The Dual Role of Hypoxia-Inducible Factor-1 in Ischemic Stroke: Neuroprotection and Blood-Brain Barrier Disruption

By

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The Dual Role of Hypoxia-Inducible Factor-1 in Ischemic Stroke: Neuroprotection and Blood-Brain Barrier Disruption

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Abstract

Stroke is a major cause of death and the leading cause of long-term disability in industrialized countries. Ischemic stroke-induced brain injury results from the interaction of complex pathophysiological processes, including energy failure, calcium overload, excitotoxicity, oxidative stress, disruption of blood-brain barrier (BBB) and inflammation. Despite the wealth of knowledge regarding the cellular and molecular mechanisms underlying neuronal death after stroke, research for several decades has failed to develop an effective and safe neuroprotective treatment. One complicating factor in the development of neuroprotective strategies is the dual nature of many of the processes that occur in the brain during stroke.

Hypoxia-inducible factor 1 (HIF-1) is a master regulator of cellular and tissue adaption to hypoxia. It plays both protective and detrimental roles in ischemic stroke by inducing a wide array of target genes involved in angiogenesis, erythropoiesis, cell survival/death, and energy metabolism. The dual face of HIF-1 in the pathophysiology of cerebral ischemia is postulated to partially depend on thedifferent functions of its target proteins in specific type of brain cells. In the current studies, we hypothesize that neuronal HIF-1 accumulation is protective whereas endothelial HIF-1 induction is implicated in BBB disruption.

We first evaluated HIF-1's role in the antioxidant N-acetylcysteine (NAC)-mediated neuroprotection in a transient cerebral ischemia animal model. The study demonstrated that pre-treatment of NAC increased the neuronal expression of HIF-1α, the regulatable subunit of HIF-1, and its target proteins erythropoietin (EPO) and glucose transporter (GLUT)-3 in the ischemic brain of rodents subjected to 90 min middle cerebral artery occlusion (MCAO) and 24 h reperfusion. Suppressing HIF-1 activity by pharmacological inhibitorsor by specific knock-out neuronal HIF-1α abolished NAC's neuroprotective effects. Furthermore, we observed that NAC increased HIF-1α stability through enhancingits interaction with heat-shock protein 90 (Hsp90) in ischemic brains.

Increased BBB permeability and associated cerebral edema formation are potentially lethal complications of ischemic stroke. Accumulating evidence has shown that admission hyperglycemia in

conjunction with ischemia/reperfusion causes exacerbated cerebrovascular endothelial cell dysfunction and increased BBB permeability, which leads to augmented brain edema and hemorrhagic transformation in ischemic stroke.The hypothesis of the second part of this dissertation is that endothelial HIF-1 is implicated in hyperglycemia-exacerbated BBB disruption after ischemia. Both *in vitro* and *in vivo* studies were undertaken to investigate the effect of hyperglycemia on (1) HIF-1α and its target genes expression; (2) ischemia-induced BBB permeability change; and (3) the effect of HIF-1α inhibition on BBB permeability after ischemia. The *in vitro*study showed that high glucose enhanced HIF-1αand its downstream factors expression in the endothelial cell culture after oxygen glucose deprivation (OGD)/reoxygenation. This was correlated withan increased paracellular permeability as well as diminished expression and disrupted continuity of tight junction (TJ) proteins. Suppressing HIF-1 activity by HIF-1α inhibitors ameliorated the alterations in paracellular permeability and expression and distribution pattern of TJ proteins induced by high glucose exposure. In *in vivo* studies, diabetic mice subjected to 90 min MCAOfollowed by reperfusiondemonstrated higher expression of HIF-1α and its target gene vascular endothelial growth factor (VEGF) in the ischemic brain microvessels than non-diabetic control mice. Diabetic mice also showed exacerbated BBB damage and TJ disruption, increased infarct volume, and worsened neurological deficits. SuppressingHIF-1 activity by specific knock-out endothelial HIF-1α ameliorated BBB leakage and brain infarction in diabetic animals.

Taken together, these present studies provide new information concerning HIF-1's function in experimental models of acute ischemic stroke. Neuronal HIF-1α is an important mediator of antioxidant NAC's neuroprotective effect in ischemic stroke, whereas endothelial HIF-1α is involved in hyperglycemia-induced BBB breakdown after cerebral ischemia. The results suggest that developing therapeutic strategies by targeting HIF-1 needs to consider its multifunctional roles and differential effects on different cell types.

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List of Abbreviations

17-AAG	17-allylamino-17-demethoxygeldanamycin
2ME2	2-methoxyestradiol
2-OG	2-oxoglutarate
AJ	Adherens junction
AM	Adrenomedullin
AMDA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AQP-4	Aquaporin-4
ARNT	Aryl hydrocarbon receptor nuclear transporter
ASA	American Stroke Association
ASA	Acetylsalicylic acid
Bax	Bcl-2-associated X protein
BBB	Blood–brain barrier
BCCAO	Bilateral common carotid artery occlusion
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BINP3	Bcl-2/adenovirus EIB 19kDa-interacting protein 3
BSA	Bovine serum albumin
BSO	L-buthionine sulfoxide
CAMKII	Ca2+ /calmodulin-dependent protein kinase II
CBF	Cerebral blood flow
ChREBP	Carbohydrate response element binding protein
CNS	Central nervous system
Co-IP	Co-immunoprecipitation
DFO	Desferrioxamine
DMEM	Dulbecco's modified Eagle medium
DMOG	Dimethyloxaloylglycine
DMSO	Dimethyl sulfoxide
EB	Evans blue
ECA	External carotid artery
ECM	Extracellular matrix
EPO	Erythropoietin
ET-1	Endothelin-1
FIH	Factor inhibiting HIF
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte colony-stimulating factor
GLUT	Glucose transporter
GSH	Glutathione
HAF	Hypoxia-associated factor
HIF	Hypoxia-inducible factor

HO	Hemo oxygenase
HRE	Hypoxia response element
Hsp90	Heat shock protein 90
i.p. injection	Intraperitoneal injection
ICA	Internal carotid artery
ICAM-1	Intercellular adhesion molecule-1
IgG	Immunoglobulin G
IL-1	Interleukin-1
JAM	Junction adhesion molecule
LDF	Laser Doppler flowmetry
LDH	Lactate dehydrogenase
MAGUK	Membrane-associated guanylate kinase-like
MCAO	Middle cerebral artery occlusion
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
Mtor	Mammalian target of rapamycin
NAC	N-acetylcysteine
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NF-κB	Nuclear factor-κB
NIX	Nip-like protein X
NMDA receptor	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NSC	Neural stem cell
NVU	Neurovascular-unit
ODD	Oxygen-dependent degradation
OGD	Oxygen glucose deprivation
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PHDs	Prolyl hydroxylases
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
Pvhl	von Hippel-Lindau
RACK1	Receptor of activated protein kinase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
STZ	Streptozotocin
SUMO	Small ubiquitin-like modifier
SVZ	Subventricular zone
TAD	Transactivation domain

TJ	Tight junction
TNF-α	Tumor necrosis factor-α
tPA	Tissue plasminogen activator
TTC	2,3,5-triphenyltetrazolium chlorid
WHO	World Health Organization
YC-1	3-(5’-hydroxymethyl-2’-furyl)-1-benzylindazole
ZO	Zona occluden

CHAPTER 1: Introduction

1.1 Overview of stroke

Stroke is the second cause of mortality worldwide and the leading cause of disability in industrialized countries. According to World Health Organization (WHO), 17 million people worldwide suffered a stroke in the year 2010, 60% of those who suffered a stroke died or became dependent on others. A report released by American Stroke Association (ASA) indicated that stroke would become the first leading cause of death in the world by 2030, with an estimated 23 million first-time strokes and an associated 7.8 million deaths [1]. Stroke remains the third leading cause of death in the U.S. It is estimated that there are approximately 800,000 primary (first-time) or secondary (recurrent) strokes occurring each year in the U.S. resulting more than 140,000 deaths[2].

The mental and physical handicaps among the patients surviving stroke are a major problem in terms of quality of life and socioeconomic cost. Approximately 20% of stroke survivors require institutional care after 3 months, and 15% to 30% are permanently disabled (AHA, 2003). In the U.S., the total direct and indirect cost of stroke has been estimated at $65.5 billion in 2008, and the mean lifetime cost of ischemic stroke is estimated at $140,048 per patient[3].

1.1.1 Defining stroke

A stroke refers to any brain injury caused by a disturbance in blood supply resulting in neurological deficits[4]. It is defined by WHOas “rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or leading to death, with no apparent cause other than of vascular origin.” The two basic types of stroke are ischemic stroke and hemorrhagic stroke. In the U.S., approximately 87% of strokes are ischemic stroke[3], which results from the development of atherosclerotic thrombi or distant emboli that decrease or completely obstruct cerebral circulation thereby causing neuronal injury and death. Hemorrhagic stroke is less prevalent but more likely to be fatal. It occurs from a rupture in cerebral vessels and can be further subclassified into intracerebral hemorrhage and subarachnoid hemorrhage. It is important to realize that a stroke is not an isolated incidence within the

brain. The primary injury to neural tissue is typically the beginning of a complex and devastating series of events that last over days and even months; these events include cytotoxic and vasogenic cerebral edema and secondary pro-inflammatory, excitotxic, oxidative stress, necrotic and apoptotic cascades that lead to progressive brain damage.

1.1.2 Risk factors

Prevention remains the most viable avenue for lessening the burden of stroke on society, particularly given the high incidence of stroke worldwide, insidious contribution of stroke risk factors, and the lack of proven acute stroke therapies[5]. Longitudinal studies have identified several characteristics or conditions that boost a person's risk for primary and recurrent stroke[6,7]. These risk factors are viewed as non-modifiable encompassing factors related to heredity or natural processes, and modifiable risk factors which are amenable to intervention for lower stroke risk. Major non-modifiable risk factors include age, sex, race-ethnicity, and genetic factors. The most significant risk factor for stroke is age; two-thirds of strokes occur in those over the age of 65[8]. Stroke is generally more prevalent in men than in women[9]. Hispanics and blacks have a higher prevalence and incidence of stroke[4]. Modifiable risk factors include those resulting from lifestyle choices and the environment, and can be modified with the help of healthcare professionals and treatment. An international case-control study showed that roughly 90% of strokes could be explained by 10 modifiable risk factors: 1) hypertension, 2) diabetes, 3) cardiac causes, 4) current smoking, 5) obesity, 6) hyperlipidemia, 7) physical inactivity, 8) heavy alcohol consumption, 9) diet, and 10) psychological stress and depression [10].

While non-modifiable risk factors are difficult to prevent or treat, the modifiable risk factors (e.g. hypertension, dyslipidemia, smoking, diet) can be managed through lifestyle adjustments and select pharmacological and surgical interventions to reduce the likelihood of developing a stroke. Diabetes is one of the aleading risk factors for stroke. It is estimated that nearly 40% of all ischemic strokes can be attributed to the effects of diabetes either alone or in combination with hypertension[11]. The association of diabetes and ischemic stroke will be discussed in the section 1.2.6.

1.1.3 Pathophysiology of ischemic stroke

Acute ischemic stroke results from sudden decrease or loss of blood circulation to an area of the brain, resulting in a corresponding loss of neurological function[12]. Within minutes of vascular occlusion, a complex sequence of pathophysiological spatial and temporal events occur, which is referred to as the ischemic cascade. Since one event in the cascade can cause or can be caused by multiple other events and since cells suffering from different severity of ischemia may go through different chemical processes, the ischemic cascade actually is a highly heterogeneous phenomenon. Nevertheless, it can be summarized as energy failure, followed by loss of cell ion homeostasis, excitotoxicity, oxidative stress, blood–brain barrier (BBB) dysfunction (reviewed in section 1.3), post-ischemic inflammation and finally cell apoptosis of neurons, glia and endothelial cells[13]**(Fig. 1-1)**.

