL-6, an inflammatory mediator, was increased upon ischemia [14, 75]. Knockdown of hepcidin rescued FPN1 from the degradation that induced upon ischemic stroke indicating that hepcidin is the main player that is responsible for ischemia-induced FPN1

degradation. Our data show that hepcidin expression significantly increased in our in-vitro models, primary cortical rat neurons and SH-SY5Y cells after exposing to OGD/R conditions. The increased hepcidin expression was associated with a significant reduction in FPN1 expression and cells/neurons viability compared with cells and neurons incubated under normal conditions. Taken together, these data indicate that hepcidin expression is upregulated by ischemic conditions not only in in-vivo models, but also in-invitro models, such as neuroblastoma cell line (SH-SY5Y) and primary neurons cultures that exposed to OGD/R conditions. The small size of hepcidin (25 amino acids) limits us to use immunoblotting analysis to detect hepcidin expression in our research models. Therefore, we used the immunocytochemistry approach to validate the upregulation of hepcidin following OGD/R conditions.

Neuronal death is considered one of the major consequences of brain ischemia. The ischemic injury is mainly determined by the duration and severity of the blood supply interruption. Prolonged ischemia could cause irreversible neuronal death. In addition, restoration blood to deprived tissues causes subsequent damage. Our study showed that exposing primary cortical neurons or SH-SY5Y cells to OGD conditions for 2 hr and followed by 24 hr reperfusion caused significant cell death. The hepcidin-FPN1 axis is an important system that mediates iron homeostasis in the numerous organs including the brain. Hepcidin binds to FPN1 and induces its internalization and degradation in lysosomes, thus resulting in accumulation of the iron inside the cells [66, 190]. The thiol cysteine C326 of the extracellular loop of FPN1 is essential for hepcidin-FPN1 interaction. Mutation of this cysteine residue of FPN1 prevents the hepcidin binding [69, 70, 191]. There is growing list of small molecules that have been identified as hepcidin antagonists. One of these antagonists is fursultiamine (Furs), the FDA approved thiamine derivative. Furs blocks the thiol cysteine C326 of FPN1 and prevents hepcidin binding. A study showed that Furs

prevented hepcidin-induced FPN1 degradation in the HEK293 cells expressing FPN1-GFP plasmid [52, 123]. As mentioned previously, hepcidin upregulation and FPN1 degradation were detected in ischemic stroke modules [14]. In addition, we demonstrated in this study that OGD/R conditions resulted in the same finding in our in-vitro cell model, SH-SY5Y cells. Our data showed that Furs rescued FPN1 from hepcidin-induced FPN1 degradation upon OGD/R conditions. Surprisingly, interfering hepcidin-FPN1 binding with Furs treatment didn't rescue SH-SY5Y cells from cell death that induced by OGD/R conditions. Our perspective here is that hepcidin may induce cell death not only through FPN1 degradation, but also via mediating other signaling pathways. Thus, we proposed that targeting hepcidin directly could be an effective therapeutic approach to prevent or reduce the cell death that induced upon ischemic stroke. We have successfully transfected SH-SY5Y cells with hepcidin shRNA plasmid and generated hepcidin knockdown cell model. Hepcidin downregulation in SH-SY5Y cells significantly reduced the cell death following OGD/R conditions, indicating that targeting hepcidin transcription directly could be a beneficial candidate to rescue cells from ischemic insult.

Due to the short half-life of natural hepcidin (*minutes*), minihepcidins, biological hepcidin mimics, are commonly used to study the effect of bioactive hepcidin [141]. In our studies, we used minihepcidin to confirm the role of hepcidin in OGD/R-induced neuronal death. Treating hepcidin knockdown cells with minihepcidin induces cell death following OGD/R conditions. This indicates that minihepcidin diminished the protective effect of hepcidin knockdown in OGD/R-induced cell death. Interestingly, we also found that minihepcidin induced cell death in SH-SY5Y cells under normal conditions. Taken together, our data revealed that hepcidin plays a critical role in cell death upon ischemia. Most importantly, targeting hepcidin directly reduced the ischemia-

induced cell death. The question has been risen upon this investigation was how knockdown of hepcidin reduced cell death following OGD/R conditions.

ROS are reactive molecules that induce damage in macromolecules such as proteins and lipids. It has been found that ischemic conditions induce ROS production [146, 147]. Iron can involve in catalytic reactions, such as Fenton reaction and leads to generation of ROS [33, 192]. Hepcidin is the main player in brain iron regulation through mediating FPN1[57]. Ischemia-induced hepcidin limits iron export and further leads to intracellular iron accumulation [14]. Our data found that hepcidin knockdown significantly reduced OGD/R-induced ROS levels. To confirm the protective role of hepcidin knockdown in ROS production, we investigated that minihepcidin induces ROS levels in SH-SY5Y cells at normal and OGD/R conditions. In addition, minihepcidin diminished the protective effect of hepcidin knockdown on OGD/R-induced ROS production. Taken together, targeting hepcidin directly protects cells from ROS production following ischemic conditions.

Mitochondria plays a critical role in energy homeostasis. Many reports showed that mitochondrial dysfunction is a fundamental hallmark of ischemia-induced neuronal death [180, 193]. Maintaining mitochondria has been one of the promising therapeutic approaches for ischemia [194]. A study showed that excessive ROS production induces mitochondrial fragmentation and dysfunction, which further leads to cell death[180]. Our research found that the OGD/R-induced ROS in SH-SY5Y cells was associated with mitochondrial dysfunction. Since we found that hepcidin knockdown significantly reduced OGD/R-induced ROS levels, whether this approach improves mitochondrial dysfunction following OGD/R conditions. Interestingly, our study demonstrated that hepcidin knockdown improves mitochondrial respiration in SH-SY5Y cells upon OGD/R conditions. This protective effect of hepcidin knockdown was confirmed with minihepcidin treatment. We demonstrated that minihepcidin diminished the protective effect of

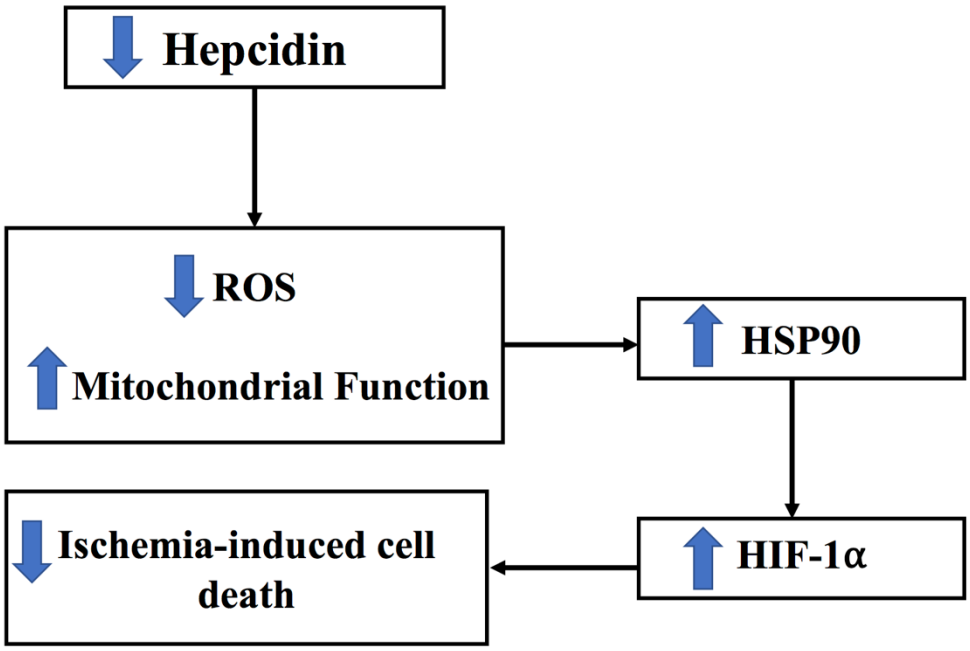
hepcidin knockdown on OGD/R-induced mitochondrial dysfunction. Taken together, reduction of ROS levels and improved mitochondrial dysfunction findings gave us a better understanding about the protective role of hepcidin knockdown in ischemic stroke.

HSP90 is a member of heat shock proteins that contributes to stability and folding of numerous protein clients. The role of HSP90 in regulation of cellular proteins has recently become a promising therapeutic target for different pathological conditions, including ischemic stroke [171, 172]. While the exact mechanisms underlying disruption of HSP90 machinery have been not fully investigated, promising evidence suggests that ROS is a major player in HSP90 degradation. HSP90 cleavage has been observed after oxidative stress [185]. A study showed that upregulation of HSP90 induces angiogenesis in ischemic mice [195]. Our current study demonstrated that hepcidin knockdown reduced OGD/R-induced ROS production and minihepcidin reversed this effect. The question of hepcidin role in ROS-induced HSP90 disruption arises. Our data indicated that hepcidin knockdown induced HSP90 expression following OGD/R conditions. However, minihepcidin diminished the protective effect of hepcidin knockdown on HSP90.

One of the important HSP90 protein clients is HIF-1α. HSP90 regulates HIF-1α stability and degradation. A previous study from our lab showed the existence of the interaction of HSP90 with HIF-1α in ischemic brains. Treating animal models of ischemic stroke with N-acetylcysteine (NAC), antioxidant, protects the brain of MCAO model from ischemic injury via upregulating HSP90 and increasing HIF-1α stability. In addition, inhibition of HSP90 attenuates the neuroprotective effect of NAC-induced HIF-1α. These findings indicate the critical role of HSP90-HIF-1α axis in neuroprotection against ischemic injury [196]. While the nature of HSP90/ HIF-1α interaction is not yet understood, PAS domain of HIF-1α is a promising target for HSP90 binding [174, 178]. Our data demonstrated the role of hepcidin in HSP90- HIF-1α axis. Hepcidin

knockdown induced HIF-1α expression following OGD/R conditions. However, minihepcidin treatment attenuated the increase of HIF-1α that induced by hepcidin knockdown. Our findings in this study suggested that hepcidin induces cell death following OGD/R conditions via increased ROS level that reduces HSP90/ HIF-1α induction. Therefore, targeting hepcidin seems to be a promising therapeutic approach for ischemic stroke injury (**Scheme 13**).

Future studies are needed to investigate the exact mechanistic effect of hepcidin on HSP90/ HIF-1α interaction. In addition, our data showed that there was harmful effect of hepcidin on mitochondria function. However, more research is required to explain the detailed mechanisms behind the mitochondrial alterations that are mediated by hepcidin. While we demonstrated in this study that hepcidin downregulation increases HSP90 and HIF-1α expression following OGD/R conditions, more research is needed to investigate whether this increase in expression is due to increased gene transcription or decreased protein degradation.



Scheme 13. Proposed mechanism to explain the role of hepcidin knockdown in ischemia-induced neuronal death. Our major findings indicate that hepcidin knockdown reduced ROS levels and improved mitochondrial function upon OGD/R condition. We proposed that reduction of OGD/R-induced ROS leads to increase HSP90 which further stabilizes HIF-1α. This collective effect reduces ischemia-induced cell death.

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