Ischemic stroke begins with dramatic blood flow reduction, but cerebral injury continues over hours or even days. The amount of permanent damage will depend on two factors: the degree and the duration of ischemia. Regions of the brain tissue exposed to severely impaired blood flow become rapidly and irreversibly injured and subsequently undergo necrotic cell death[14]. This region is referred as the ischemic core. The core is surrounded by a zone of less severely affected tissue which is known as penumbra. The penumbra is rendered functionally impaired by reduced blood flow but remains structurally intact and metabolically active[15]. It is the battle field where the ischemic cascade with several deleterious mechanisms is triggered, resulting in ongoing cellular injury and infarct progression. While necrosis is more dominant in the core tissue, penumbral cells are more likely to undergo apoptosis after several hours or days[16], and thus they are potentially recoverable for some time after the onset of stroke. Since salvage of this tissue is associated with neurological improvement and recovery, it is the target for acute stroke therapy[17].

Brain tissue has a relatively high consumption of oxygen and glucose, and depends almost exclusively on oxidative phosphorylation for energy production. The first consequence of cerebral blood flow (CBF) reduction is the depletion of substrates, particularly oxygen and glucose, which causes

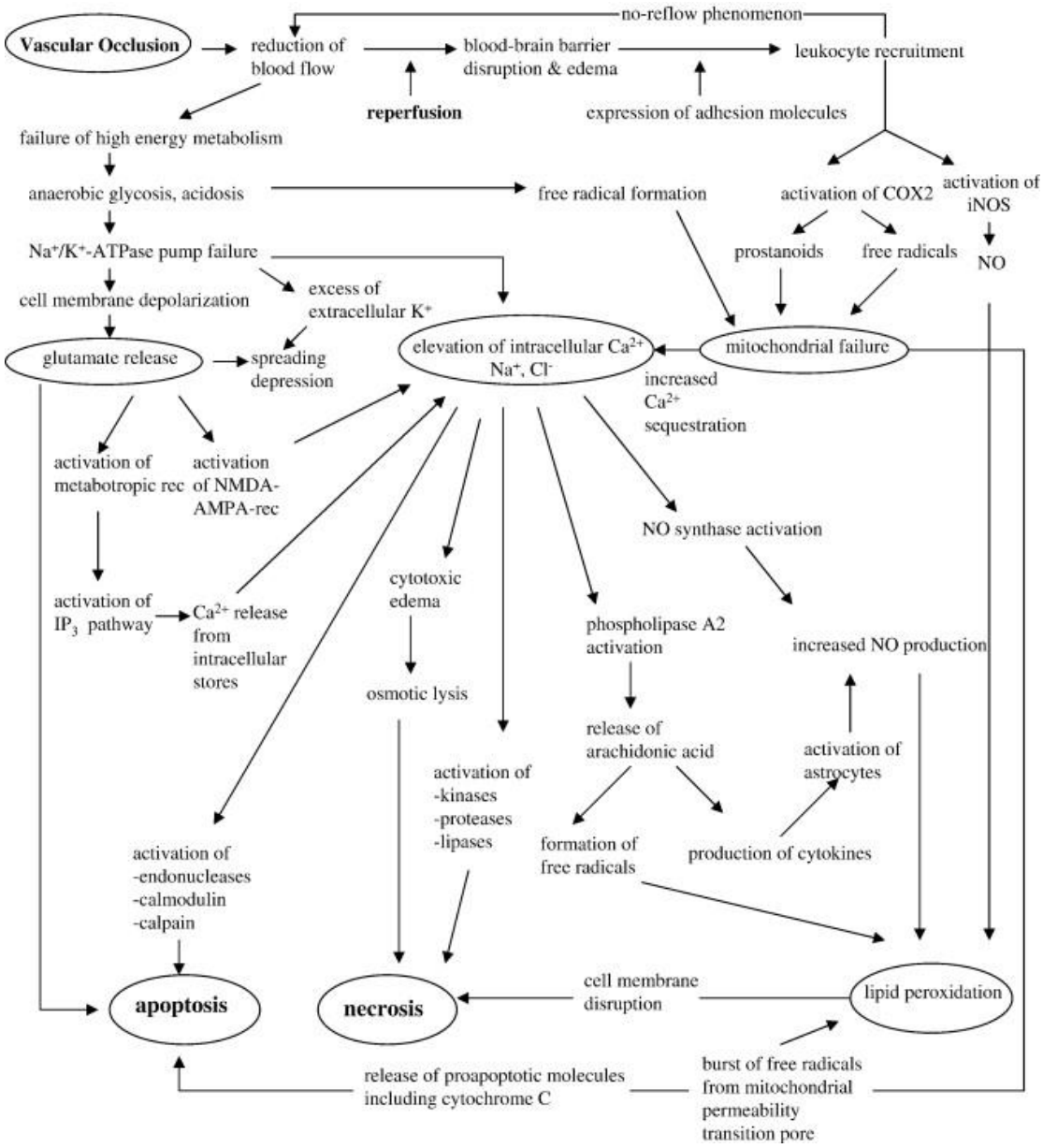

Fig. 1-1 Diagram depicting major events that occur after ischemic stroke. The figure is not fully comprehensive of all events (cited from Aysan Durukan*et al.*, 2009).

accumulation of lactate via anaerobic glycolysis. Acidosis may enhance free radical formation, interfering with intracellular protein synthesis and worsen ischemic brain injury[18]. Energy failure leads to dysfunction of energy-dependent ion transport pumps and depolarization of neurons and glia[19]. Consequently, voltage-dependent Ca^{2+} channels become activated and excitatory amino acids are released into the extracellular space. At the same time, reuptake of excitatory amino acids by presynaptic axons and astrocytes is impeded due to energy failure[20]. This further increases the accumulation of glutamate in the extracellular space. The build-up of glutamate results in activation of its receptors (e.g. AMPA-, NMDA-type glutamate receptors) on other neurons, with consequent influx of Na^{+}, Cl^{-} and Ca^{2+} ions through the channels gated by these receptors[21]. These neurons then become depolarized, causing more calcium influx and more glutamate release leading to local amplification of the initial ischemic insult. This spreading depression-like depolarization starts from ischemic core and extending outwards to surrounding tissue, leading to the recruitment of penumbral tissue into the infarct[22]. Additionally, water passively follows the ion influx, resulting in cytotoxic edema. Intracellular increase of Ca^{2+} causes generation of free radicals and activation of Ca^{2+}-dependent enzymes including protein kinase C (PKC), phospholipase A2, phospholipase C, cyclooxygenase (COX), calcium-dependent nitric oxide synthase, calpain and various proteases and endonucleases[23]. Both necrotic and apoptosis are triggered by excess of intracellular Ca^{2+}as a result of formation of cytotoxic products such as free radicals, irreversible mitochondrial damage, and inflammation.

Numerous experimental and clinical observations have shown that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are important mediators of tissue injury in acute ischemic stroke[24]. The primary sources of ROS during ischemic stroke injury are the xanthine oxidase and mitochondria which produce superoxide anion radicals during the electron transport process[24]. Another potentially important source of superoxide in post ischemic neurons is the metabolism of arachidonic acid through the COX and lipoxygenase (LOX) pathways[25]. Oxygen free radicals can also be generated by activated microglia and infiltrating peripheral leukocytes via the NADPH oxidase system following reperfusion of ischemic tissue[26]. In addition, nitric oxide (NO) is generated from L-arginine through one of several

isoforms of NO synthase (NOS)[27]. The activity of neuronal NOS (nNOS) and inducible NOS (iNOS) is damaging to the brain under ischemic conditions[28]. NMDA receptor activation has been shown to stimulate NO production by nNOS, which possibly plays a role in excitotoxic-mediated injury in ischemic stroke[29]. NO diffuses freely across membranes and can react with superoxide to produce peroxynitrite anion, another highly reactive free radical[30]. Both ROS and RNS exhibit a spectrum of cellular effects including inactivation of enzymes, release of Ca^{2+} from intracellular stores, protein denaturation, lipid peroxidation, and damage to the cytoskeleton and DNA. Mitochondrial function is impaired by free radical-mediated disruption of the inner mitochondrial membrane and the oxidation of proteins that mediate electron transport, H^+ extrusion and ATP production. Cytochrome C is released from mitochondria and provides a trigger for apoptosis[13]. Severe oxidative stress causes cell death through necrosis while moderate oxidation can result in apoptosis.

Ischemic injury triggers inflammatory cascades in the brain parenchyma that may further amplify tissue damage by many mechanisms. There are several resident cell populations within brain tissue that are able to secrete proinflammatory mediators after an ischemic insult. First of all, microglia and astrocytes are activated by ROS and are capable of secreting inflammatory factors such as cytokines (e.g. interleukin-1, tumor necrosis factor-α) and chemokines (e.g. monocyte chemoattractant protein-1)[31]. Within hours after stroke onset, adhesion molecules expression on endothelial cells and leukocytes increases upon stimulation by proinflammatory factors. The adhesion molecules mediate the interaction between leukocytes and the vascular endothelium and promote leukocyte recruitment[32]. Circulating leukocytes adhere to vessel walls and migrate into the brain. This leads to the release of more pro-inflammatory mediators, especially excessive production of ROS, that amplify the brain-inflammatory responses by causing more extensive activation of resident cells. The infiltration of leukocytes eventually leads to secondary injury of potentially salvageable tissue in the penumbra[33]. Neutrophils are the first inflammatory cells to infiltrate areas of brain ischemia as early as within hours after reperfusion[34]. Macrophages and monocytes arrive within few days. Whereas microvascular obstruction by neutrophils (no-reflow phenomenon) can worsen the degree of ischemia, production of toxic mediators by activated

inflammatory cells and injured neurons (cytokines, NO, superoxide and prostanoids) can amplify tissue damage by inducing BBB disruption, brain edema, neuronal death, and hemorrhagic transformation. In addition, the inflammatory reaction might also be linked to apoptosis[19]. The overall role of inflammation may differ at different time points following stroke insult, with protective or regenerative activities occurring days to weeks after the onset of ischemia[35].

Necrosis is the predominant mechanism of cell death that follows acute, permanent vascular occlusion, whereas milder injury, particularly within the ischemic penumbra often results in apoptosis. If the cell dies through necrosis, it releases more glutamate and toxins into the environment, affecting surrounding neurons. In parallel, apoptosis is a genetically regulated cell program that allows cells to die without eliciting an inflammatory reaction[36]. Several factors determine which process predominates, including the local degree of ischemia, cell maturity, the concentration of intracellular free Ca^{2+} and the cellular microenvironment[37]. Activation of glutamate receptors, early mitochondrial production of ROS, and reduction of intracellular K^{+} may trigger apoptosis. Following ischemia, caspase-mediated apoptosis occurs in response to pro-apoptotic signals such as release of cytochrome Cfrom mitochondria, and downregulation of Bcl-2 and upregulation of the Bax/Bid and Death receptor family[38]. Activated caspases are protein-cleaving enzymes that modify crucial homeostasis and repair proteins. Especially caspases 1 and 3 seem to play a pivotal role in ischemia-mediated apoptosis but other caspase-family members might be important in the late stages of cell death[39]. Apoptosis is an energy-consuming process, so reperfusion could potentiate apoptosis by restoring cellular energy[40].

Prompt restoration of the blood supply can reduce infarct size through salvation of the penumbra and can improve clinical outcome in patients with ischemic stroke[40]. Paradoxically, reperfusion may exacerbate the brain injury and produce a so-called cerebral reperfusion injury[41]. Reperfusion injury triggers alterations in production of various cytotoxicsubstances, including free radicals, excitatory amino acids, free fatty acids, proinflammatory cytokines, and adhesion molecules. Leukocytes appear to play a critical role in reperfusion injury through damaging of the endothelium, obstruction of the microcirculation, disruption the BBB and infiltration in the brain tissue where they release cytokines and

propagate inflammation[42]. Platelets play a synergistic role with leukocytes in reperfusion injury via the "no-reflow phenomenon" and release a variety of biochemical mediators that may lead to vasospasm and exacerbation of oxidative stress and the inflammatory cascade[43].

1.1.4 Current treatment of ischemic stroke

Today, the only Food and Drug Administration(FDA)-approved stroke treatment is intravenous application of recombinant tissue plasminogen activator (rtPA), which induces the thrombolysis of occluded vessels. Thrombolysis is the breakdown of blood clots by pharmacological means. Clearing the cross-linked fibrin mesh (the backbone of a clot) makes the clot soluble and subject to further proteolysis by other enzymes, thereby restoring blood flow in the occluded blood vessel. This treatment was first described in 1995 in the NINS-study (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group (NINS 1995)) showing thatdespite a slightly increased incidence of symptomatic intracerebral hemorrhage, treatment with intravenous rtPA within 3 h of the onset of ischemic stroke improved clinical outcome at three months. The time window of application was extended to up to 4.5 h after acute stroke in 2008 (ECASS III). Major limitations of rtPA are the limited time window in which reperfusion therapies can be initiated and the presence of exclusion criteria (e.g., medically uncontrollable hypertension, anticoagulation, presence of signs of hemorrhage). As such, only < 5% patients get benefit from the clot dissolving agent. Almost two decades after FDA approval, intravenous rtPA remains the only approved treatment for stroke with no increase in the risk of death. However, the associated side effects, specifically symptomatic intracerebral hemorrhage complications have to be taken into account critically.

Long-term stroke therapy is restricted to secondary prevention, symptomatic therapy, rehabilitation as well as psychosocial support. Medications that are considered 24h after the treatment with rtPA or in patients who were not thrombolyzed include acetylsalicylic acid (ASA), clopidogrel, ticlodipine or the combination of dipyridamole-ASA. Secondary prophylaxis includes the antihypertensive treatment with ACE inhibitors, angiotensin II AT1 receptor antagonists, beta blockers, diuretics and calcium entry blockers, the maintenance of a normal blood glucose level with oral

antidiabetics in case of patients suffering from type II diabetes, the treatment of hypercholesterolemia with HMG-CoA reductase inhibitors (statins) and the treatment of hyperhomocysteinemia with folic acid. Statins, specifically high-dose atorvastatin, have been demonstrated to reduce the recurrence of stroke in patients with previous stroke or transitory ischemic attack and are yet established in the clinic.

There is a wide variety of pharmacological tools available for the prevention and symptomatic treatment of stroke, however, an effective, safe and well-tolerated compound that directly interacts with the ischemic cascade and thus minimizes neurological deficits, does not exist on the market so far. This fuels the interest in the development of neuroprotective therapies.

1.1.5 Animal models of stroke

Animal research remains critical to the understanding ofthe basic mechanisms of ischemic damage and functionalrecovery thereafter. The use of appropriate animal models is essential to predict the value and effect of therapeutic approaches in diseases that are common in human subjects. Rodents are mostly often utilized in experimental stroke research because of several reasons: the resemblance to humans in cerebrovascular anatomy, moderate size allowing easy manipulations, low costs, the relative homogeneity within strains, and the accessibility for use by transgenic technology. There are two fundamentally different rodent models of cerebral ischemia which lend important insights into the pathophysiology of ischemic stroke[18]. One model is *focal* cerebral ischemia which clinically corresponds to ischemic stroke. The other model, *global* cerebral ischemia,mimics the consequences of cardiac arrest. The central goal of these cerebral ischemia models is to reduce oxygen and nutrient/glucose supply to brain tissue. This section mainly focuses on models of focal cerebral ischemia with an emphasis on highlighting a few examples of rodent models.

In focal ischemic stroke models, the middle cerebral artery (MCA) is the most commonly occluded vessel because majority of all strokes occur in the territory of this artery[44]. There are two models of focal ischemic stroke, 1) transient focal ischemia and 2) permanent focal ischemia. In transient focal ischemia models, vessels are blocked for periods of up to 3 hours, followed by prolonged reperfusion; whereas, in permanent focal ischemia, the arterial blockage is maintained throughout an experiment,

usually for one or more days. Ischemic lesion size varies greatly according to the ischemia duration. To obtain reproducible infarct volumes, minimum 60 to 90 min of ischemia is required and for this reason, transient focal ischemia models are usually based on 90–120 min of ischemia. It is well known that lesions induced by more than 3 h of focal ischemia are not reversible[21]. Permanent stroke models permit the study of cerebral ischemia without the effect of reperfusion. When an occluded artery is recanalized in a model of transient cerebral ischemia, as it happens in most of human stroke, consequences of reperfusion to the ischemic territory (i.e. reperfusion injury) can be evaluated[45,46].

There is a rich diversity of focal ischemia models, among which none is capable in mimicking all aspects of human stroke, but the most appropriate model can be chosen to address a specific question. Among different animal models available for focal cerebral ischemia induction, those meeting thefollowing criteria may be moresuitable: (1) the ischemic processes and pathophysiologic responses should be relevant to human stroke, (2) the ischemic lesion size should be reproducible, (3) the technique used to perform the modeling should be relatively easy and minimally invasive, (4) physiologic variables can be monitored and maintained within normal range, (5) brain samples should be readily available for outcome measurements, such as histopathological, biochemical, and molecular biological evaluation, and (6) the cost and effort should be reasonable[21].

Suture occlusion of the MCA

Among experimental ischemic stroke models, the intraluminal suture MCAO in rats and mice is the most frequently used model. This model allows strict control on the timing of the reperfusion and is less invasive and easy to perform both permanent and transient ischemia in a controlled manner. The intraluminal suture MCAO model involves inserting a monofilament into the internal carotid artery and advancing it until it blocks blood flow to MCA **(Fig 1-2)**. This model provides reproducible MCA territory infarctions involving both frontoparietal cortex and striatum and allows reperfusion by retracting the suture. Lesion reproducibility and size seem to be affected by many specific factors in this technique such as suture diameter[47], coating of the suture (with silicone or poly-l-lysine), or insertion length of the thread[48,49]. In contrast to uncoated suture, silicone-coated suture was shown to cause larger and more

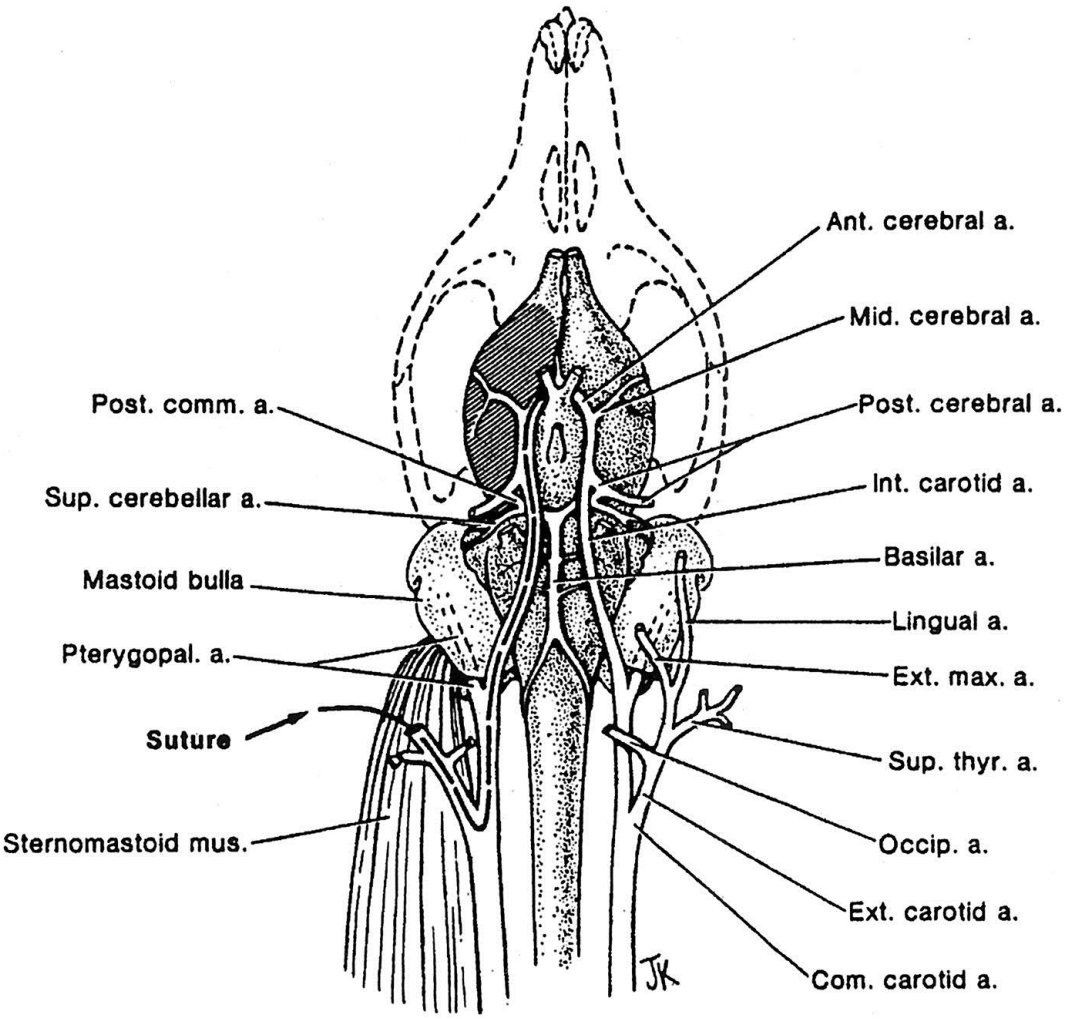

Fig. 1-2 Anatomy of MCAO.This illustration shows occlusion on the left common external and internal carotid arteries and their branches with the approximate region of MCAO shaded in gray. This illustration was cited from Zea Longa *et al.* (Zea Longa *et al.*, 1989).

consistentinfarctswith good reproducibility and reliability even among investigators of varying experience[50-52].

Disadvantages of the suture occlusion method include: (1) vessel rupture and subsequent subarachnoid hemorrhage, (2) hyperthermia, and (3) inadequate MCAO. Silicone coating of the suture and laser Doppler-guided placement of the suture could reduce the incidence of subarachnoid hemorrhage[53]. Spontaneous hyperthermia can be avoided by limiting ischemia duration to 90 min or less or adjusting suture tip to a size that does not occlude the hypothalamic artery[54].

Thromboembolic models

Thromboembolic models use two main strategies to induce stroke: injecting clots that are formed *in vitro*[55] or endovascular instillation of thrombin for in situ clotting[56]. It is the closest to the pathophysiology of human stroke since most of the human strokes are caused by thromboembolism. Other advantages of thromboembolic models are their potential to test new thrombolytic agents and combination therapies of thrombolysis and neuroprotection for acute stroke[57,58].

Thromboembolic ischemia is induced most commonly by the injection of autologous thrombi into extracranial arteries to reach the more distal intracranial arteries[59,60]. Originally, human blood clots or suspensions of homologous small clot fragments were used to produce embolism[61]. In these earlier embolism models, infarcts induced by these methods were variable in size and early spontaneous recanalization took place[62,63]. Autolysis resistant fibrin-rich emboli resembling human arterial thrombi were developed to achieve a consistent reduction of CBF and infarcts without any spontaneous recanalization[64,65].

Many compounds and artificial embolic materials, such as viscous silicone[66], collagen[67], polyvinylsiloxane[68], retractable silver ball[69], and heterologous atheroemboli [70]have been used to induce ischemia by injection into common carotid artery or internal carotid artery. When injecting spheres into the cerebral circulation, their size determines the pattern of brain infarction. Macrospheres induce infarcts similar to those achieved by occlusion of the proximal MCA[71], whereas the injection of microspheres results in distal, diffuse embolism and the multifocal and heterogenous developing lesions[72].

Other models

In **photothrombosis models,** the animals were intravenously injected with a photoactive dye (most often Rose Bengal) and a cortical brain area is irradiated by a light beam at a specific wavelength through the intact skull[73].Light activation of the dye causes formation of freeradicals and endothelial cell damage, aggregation ofplatelets and eventually occlusion of the vessel[74].The region of irradiation can be determined so as to induce ischemic lesion in any desired cortical area.The main advantage of the photothrombosis models istheir noninvasive operation, which produces a consistentinfarct with a precise location and sizeand low mortality. The unique feature in the photothrombotic models is occlusion of smallcortical vessels, so major arteries or branches are notaffected. Photothrombosis induces vasogenic edema and BBB breakdown in the lesion within minutes, thus, there is not much penumbral area or collateral flow which is deemed important for neuroprotectionand recovery processes[75]. Therefore, the model is undesirable for preclinical therapies if the chief target is penumbra or enhancement of collateral perfusion.

Endothelin-1 (ET-1) is a potent vasoactive peptide, which produces a marked vasoconstriction[76]. ET-1 application onto MCA provides significant decreases in CBF in the MCA territory, resulting in an ischemic lesion pattern similar to that induced by direct surgical MCAO[77,78]. Direct cortical application of ET-1 provides sufficient reduction in blood supply of frontoparietal cortex and induces a semicircular infarct involving all layers of the neocortex[79]. Less invasiveness and ability to induce ischemia in any desired region of the brain are the chief advantages of endothelin-1 application, but dose dependent action of endothelin-1 reduces the control on ischemia duration and intensity.

Measurement of ischemic stroke damage in animal models

The size of the brain infarct in focal cerebral ischemia increases during the period of reperfusion. This has been shown in animal models of stroke and in human stroke patients[80]. The infarct volume is normally analyzed after 12-24 hours in transient and permanent focal ischemia models. The brain is removed and coronal sections are cut (2 mm-thick slices in rats or 1-2 mm thick slices in mice) through the entire rostro-caudal extent of the cerebral cortex. The slices are immersed in a solution of 2,3,5-

triphenyltetrazolium chloride (TTC). Image analyzing systems allow manual or automated delineation of the lesion area, after which lesion volume is calculated by multiplying by the slice thickness. The enlargement of the injured tissue by edema results in overestimation of the infarct volume. Thus, the actual infarcted lesion size should be calculated with a correction for edema[81].

In vivo magnetic resonance imaging (MRI) enables monitoring lesion progression by repeated imaging. With DWI sequence ischemic lesion can be identified as early as 3 min after the onset of ischemia[82] and MRI-based lesion volume correlates well with the TTC-based infarct volume[83].

The functional consequences of focal ischemic stroke injury are evaluated using a 7-point scale neurological deficit score (0=no neurological deficit; 1=failure to extend right forepaw fully; 2=decreased grip of the right forelimb while tail gently pulled; 3=spontaneous movement in all directions, contralateral circling only if pulled by the tail; 4=circling or walking to the right; 5=walks only when stimulated; 6=unresponsive to stimulation with a depressed level of consciousness)[84]. More recently a 14 point neurological scoring system was developed. This new scoring method includes the results of motor, reflex and balance tests; a single point is awarded for the inability to perform the test or for the lack of a tested reflex[85].

1.1.6 Lost in translation: taking neuroprotection from animal models to clinic

Neuroprotection for ischemic stroke refers to any strategy, or combination of strategies, thatantagonizes, interrupts or slows the sequence of injurious biochemical and molecular events that eventuate in irreversibleischemic injury[86]. A large number of potentially neuroprotective agents directed at different harmful mechanisms in the ischemic cascade have been investigated in experimental animal stroke studies. The majority of the substances which were found to be neuroprotective in animals have failed in clinical trials. The inconsistency between animal results and clinical trials may be due to several factors including: the heterogeneity of human stroke, morphological and functional differences between the brain of humans and animals, the relatively long post-stroke delay in administration of the drugs in clinical trials, the better control of physiological variables such as temperature, blood pressure, and differences in

evaluating efficacy in animal models. A brief summary of some of the key neuroprotective agents that have been tested in clinical trials is provided below.

It is well-established that ischemia causes the release of glutamate and excess glutamate release, with activation of NMDA receptors, is a pivotal event in the evolution of irreversible ischemic damage in animal models of ischemia. Cell death cascades in ischemic stroke are mediated, in part, by excessive Ca^{2+} influx resulting from activation of glutamate receptors and voltage-dependent calcium channels. In addition, the function of Ca^{2+}-ATPase is compromised, resulting in prolonged elevation of the intracellular Ca^{2+} concentration. Several compounds that block glutamate receptors(e.g. MK-801) or voltage-dependent calcium channels (e.g. nimodipine) have been developed and tested against experimental animal models of stroke as well as in human clinical trials. **Nimodipine** is a 1,4-dihydropyridine calcium channel antagonist. At least 14 clinical trials of nimodipine in ischemic stroke were conducted, with nine trials finding no effect, one trial finding short-term worsened outcome with treatment, and four trials finding positive outcomes[87]. Both **MK-801** and **dextromorphan**, two noncompetitive NMDA receptor antagonists, exhibited protective effects in experimental studies, but clinical trials were terminated early because of phencyclidine-like psychotic side effects and lack of efficacy against stroke injury[88]. Some other noncompetitive (**aptiganel**) or competitive (**selfotel, eliprodil**) NMDA receptor antagonists were tested in clinical trials and have shown no efficacy or an increase in the adverse event to benefit ratio[87]. **Zonampanel** (YM-872) is an AMPA antagonist tested in human phase II clinical trials in conjunction with tPA thrombolysis[89]. **Magnesium** may play a protective role in a variety of ways, including the NMDA receptor blockade, the inhibition of excitatory neurotransmitter release and the blockade of calcium channels[90]. Giving magnesium to stroke patients soon after symptoms began was shown to be safe in a small, open-label pilot trial. Good functional outcomes after 90 days were achieved in 69% of all patients and in 75% of those treated within 2 hours[91]. A large randomized trial did not show any beneficial effect of magnesium sulfate on death and disability; however, it slightly increased mortality[92]. Interestingly, the investigators revealed a beneficial effect in a subgroup of patients with lacunar strokes.

As mentioned above, free radicals are produced in ischemic brain following stroke injury and have a significant pathogenetic role in cerebral tissue damage. Removal of pathologically produced free radicals is therefore a viable approach to neuroprotection. **Tirilazad** is a free radical-induced lipid peroxidation inhibitor. The clinical trials showed no benefit of tirilazad despite of broad evidence of acting as a neuroprotective drug in animal models of stroke[93]. **Edaravone** is a free radical scavenger which has been approved for ischemic stroke treatment in Japan since 2001. It has been studied in a major phaseIII clinical trial on 252 patients. The study reported an enhanced functional outcome of patients treated with edaravone at three months. Further studies have been performed yielding controversial results from large to only modest or no clinical improvements[94]. **Ebselen** is a selenium compound with glutathione peroxidase-like activity; it also reacts with peroxynitrite and inhibits a variety of enzymes. A clinical trial of 300 acute ischemic stroke patients revealed that ebselen treatment achieved a significantly better outcome than placebo at 1 month but not at 3 months[95]. A phase III trial exploring the efficacy of esbelen in patients with a cortical infarct is currently ongoing[96]. **NXY-059** is a nitrone-based free-radical-trapping agent [94]. The first clinical trial of NXY-059 (SAINT I) has reported a small but significant benefit. However, the subsequent SAINT II which included a higher number of patients failed to show substantial efficacy[93]. **Lubeluzole** is thought to act by inhibiting NO production[97]. A series of phase I to III trials were conducted to examine its therapeutic efficacy for ischemic stroke.A recent phase III trial failed to show significant benefits compared to placebo[98].

Inflammation in stroke is characterized by the accumulation of leukocytes and activation of resident microglial cells. Cell adhesion molecules such as selectins, integrins, and intercellular adhesion molecule (ICAMs) permit endothelial-inflammatory cell interactions. **Enlimomab** is a murine ICAM-1 antibody that reduces leukocyte adhesion. A recent clinical trial using enlimomab showed worsened neurologic score and mortality in patients, which was probably due to immune activation in response to the foreign mouse protein[99,100]. In a related approach, neutrophil activation was blocked by a recombinant protein inhibitor of the CD11b/CD18 receptor, **UK 279276**. The clinical trial of this compound was terminated early for futility[101]. **Minocycline** is an oral antibiotic with proven safety over years of use. In

addition to its antibiotic properties, minocycline also has anti-inflammatory and anti-apoptotic effects that have been shown to be neuroprotective in animal models of stroke and in previous human trials. Patients receiving minocycline showed significant improvement after 30 days. Larger phase II and phase III trials are awaited[102].

Citicoline is an essential precursor in the synthesis of phosphatidylcholine, a key cell membrane phospholipid, and is known to have neuroprotective effects in acute ischemic stroke by reducing lipid metabolism[103]. It is able to attenuate the production of free radicals in ischemic conditions, while it also stimulates glutathione synthesis and the activity of glutathione reductase. A pooled analysis of individual patient data from the 4 randomized trials of oral citicoline revealed a statistically significant effect of citicoline on global recovery[104].

Albumin is thought to be neuroprotective by multiple pathways: (1) it is the major plasma antioxidant and can fight endogenous and exogenous oxidative stress products; (2) it can mediate hemodilution at pharmacological doses; (3) it reacts with nitric oxide to form a stable S-nitroso-sulfur anhydride, which is the endothelial cell-derived relaxing factor; and (4) it decreases the deposition of red blood cells and improves microvascular blood flow in the ischemic cortex [105]. The clinical trial of albumin therapy-ALIAS Pilot Trial suggested that human albumin was well-tolerated by patients and tPA therapy did not affect the safety profile of albumin [106]. Additionally, tPA-treated patients who received higher-dose albumin were three times more likely to achieve a good outcome than subjects receiving lower-dose albumin, suggesting a positive synergistic effect between albumin and tPA [107]. However, in phase III study, albumin given within 5 h of the onset of acute ischemic stroke showed no clinical benefit [108].

Brain's response to stroke includes multiple processes of endogenous repair and remodeling. It is suggested that candidate drugs with regenerative mechanisms may achieve sustained neurological improvements. Among potential neurorestorative and neuroregenerative compounds, granulocyte colony-stimulating factor (**G-CSF**) has been extensively studied. G-CSF is a neuroprotective and anti-inflammatory agent with an anti-apoptosis mechanism[109]. The AXIS trial has shown that G-CSF is well-tolerated even at high dosages in patients with acute ischemic stroke[110], but the phase II trial showed no

significant benefit with respect to either clinical outcome or imaging biomarkers[111]. Basic fibroblast growth factor (**bFGF**) increases neuronal survival, has trophic effects on brain glial and endothelial cells and is a potent systemic and cerebral vasodilator. Phase II trial of bFGF was halted because patients that received the drug did worse compared to the placebo group[98].

It has been well documented that mild (34°C) to moderate (32°C) systemic **hypothermia** protects brain from ischemic damage in various animal models. Although the exact mechanisms are unknown, a reduction of body temperature, especially brain temperature, may lead to reduced cerebral oxygen consumption, decreased intracellular lysosomal enzyme activity, suppressed free radical formation, protection of the fluidity of the cell membranes, reduced intracellular acidosis, and inhibition of cell damage mediated by excitatory neurotransmitters [98]. Induction of hypothermia is currently used in clinical practice to prevent secondary brain injury after cardiac arrest and resuscitation, perinatal or neonatal asphyxia, and head trauma. The transfer of hypothermia treatment from laboratory to the clinic undertaken in two pilot studies showed the feasibility and safety of induced hypothermia in acute ischemic stroke patients[112,113]. A large trial (The Intravascular Cooling in the Treatment of Stroke 2/3 Trial) is currently in progress to test the possible neuroprotective effect of mild hypothermia [114].

Neural stem cells are able to regenerate and restore loss of brain function in injuries like stroke. Stem cell therapies could act in a trophic, neuroprotective capacity, reducing the damage site and aiding in endogenous neurogenesis. Cells could also be administered at a later stage to replace nonviable tissues and restore function [115]. In animal models, transplanted stem cells have been shown to migrate into the injured regions, promote revascularization, enhance plasticity and regulate the inflammatory response, thereby minimizing injury[93]. Different clinical studies, the majority of which were small, nonrandomized and uncontrolled, have now been reported and indicate that stem cell therapy seems safe, feasible, and potentially efficacious. The increasing number of ongoing studies, including large randomized double-blind studies, have the potential to determine the efficacy of cell therapy for stroke and to translate the preclinical findings into clinical practice [116].

In summary, despite encouraging data from experimental animal models, most clinical trials of neuroprotective therapies have been unsuccessful. Some arguments have been suggested to explain why neuroprotection works in pre-clinical experimental models but not in human trials. (1) One possible explanation may be the discrepancies of time window for the administration of the agent. In many animal models the drug was givenbefore or shortly after stroke is induced, which is not feasible in most patients.(2) The outcomes have been evaluated almost exclusively at 24 h after the stroke and efficacy of neuroprotective agents is detected by reduction of histological infarction, whereas long-term recovery and behavioral measures have been used in clinical investigations. (3) Most of the experimental models used young and healthy animals. However, the stroke patients are usually old and suffer from multiple chronic diseases. Co-morbidities of patients can affect their functional outcome, thus altering the measurements of drug efficacy and safety. (4)Animal studies often useMCAO to induce ischemia. The homogeneity of MCAO is fairly good. The human cerebral ischemia is relatively complex and the embolus properties are diverse. Therefore the experimental studies do not mimic the pathophysiological heterogeneity of different stroke types included in clinical trials. (5) More than 90% of rodent brain is composed of grey matter, whereas in humans, gray matter makes up about 50% of the brain. The damage to white matter in humans will be significantly larger than in rodent models. This may cause a problem in neuroprotective agents which have differential effects in white and gray matter. (6) The effective dose of a neuroprtective agent in an animal study is quite large when it is converted to a human dose. The adverse effects will increase accordingly. Also, some drugs, like NXY-059 has poor BBB penetration, which may restrict the clinical application. Therefore, in animal models, the rescue of vulnerable ischemic brain tissue might be achieved by more meticulously designed studies, i.e. different animal models and species, aged animals, proper drug dosage, feasible therapeutic window, physiological monitoring, delay between the occlusion and the outcome analysis, behavioral tests; and in humans by more aptly designed clinical trials, i.e. temporal window of efficacy, selection of patients, outcome measures.

1.2 The blood-brain barrier (BBB)

The central nervous system (CNS) is the most critical and tightly regulated physiological system in the human body. Stringent regulation of the extracellular environment and the maintenance of ion concentrations within narrow ranges are necessary in order to maintain proper neuronal survival and function. Additionally, the metabolic demands of CNS tissue are considerable with the CNS accounting for approximately 20% of oxygen consumption in humans [117]. Therefore, the interface between the CNS and the systemic circulation must possess highly selective and effective mechanisms that can facilitate nutrient transport, exactly regulate ion balance, and provide a barrier to toxic substances that may be present in the systemic circulation. The requirement for a physical and metabolic barrier is further emphasized by the extreme sensitivity of CNS tissues to a wide range of chemicals, xenobiotics, plasma composition fluctuations and metabolites in the blood [118]. The interface that tightly controls brain homeostasis and narrowly regulates brain microenvironment is the BBB.

1.2.1 Brief history of the BBB

The concept of the BBB originated with the studies of Paul Ehrlich in 1885 who observed that water-soluble dyes injected into the circulatory system stained all organs except for the brain and spinal cord [118]. Ehrlich's explanation was CNS had low affinity to this water-soluble dye. In subsequent experiments, an Ehrlich's student, Edwin Goldmann noticed that injection dyes into the cerebral spinal fluid stained the brain but not the rest of the body[118]. These observations suggested that a barrier must exist separating the CNS from the peripheral circulation. In 1900, Lewandowsky was the first to introduce the term *bluthirnschranke* (blood-brain barrier) while studying the limited permeability of sodium ferrocyanide into the brain via intravenous injection. However, it was not until the1960s when scientists were able to confirm the existence of a barrier at the structural level. By using electron microscopy, Reese and Karnovsky (1967) showed for the first time that at an ultrastructural level, the endothelium of mouse cerebral capillaries constituted a structural barrier to horseradish peroxidase[119]. They determined that the barrier was composed of the plasma membrane, the cell body of endothelial cells, and the presence of tight junctions (TJs) between adjacent cells. Furthermore, they observed that the horseradish peroxidase

entered up to the interendothelial spaces from the lumen of the capillary but was unable to penetrate the interendothelial TJs in cerebral capillaries and beyond. This finding led to the conclusion that TJs probably were responsible for preventing intercellular passage of peroxidase. Brightman and Reese (1969) demonstrated in separate experiments using intravascular injections of peroxidase and lanthanum hydroxide that TJs were present, separating the vascular lumina from the underlying spaces[120].

1.2.2 Anatomy of the BBB

The current understanding of the basic structure of the BBB is built primarily upon work by Reese, Karnovsky, and Brightman from the late 1960s.The BBB exists as a selective diffusion barrier at the level of the cerebral microvascular endothelium and is characterized by the presence of TJs (as reviewed in subsequent sections) [121]. **Figure 1-3**shows a schematic cross-sectional representation of a typical cerebral capillary. The capillary lumen circumference is surrounded by endothelial cells. Additionally, pericytes attach to the abluminal membrane of the endothelium at irregular intervals [122]. Pericytes and endothelial cells are ensheathed by the basement membrane (basal lamina). Astrocytic endfeet surround the cerebral capillaries to form the structural basis of the BBB [123].

Basic cellular organization of the neurovascular unit

A critical concept in BBB biology is that brain microvascular endothelial cells (BMECs) are not intrinsically capable of forming a “barrier.” In fact, in addition to endothelial cell, formation and function of the BBB requires support of astrocytes, pericytes, neurons, and extracellular matrix (ECM), which have been collectively redefined as neurovascular-unit (NVU) [123]. The individual components of the NVU work in concert to regulate microvascular permeability, ion gradients, nutrient uptake, toxin removal, and cerebral hemodynamics [124]. Likewise, a breakdown in any of the individual components may contribute to BBB dysfunction [125]. During ischemic stroke, various NVU cell types are triggered by pathological stimuli that disrupt the BBB. Understanding the NVU responses that are involved in modifying the brain microvaculature in the context of ischemic stroke will provide an opportunity to protect BBB integrity during pathological insult. Furthermore, The NVU serves to repair the brain after stroke by restoring bloodsupply to affected area of the brain through mechanism of angiogenesis[126].

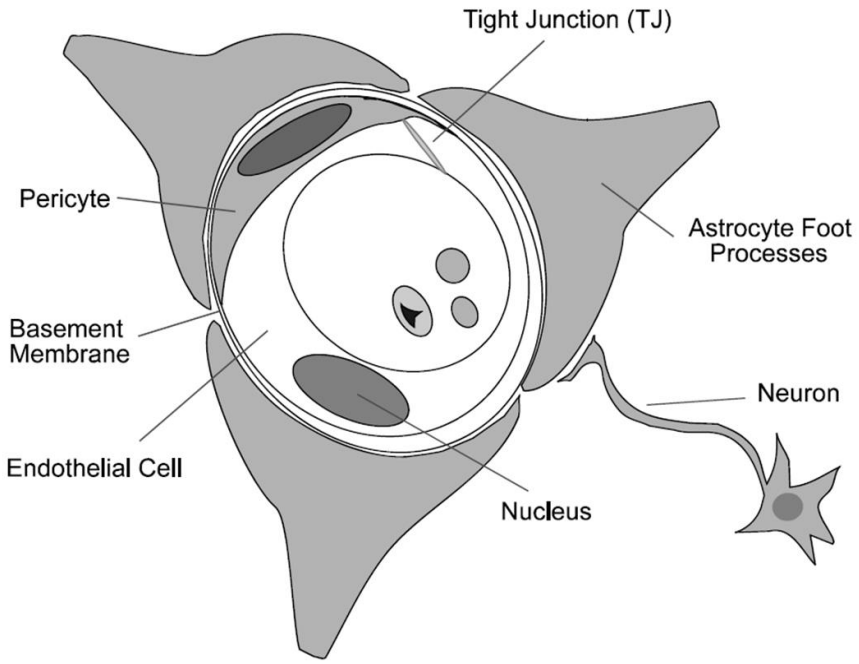

Fig. 1-3 Schematic diagram of neurovascular unit.The circumference of the capillary lumen is surrounded by endothelial cells, which are connected via tight junctions. Pericytes are attached to the abluminal surface of the endothelial cell, and both are surrounded by the basement membrane. Astrocytic endfeet processes surround the cerebral capillary to form the basis of the BBB, with neuronal signaling also mediating capillary function. (Source: Sharon Hom, 2006)

The first line of defense between the systemic circulation and the brain is the endothelium. The **endothelial cells** of the BBB differ significantly from non-brain endothelial cells by (1) the absence of fenestration correlating with the presence of intercellular TJs; (2) the minimal activity of pinocytosis and severe restriction of the paracellular diffusion of hydrophilic compounds; (3) a high number of mitochondria, associated with a strong metabolic activity; and (4) the polarized expression of membrane receptors and transporters which are responsible for the active transport of blood-borne nutrients to the brain or the efflux of potentially toxic compounds from the cerebral to the vascular compartment[127-129]. In brief, the hallmark of brain endothelium is its highly restricted and controlled permeability to plasma compounds and ions, reflected by a very high trans-endothelial electrical resistance (1500-2000 Ωcm^2)[130,131]. Maturation of the BBB necessitates endothelial cell expression of specific molecule. Specific transport systems selectively expressed in the membranes of brain capillary endothelial cells mediate the directed transport of essential nutrients into the CNS or of toxic metabolites out of the CNS[132].Transendothelial transport occurs, among many others, for glucose, amino acids, purines, and nucleosides. A receptor-mediated transport system resides in brain endothelial cells for many substrates, including low-density lipoprotein, insulin, immunoglobulin G (IgG), and transferrin[133]. Active efflux pumps are also expressed in endothelial cells. Three classes of transporters are implicated in the efflux of drugs from the brain: (1) monocarboxylic acid transporters, (2) organic ion transporters, and (3) multidrug resistance transporters (prototype is P-glycoprotein) [134].Enzymatic roles of the endothelial cells comprise another level of barrier between cerebral circulation and brain ("metabolic BBB"): ecto-enzymes such as peptidases and nucleotidases are capable of metabolizing peptides and ATP, respectively, whereas intracellular enzymes such as monoamine oxidase and cytochrome P450 (1A and 2B) can inactivate many neuroactive and toxic compounds[123].

Astrocytes localize between neuronal cell bodies and endothelial cells and ensheath over 99% of cerebral capillaries with their end-feet[135]. Studies have shown that astrocytes are necessary for maintenance and maturation of the BBB[136-138]. Astrocyte end-feet contacts have also been shown to mediate transient regulation of cerebral microvascular permeability[139]. A number of astrocyte-produced

factors (e.g. transforming growth factor-β, basic fibroblast growth factor, glial-derived neurotrophic factor, and angiopoetin-1) have been found to support cerebral endothelial cell TJs, induce angiogenesis and the expression of BBB transporter proteins[140,141]. In addition to the role of perivascular astrocytes, direct neuronal innervation of the neurovascular unit has also been implicated in regulating blood flow through projecting neuronal inputs and cortical interneurons to communicate and translate these signals into integrated microvascular responses[125].

In addition to astrocytes, **pericytes** also play a crucial role in maintenance of BBB homeostasis[142]. Pericytes are flat, undifferentiated, contractile cells that have a close physical association with the endothelium [143]. They share the same basement membrane with the endothelial cell and cover 22 to 32% of the abluminal endothelial surfaces[144]. The recruitment and interaction of pericytes with the microvascular endothelium is essential for the formation, maturation, and maintenance of the BBB [145]. Pericytes and endothelial cell communicate with each other through several apparatuses such as gap junctions, TJs, adhesion plaques and soluble factors [146]. The association of pericytes to blood vessels has been suggested to regulate endothelial cell proliferation, migration and differentiation[125]. It has been reported that these cells induce expression of occludin at the BBB via secretion of pericyte-derived angiopoetin, which suggests that pericytes are directly involved in induction and/or maintenance of barrier properties[147]. Pericytes have also been shown to migrate away from brain microvessels in rapid response to hypoxia and traumatic brain injury; both of these conditions are associated with increased BBB permeability[148,149].

Anatomical evidence has been found for direct innervation of the microvascular endothelium and/or associated astrocytic processes by, serotonergic, cholinergic, and GABA-ergic **neurons**, among others [118]. Little is known about the developmental role that neurons have on the BBB phenotype. However, there is some evidence that neurons can regulate the blood flow in CNS, as well as cerebral microvascular permeability, particularly via dynamic Ca^{2+} signaling between astrocytes and the endothelium[134]. Moreover, mature endothelium has a reciprocal function in inducing a stable brain microenvironment that enables proper neuronal activity[134].

In addition to cellular components of the neurovascular unit, the **extracellular matrix** (ECM) of the basal lamina also interacts with the BBB endothelium. The ECM is composed of structural proteins (i.e. collagen type-IV, laminin, fibronectin, elastin, trombospondin, and various proteoglycans) which are susceptible to enzymatic degradation[150]. Degradation of the ECM is associated with increased BBB permeability during pathological states including stroke[151]. The ECM seems to serve as an anchor for the endothelium via interaction of laminin and other matrix proteins with endothelial integrin receptors[152]. Such cell-matrix interactions can stimulate multitude intracellular signaling pathways[153]. Matrix proteins can also influence the expression of TJ proteins, indicating that the proteins of the basal lamina are intimately involved in maintaining the restrictive nature of the BBB TJs[154].

It has been proposed that the microvascular endothelium, astrocytes, pericytes, neurons, and ECM constitute a NVU and that this view of the BBB is critical to understanding its development and physiology. Furthermore, the concept of the neurovascular unit establishes a framework for an integrative approach to understanding how the brain responds to cerebrovascular pathology. Finally, the neurovascular unit concept provides a basis for understanding the multiple pathways by which cerebral microvascular permeability could be regulated by drugs or disease.

1.2.3 BBB tight junctions

At the junctional complex of the cerebral microvasculature, the interendothelial space is characterized by the presence of adherens junctions (AJs) and TJs. AJs form a continuous belt localized near the apical end of the junctional cleft, just below the TJ [155]. While the TJs are identified as the primary paracellular barrier, AJs appear to play a key role in the localization and stabilization of the TJs [156]. AJs are primarily composed of vascular endothelial (VE)-cadherin, which is linked to cytoskeleton via scaffolding proteins α-, β-, and γ-catenins [157,158]. The AJs hold the cells together giving the tissue structural support. They are essential for formation of TJs, and disruption of AJs leads to barrier disruption [159].

At the BBB, TJs are the main structures responsible for the barrier properties. TJs are the most apical structure within the intercellular cleft, limiting the paracellular flux of hydrophilic molecules across the BBB[160]. TJs, along with AJs, form a circumferential zipper-like seal between adjacent endothelial

cells, maintaining distinct tissue spaces through separation of the luminal side from the abluminal side of the plasma membrane. The preservation of the TJ is governed by three essential transmembrane proteins: **claudins, occludin,** and **junction adhesion molecules** (JAMs)[161]. The cytoplasmic regions of these transmembrane proteins are linked to actin cytoskeleton though interactions with accessory proteins (i.e., zonula occluden (ZO)-1, -2, and -3). ZO proteins act as a scaffold for multiple intracellular signaling pathways and are involved in regulation of TJs function[127,162]. Generally, movement of these proteins away from the cellular borders or decrease in their expression at the TJ cleft indicates a loss of junctional integrity and increased paracellular permeability. In subsequent sections, key features of the TJ proteins are highlighted (see **Figure 1-4**).

Integral transmembrane proteins

Occludin is a 60- to 65-kDa protein that has four transmembrane domains with the carboxyl and amino terminals oriented to the cytoplasm and two extracellular loops that span the intercellular cleft [163]. It is highly expressed and consistently stains in a distinct, continuous pattern along the cell margins in the cerebral endothelium. It has been shown that high levels of occludin ensure low paracellular permeability and high electrical resistance of the BBB [130]. Multiple phosphorylation sites were identified on occludin serine and threonine residues, and the phosphorylation state of occludin regulates its association with the cell membrane and barrier permeability[164]. The occludin's cytoplasmic C-terminal domain binds to the ZO-1 and ZO-2[165], which in turn binds to the cytoskeleton, localizing it to the cellular membrane. Although several knockout and knockdown experiments provided evidence that occludin is not essential for the formation of TJ[118], diminished occludin expression is associated with BBB dysfunction in a number of disease states including ischemic stroke.

Claudins (20–24-kDa proteins) share very similar membrane locations with occludin without having any sequence homology[166]. Up to 24 claudins have been identified in mammals, while only claudin-1, -3, -5 and -12 are present within BBB endothelial cell [167]. The extracellular loops of claudins interact via homophilic and heterophilic interactions between cells, forming primary seal of the TJs[168].

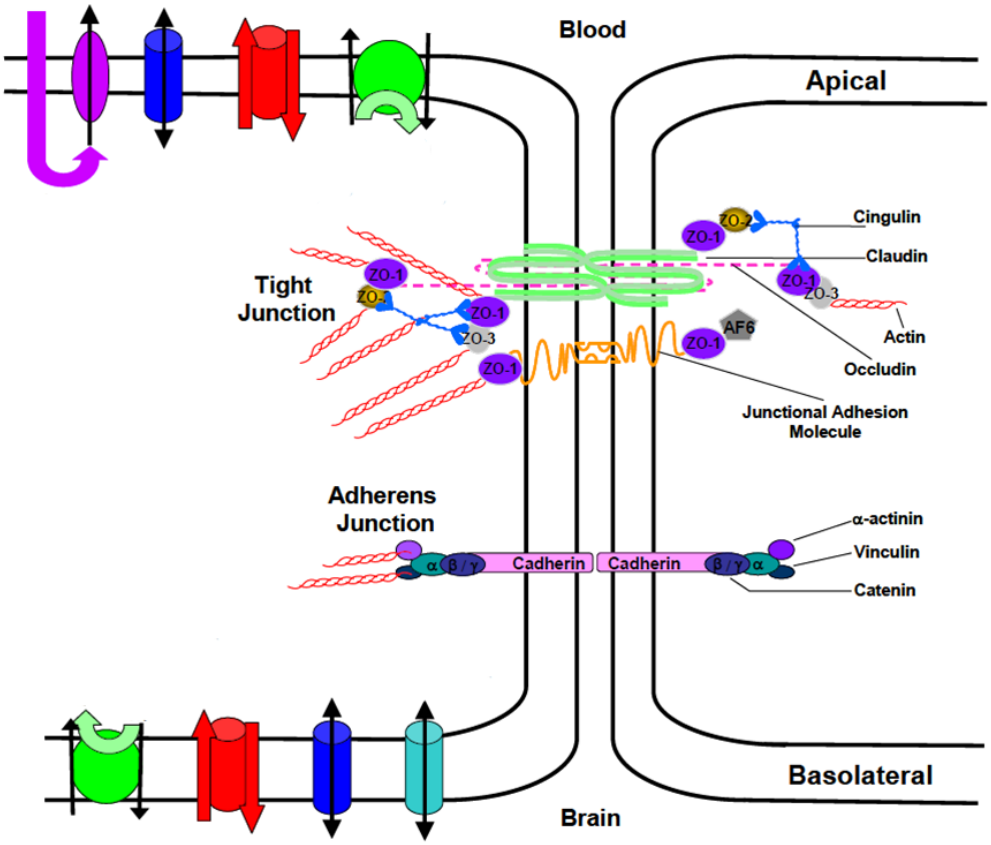

Fig. 1-4 Schematic representation of the basic structural transmembrane components of TJs.The TJ is established by the interaction between the transmembrane proteins (claudins, occludin, and junction adhesion molecule) on adjacent endothelial cells. The C terminal of these transmembrane proteins is linked to cytoskeletal actin through ZO-1/2. This figure was modified from Fleegal MA *et al.* (Fleegal MA *et al.*2005).

Overexpression of claudin isoforms in fibroblasts can induce cell aggregation and formation of TJ-like strands. Conversely, occludin only localizes to TJs in cells that have already been transfected with claudins [169]. Thus, it appears that claudins form the primary "makeup" of the TJ, and occludin further enhances TJ tightness. Evidence indicates that claudin-5 is specifically involved in the active regulation of small molecule paracellular permeability at the BBB. Mice lacking the claudin-5 gene are characterized by a size-selective BBB defect[170], whereas reduced expression of claudin-5 and disruption of interaction between claudin-5 and occludin has been reported in experimental model of ischemic stroke.

Several **JAM** isoforms have been identified at the mammalian BBB including JAM-1, JAM-2, and JAM-3. These are 40-kDa proteins from the IgG superfamily and composed by a single membrane spanning chain with a large extracellular domain that mediates homophilic and probably also heterophilic interactions in the tight junctional region [171]. JAM-1 is involved in cell-to-cell adhesion, organizing the tight junctional structure, and taking part in the formation of TJ as an integral membrane protein together with occludin and claudins [160]. JAMs also play a role in developmental processes and regulate the transendothelial migration of leukocytes [172]. Additionally, loss of JAM protein expression is directly correlated with BBB breakdown[173,174]. Interestingly, serum levels of JAM-1 were unchanged over time in 13 patients with acute ischemic stroke, suggesting that this JAM isoform is not a suitable biomarker of BBB breakdown in ischemic stroke[175].

Cytoplasmic proteins

Proper physiological functioning of the BBB, particularly restriction of paracellular solute transport, requires association of transmembrane constituents of TJ protein complexes with accessory proteins localized within the endothelial cell cytoplasm. These include members of the membrane-associated guanylate kinase-like (MAGUK) family[176]. MAGUK proteins are characterized by multiple postsynaptic density protein-95/discs-large/ZO-1 binding (PDZ) domains, an Src homolog-3 (SH3) domain, and a guanylate kinase-like (GuK) domain [177]. The PDZ domains mediate specific binding to carboxy-terminal cytoplasmic ends of transmembrane proteins; the SH3 domain binds signaling proteins and cytoskeletal

elements; and the GuK catalyzes the ATP-dependent transformation of GMP to GDP[161]. The SH3-GuK region is further involved in binding to TJ and AJ proteins[177]. In BMECs, MAGUK proteins are involved in coordination and clustering of TJ protein complexes to the cell membrane and in establishment of specialized domains within the membrane [176]. Three MAGUK proteins have been identified at the TJ: ZO-1, -2, and -3.

ZO-1 is a 220-kDa phosphoprotein expressed in endothelial and epithelial cells that normally form the TJ assembly [178]. ZO-1 connects transmembrane proteins of the TJ to the actin cytoskeleton. This interaction is critical to the stability and function of the TJ, since loss or dissociation of ZO-1 from the junctional complex is often associated with increased permeability[134]. Furthermore, ZO-1 has been shown to localize to the nucleus under conditions of proliferation and injury[179]. ZO-1 may also act as a signaling molecule that communicates the state of the TJ to the cellular interior, or vice versa. **ZO-2**, a 160-kDa protein, has high sequence homology to ZO-1 [180]. Very much like ZO-1, ZO-2 binds to structural TJ constituents, signaling molecules, and transcription factors, and it localizes to the nucleus during stress and proliferation [118]. More recently, ZO-3, a 130-kDa protein, has been identified at the BBB but its exact role in TJ formation and/or function has not been elucidated. There is evidence that ZO-3 binds to occludin and ZO-1 directly [131].

In addition to MAGUK family members, other accessory proteins have been identified at the TJ. These include cingulin, AF-6, and 7H6 [181]. Cingulin (140–160 kDa) has been shown to bind to the ZO proteins, myosin, JAM-A, and AF6, and has been suggested to transduce the mechanical force generated by the contraction of the actin–myosin cytoskeleton, regulating TJ permeability [181]. The AF-6 (180 kDa) protein participates in the regulations of TJs, via direct interaction with ZO-1[182]. 7H6 (155 kDa) is a phosphoprotein that reversibly dissociates from the TJ under conditions of ATP depletion, which is associated with increased paracellular permeability[183]. To date, several other accessory proteins found in epithelial and peripheral endothelial cell TJs have been implicated as potential mediators of paracellular regulation (e.g. EMP-1) [184], yet confirmation of their existence or activity within BBB endothelial cells is presently lacking.

1.2.4 Experimental models of BBB

BBB dysfunction has been described in a variety of neurological diseases as putatively involved in the early steps of disease progression. In fact, studies have shown a faulty BBB clearance of potential brain toxins in Alzheimer's and Parkinson's diseases, increased transport of leukocytes across the activated BBB in AIDS dementia and BBB breakdown after an ischemic insult or traumatic brain injury. BBB impairment may thus initiate and/or contribute to progressive neuronal dysfunction in such disorders. Novel therapeutic approaches are required to abrogate such disease processes, namely by modulation of TJ, or of the transport systems. Given the large population that suffers CNS disorders worldwide, great attention has been attracted to the study of BBB. Several experimental systems have been used, depending on the purposes of the study and on the expertise and resources of the researchers. The *in vitro* BBB model and *in vivo* animal use are highlighted in the subsequent sections.

In vitro

In vitro modeling of the BBB is a simplification of the *in vivo* situation, which allows investigation and more accurate interpretation of experiments that are difficult, or even impossible, to be carried out *in vivo* [185]. An ideal *in vitro* model should be simple, reproducible, and mimic as closely as possible the *in vivo* barrier either functionally or anatomically in order to allow the study of BBB-related issues in normal and pathological states, as well as drug delivery to the CNS. In particular, the cell model must display a restrictive paracellular pathway, possess a realistic cell architecture, display functional expression of transporter mechanisms and cell cultures should be easy [186].

In vitro models of the BBB essentially rely on cell cultures of endothelial cell as these cells are considered to be the anatomic basis of the BBB. Although limited by the absence of *in vivo* signaling and intercellular contacts, and subjected to the *in vitro* differentiation and phenotypic modification occurring when cells are isolated and kept in culture, cell culture systems are still privileged systems. In fact, they allow the assessment of a large number of cell functions, biologic processes and disease mechanisms, and may constitute the first approach in routine toxicity and pharmacological testing, thus reducing the number of animals used.

Different categories of brain endothelial cell cultures, comprising primary cell cultures and cell lines, can be used to analyze features such as cell morphology, energy metabolism and receptor interaction under the direct effect of the substances of interest. Primary cell cultures represent the closest possible phenotype to the *in vivo* BBB but require considerable technical resources and are more time consuming. Although primary cultures have been shown to retain some phenotypic characteristics of brain endothelium, they rapidly lose their specific characteristics in culture undergoing de-differentiation and senescence even upon limited passaging, thus hampering usefulness as *in vitro* models of the human BBB[187,188]. In order to circumvent the disadvantages of primary cultures, several immortalized endothelial cell lines were created with the potential to provide a stable source with high yield and homogeneity throughout numerous passages. Cell lines of human BMEC became the most obvious alternative to perform studies in human BBB models, due to the rarity of samples from living individuals. The study in Chapter 3 utilized **hCMEC/D3** cell line. hCMEC/D3 stably maintains in culture most of the unique structural and biochemical properties of brain endothelium *in vivo*[188]. The cell monolayers, even in the absence of co-culture with glial cells, possess functional intercellular junctions with highly restrictive permeability. hCMEC/D3cells express telomerase and grow indefinitely without phenotypic dedifferentiation. These cells express chemokine receptors, efflux transporters, and demonstrate BBB characteristics, including TJ proteins and the capacity to actively exclude drugs. Therefore, this cell line constitutes a reliable *in vitro* model of human BBB [189].

The recognition of the importance of astrocytes to the induction of BBB properties, as well as of the interplay between different cellular components of the NVU on BBB has led to the establishment of more complex *in vitro* models[123,127,185]. Research on BBB functionality has been very much enhanced by the availability of *in vitro* BBB co-culture systems[190]. Co-culture systems have been developed in which brain endothelial cells are grown on microporous filter inserts in the presence of primary cultures of astrocytes in order to mimic the anatomical and functional relationship between brain endothelium and surrounding astrocyte foot processes[191-194]. With co-cultures it has also been possible to evaluate effects on endothelial cells in the presence of other types of cells, such as astrocytes, pericytes and neurons [195].

By incorporating the cross-talk between endothelial cells and neighboring elements of the NVU, these co-culture systems allow the closest reproduction of the *in vivo* condition. However, its complexity limits the wide utilization.

Methods to evaluate BBB permeability in vitro

Elevated permeability of the normally highly restrictive BBB accompanies a variety of CNSafflictions[196,197]. Transcellular permeability to small molecule tracers yields valuable informationregarding barrier integrity[198]. Most tracers are labeled by a fluorescent dye or isotope that helps the quantification of the molecule[199,200]. To determine the limiting size for permeability, different molecular weight tracers can be used, such as fluorescent-conjugated dextran (70-,40- and 10-kDa FITC-dextran). In addition to fluorescent labels, the permeability can also be measured by the use of radioactive labels such as [^{3}H]-sucrose [201] or [^{3}H]-mannitol [202]. The tightness of the barrier and permeability to polar molecules is less stringent *in vitro* than *in vivo*, allowing compounds that would normally poorly penetrate across BBB *in vivo* to readily diffuse across the endothelial monolayer in the static model[203].

In vivo

In vivo studies provide the most reliable reference information for testing and validating other models. They take into account not only a section but the whole brain microenvironment and biological processes in live animals. BBB properties can be assessed using fluorescence microscopy. Since BBB perturbation is likely to be subtle, studies require large sample sizes and appropriate controls to detect modest but clinically relevant BBB changes in cerebral microvascular disease [204]. *In vivo* studies have been used to assess brain metabolism, BBB disruption and transport, as well as neurological disease progression [205-207]. Based on *in vivo*models, studies have demonstrated the degradation of the TJ proteins and increased BBB permeability in ischemic stroke.

Methods to evaluate BBB permeability in vivo

Evans Blue (EB) is most often used as an indicator of BBB permeability in *in vivo* studies. It is an azo dye with high affinity to plasma albumin[208]. The EB-albumin complex extravasates from blood vessels into the brain parenchyma when the BBB is disrupted. EB is intravenously injected in saline and

circulates in the vasculature[209]. Before terminating the experimental animal, the dye is cleared from the bloodstream by transcardiac saline perfusion. Macroscopically, blue-stained tissues indicate areas of BBB disruption. The extravasated EB can be quantitative determined in brain homogenate by fluorometric method [210]. The integrity of BBB can also be assessed in *in vivo* studies using radiolabeled tracers, fluorescent-labeled tracers[211,212] and an electron-dense compound as a flux tracer and transmission electron microscopy analysis[213].

1.2.5 BBB in ischemic stroke

Ischemic stroke consists of two distinctive periods of pathological impact, ischemia and reperfusion. Both ischemia and reperfusion can be further delineated into a series of interdependent biochemical and cellular events that evolve over minutes to days. With this understanding, BBB TJ alterations can be divided into time-dependent phases, based on states of paracellular permeability over the time-course of ischemia/reperfusion.

Ischemia

The ischemic phase of stroke is denoted by a loss of regional CBF and increased vascular resistance owing to mechanical plugging of a vessel via a thrombus or embolus, resulting in loss of oxygen and nutrients to the surrounding tissue.One of the major events of cerebral ischemia is energy failure due to a lack of glucose and oxygen. Energy failure, in turn, leads to a cascade of events, including depletion of ATP, a decrease in Na^+-K^+ ATPase activity, a rise in intracellular Na^+, Ca^{2+}, lactic acidosis, release of intracellular glutamate to the extracellular environment, oxidative stress, and activation of inflammatory processes. These mechanisms demonstrate over-lapping and redundant features, all of which can result in BBB disruption[214]. Endothelial swelling may occur within minutes to hours of ischemic onset, leading to narrowing of the internal diameter of the blood vessel. Lactic acidosis also directly contributes to swelling of endothelial cells, neurons, and astrocytes. Experimentally it has been shown that after microsphere-induced cerebral embolism, there is a decrease in occludin and ZO-1 at the level of the TJs, contributing to increases in paracellular permeability[215]. Furthermore, induction of proteases (i.e., tPA, matrix metalloproteinases (MMPs), cathepsins, and heparanases) contributes to BBB ECM degradation[216].

NOmay add to further insult by forming RNS thus exacerbating DNA damage and endothelial injury. The astrocytes themselves express NO synthase during cerebral ischemia, which in turn contributes to peroxynitrate formation and BBB breakdown[214]. Microvascular leukocyte accumulation has been shown to occur as early as 30 minutes after permanent MCAO[217]. Proinflammatory cytokines are induced and followed by chemokines,which are associated with an activated endothelium expressing adhesion molecules[218]. This leads to leukocyte recruitment and extravasation, thereby further enhancing inflammatory activity and toxic free radical production[218,219]. Therefore, the various previously discussed mechanisms initiated during the ischemic cascade have a significant impact on the BBB. The observed increases in paracellular permeability generally correlate with the loss of TJ protein localization and/or expression along the cellular membrane.

Reperfusion

Reperfusion is denoted by the reestablishment of CBF to the ischemic and hypoperfused brain. Although it is essential for brain tissue survival; it also contributes to additional tissue damage and has the potential for hemorrhagic transformation (i.e. phenomenon in which blood vessels weakened by ischemic stroke rupture to cause brain hemorrhage)[220]. Reperfusion injury has been defined in numerous ways, including activation of endothelium, excess production of free radicals, inflammatory responses and leukocyte recruitment, increase in cytokine production, and edema formation[221]. Common among these mechanisms is BBB disruption.

Depending upon the duration and severity of ischemia, degree of reperfusion, and type of stroke animal model, it is proposed that there are 3 stages of paracellular permeability after reperfusion. There is an initial reperfusion permeability associated with acute elevations in regional CBF, which is then followed by a biphasic permeability response. The initial reactive hyperemia phase constitutes a loss of cerebral autoregulation, increased BBB permeability, and acute elevation in regional CBF [222]. This acute phase is passively dependent on perfusion, and is often concurrent with a sharp increase in blood-pressure. Following the initial hyperemia, hypoperfusion of the ischemic area occurs (i.e. no-reflow effect), which is attributed to continued cerebral metabolic depression, microvascular obstruction,

occlusion via swelling of endothelial cells and astrocytic end-feet, and formation of endothelial microvilli[223]. This causes nutritional deficiency in brain tissue and enhances neutrophil adhesion, with subsequent inflammatory activity. These events directly contribute to the next period of increased BBB paracellular permeability, which occurs as a biphasic response. The first phase of the biphasic permeability occurs at 3 to 8 hours post-reperfusion and has been attributed to increased inflammatory and oxidative stress on the BBB, in conjunction with enzymatic degradation of the ECM[224,225]. The second phase occurs at 18 to 96 hours post-reperfusion dependent on ischemic severity and brain region evaluated. It coincides with the increased vasogenic edema and angiogenesis[226-230]. However, neurovascular remodeling may continue weeks after an ischemia/reperfusion event[231,232].The duration of the last phase of opening seems to depend on the severity of the initial insult, and 2 h of MCAO results in the barrier being open to proteins for as longas 3 weeks. This phase is more complicated and results in greater tissue damage through leukocyte infiltration and marked MMP-9 release from neutrophils transmigrated to the ischemic brain, reducing BBB TJ integrity[233,234].

Disruption of the BBB allows leakage of blood components into the brain parenchyma. Extravasation of high molecular weight molecules is followed by water due to osmosis and leads to vasogenic edema, which may cause secondary damage through intracranial hypertension. Additionally, extravasation of red blood cells leads to hemorrhagic transformation of the infarcted area. Finally, the leaky BBB facilitates transmigration of inflammatory cells, promoting the post-ischemic inflammatory response[235].

Edema

Edema is one of the primary causes of clinical deterioration, and a leading cause of death subsequent to ischemic stroke. There are two major types of edema associated with ischemia/reperfusion, cytotoxic and vasogenic[236]. Cytotoxic edema occurs soon after ischemic onset, and is caused by translocation of interstitial water into the intracellular compartment[237]. During ischemia, the ATP depletion together with the compensatory activation of anaerobic glycolysis results in cellular acidosis, which induces neuronal membrane depolarization. Cellular swelling is initiated by an increase in

intracellular Na^+ and water influx[238]. Moreover, the excessive extracellular glutamate due to inefficiency of glutamate uptake causes a prolonged activation of NMDA receptors, further enhancing Na^+ influx[239]. NMDA receptor-gated ion channels are highly permeable to Ca^{2+}, thus further enhancing Ca^{2+} influx. In normal physiologic conditions, influx of osmotically active solutes such as Na^+ is counteracted by active energy-requiring pumps, such as Na^+/K^+-ATPase, resulting in zero net solute shift[240]. The energy failure following ischemia impairs active Na^+ and Ca^{2+} export via energy-requiring pumps. Enhanced Na^+ and Ca^{2+} influx followed by Cl^- and water influx precipitates cell swelling[241]. All cellular elements (neurons, glia, and endothelial cells) take in fluid and swell but do not contribute to a net increase in brain volume because a corresponding reduction in the extracellular fluid space also occurs. Cytotoxic edema depends primarily on the duration and severity of ischemia and is an important indicator of ultimate infarct size and stroke outcome.

Vasogenic edema occurs as a result of the movement of water from the intravascular to the extravascular compartment. Perturbation of the physiological barrier function of microvessels causes uncontrolled leaks of fluid from vessels to the surrounding parenchyma. Vasogenic edema is usually formed later than cytotoxic edema, after the cessation of CBF. However, the disruption of the BBB can be demonstrated as early as 20 min after transient global forebrain ischemia. Several mechanisms and mediators have been associated with the pathological opening of the BBB. Vasogenic edema is an ultimate cause of brain volume increase after ischemic stroke, which may result in clinical deterioration by its mass effect [224]. The compression of surrounding structures and the resultant dysfunction of the compressed structure are consequences of this mass effect.

Pathways of mediation

Although several factors have been identified in the regulation of BBB TJ permeability, no single molecular/cellular pathway independently predominates over the course of an ischemia/reperfusion event, because of the complexity of events surrounding the different phases of TJ permeability. Nevertheless, a certain inter-relation of mechanisms directing these alterations has been identified.

Oxidative and nitrosative stress

Oxidative stress is aputative mediator of the BBB disruption and brain edema, particularly during the reperfusion[224]. In physiological conditions, ROS are tightly controlled by superoxide dismutase (SOD) enzymes. During cerebral ischemia/reperfusion, highly potent ROS such as superoxide and hydroxyl radicals are produced at such high levels that the ability of SODs to scavenge it is overwhelmed[242]. ROS damage cellular macromolecules (lipids, proteins, and nucleic acids) and also mediate the cell signaling involving mitochondria, DNA repair enzymes, and transcription factors that may lead to apoptosis during reperfusion. ROS are generated as part of mitochondrial electron flow. Mitochondrial oxygen radical production can be stimulated by elevated intracellular Ca^{2+}, Na^{+}, and ADP levels in ischemic cells[242]. Also, there are a number of other possible sources of free radical generation, including xanthine oxidase, COX, LOX, cytochrome P450 (CYP450), endothelial NOS (eNOS), and the NADPH oxidase family.

Experimental animal stroke models have demonstrated that ROS are capable of directly injuring the endothelium and thatantioxidants provide a protective effect. After ischemia, free radical scavengers significantly reduced infarction size and BBB leakage.Treatment with CuZnSOD, recombinant SOD, or polyethylene glycol-SOD attenuated either ischemia-induced edema or increased permeability[243]. In *in vitro* rat brain capillary endothelial cells, oxygen radicals were found to increase the permeability of cell monolayer. Moreover, these permeability changes preceded the onset of cell death and were blocked by SOD and catalase[244]. Transgenic overexpressing SOD significantly reduced BBB leakage and infarct sizes in mouse photothrombotic ischemia model[245]. On the other hand, mice deficient in SOD were highly susceptible tofocal cerebral ischemia/reperfusion, with exacerbated vasogenic edema and higher mortalitythan wild-type animals[246].

Evidence indicates that oxidative stress disrupts endothelial TJs, resulting in increased paracellular permeability[247-249]. Oxidative stress-induced endothelial cell permeability has been shown to associate with tyrosine phosphorylation of occludin and ZO-1, which is mediated through Src [247]. Oxidative stress has also been reported to induce F-actin redistribution and stress fiber formation[162,250]. In an *in vitro* BBB model, ROS selectively activated signaling cascades involving RhoA, phosphatidyl

inositol-3 kinase (PI3K), and protein kinase B (PKB/Akt), leading to rearrangements of the actin cytoskeleton and spatial redistribution and downregulation of occludin and claudin-5, inducing altered BBB integrity. The effects of ROS on endothelial monolayer could be blocked through inhibition of PI3K or PKB/Akt [248]. In another study, hydrogen peroxide induced hyperpermeability in BMECs and delocalization of the TJ proteins occludin, ZO-1, and ZO-2 via p44/p42 MAP kinase activation [251]. Oxidative stress also contributes to the activation of enzymes, impacting BBB permeability. For example, free radicals activate MMP-9, leading to TJ and basement membrane degradation[46]. Moreover, ROS may mediate inflammatory response after ischemia by stimulating cytokines generation and protease secretion from leukocytes and resident cells of the NVU, which induce further vascular leakage.

In cerebral ischemia, NO has been suggested to have both protective and deleterious effects on cerebral vessels, depending on the cellular source of NO, the amounts produced, and the time after ischemia[252]. eNOS production of NO has been shown to be beneficial by increasing CBF and decreasing platelet aggregation and leukocyte adhesion to the endothelium. However, the protective effects are reportedly short-lived (less than 2 h)[253].

There is general agreement that NO increases microvascular permeability in cerebral ischemia and that NO donors increase BBB permeability. Increased expression of nNOS has been shown in the ischemic core and penumbra 24–48 h following permanent MCAO[254]. Cerebral ischemia also enhances iNOS expression, which is localized to capillaries in the ischemic area and has been suggested to contribute to ischemia/reperfusion-induced BBB damage[46]. The nonselective NOS inhibitors have been shown to reduce brain edema, BBB damage, and infarct size in experimental MCAO models[255]. Additionally, genetic depletion of nNOS or iNOS gene has shown beneficial effects following cerebral ischemia in mouse models[256,257]. The mechanism of the BBB damage is associated with generation of the peroxynitrite[258]. NO produced in the endothelium rapidly conjugates with superoxide to form peroxynitrite, which is a potent cytotoxic and proinflammatory molecule. Peroxynitrite is well-known to induce cellular damage by its ability to nitrosylate tyrosine, leading to functional modifications of critical proteins[259]. Breakdown of peroxynitrite into nitrogen dioxide and hydroxyl radicals is also known to

contribute to endothelial injury and BBB disruption in cerebral ischemia[260].NO has also been suggested to activate MMP-9[261], with NO-sensitive transcription factors (i.e. NF-κB, activated protein-1) being transcription factors for MMP-9[262]. Overall, oxidative stress contributes to endothelial dysfunction and BBB disruption by promoting redistribution and/or disappearance of critical TJ proteins such as claudin-5 and occludin[248].

Inflammation

Postischemic inflammation is a progressive and interactive process, which involves the induction of cytokines and adhesion molecules at the level of the endothelium, in coordination with the activation and migration of neutrophils and microglia[263]. Previous research has demonstrated that inflammatory responses in focal cerebral ischemia are primarily mediated through pro-inflammatory cytokines interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α), which appear within several hours following ischemic insult[264].

Cytokines stimulate the production and release of chemokines chemoattractant proteins monocyte chemoattractant protein-1 (MCP-1) and cytokine-induced neutrophil chemoattractant [218]. Chemoattractants released at the site of injury are believed to provide driving force for leukocyte movement across BBB[265,266].MCP-1 is a major factor associated with leukocyte infiltration into the brain parenchyma in a variety of inflammatory conditions, including stroke. An *in vitro* examination identified a biphasic increase in permeability during post-hypoxic reoxygenation, which coincided with increased secretion of MCP-1 by both astrocytes and brain endothelial cells[267]. In a subsequent study, MCP-1 receptor knock-out mice showed a decrease in BBB permeability, infarct size, brain edema, leukocyte infiltration, and inflammatory mediator expression after ischemia[268].

Signaling mediated by cytokines/chemokines induces an upregulation of endothelial (P-selectin, ICAM-1, E-selectin) and leukocyte (L-selectin, β2-integrin) adhesion molecules[269]. TNFα has been shown to increase the expression of the ICAM-1 at the surface of cultured endothelial cells[270,271]. Such induction of ICAM-1 can be mediated through TNFα activation of NF-κB[272,273]. Upregulation of TNFα expression by neurons and astrocytes in ischemic regions has been shown to precede BBB permeability[274,275]. IL-1 is

also associated with induction of endothelial cell adhesion molecules expression after stroke. Rats receivedintraventricular injection of IL-1β after MCAO showed increased BBB permeability andbrain edema formation, as well as an increased influx of neutrophils[276]. In another study, the IL-1 receptor antagonist significantly reduced infarct volume and BBB permeability following 24 h of reperfusion in mice. IL-1β-induced neutrophil adhesion andBBB paracellular permeability increase are associated with TJ disorganization with a breakdown of key components such as occludin and ZO-1[277].

As a consequence of the up-regulation of cytokines and adhesion molecules, leukocyte recruitment and transmigration to the extravascular space occurs. In cerebral ischemia, neutrophils are found in the brain soon after MCAO and this infiltration peaks at 24–72 h[278]. Infiltrating neutrophils are an exogenous source of molecules deleterious to the ischemic area, i.e., free radicals, NOS, MMPs, COX-2, VEGF, platelet-activating factor (PAF) and other mediators[224,233]. Neutrophil infiltration into tissues causes edema, and mediators of inflammation such as TNF-α, IL-1β, ICAM-1, and β_2-integrins also increase endothelial permeability. In addition, BBB disruption is accentuated by MMP-9, superoxide, and PAF, which are secreted by activated neutrophils[279]. The molecular mechanisms of BBB opening are associated with alterations in TJs and the endothelial cytoskeleton. Leukocyte adhesion to the endothelium triggers signal transduction cascades, leading to the loss of the TJ proteins and the redistribution of the AJ proteins[280]. Moreover, glutamates derived from activated neutrophils may contribute to endothelial barrier opening by acting on the metabotropic glutamate receptors expressed by endothelial cells.

Vascular endothelial growth factor (VEGF)

The vascular endothelium-specific growth factors include members from the VEGF, angiopoietins, and ephrin families, which act in coordination during angiogenic remodeling[281]. To date, the majority of growth factor related studies affiliated with ischemia/reperfusion and BBB TJ permeability have focused on VEGF and associated intracellular mechanisms. VEGF is a vascular endothelial cell mitogen and a major inducer of angiogenesis. In addition, it is a potent vascular permeability factor that increases BBB leakage in various types of brain injury[282]. During cerebral ischemia, VEGF is transcriptionally activated

by hypoxia inducible factor-1 (HIF-1) [283]and expressed in various cell types including astrocytes, pericytes, vascular endothelial cells, and neurons[282]. Upregulation of endogenous VEGF interacts with two tyrosine kinase receptors, VEGF receptor-1 (flt-1) and VEGF receptor-2 (flk-1/KDR) on the ischemic vessels and contributes to the disruption of BBB[284]. Focal ischemia in the rodent induces VEGF expression at 1–3 h, with a sustained peak lasting up to 24–48 h, and then persisting for ~7 daysafter the onset of ischemia[285]. The majority of studies with early intravenous administration of VEGF have exhibited detrimental effects, reflected by an increase in brain injury, hemorrhagic transformation, and increased microvascular injury and inflammation[286-289]. The inhibition of VEGF using a VEGF antagonist significantly decreased brain edema and infarction in a focal ischemia model in mice[290]. Yet, delayed administration of VEGF (2 or 3 days after stroke) [291] or local (ICV or brain) application of VEGF[292] appear to beneficially enhance angiogenesis, neuronal survival, and may even decrease infarct size. This suggests that the time point and route of delivery plays a crucial role in the actions of VEGF in the ischemic brain.

VEGF has been shown to increase paracellular permeability, reduce the expression of ZO-1 and occludin and disrupt the organization of both proteins, as well as change the distribution of actin cytoskeleton in endothelial cells[293]. VEGF has also been found to down-regulate both the mRNA and protein level of claudin-5 and occludin, leading to BBB dysfunction. Activation of the flk-1 has been proposed to be the major mediator in VEGF-induced BBB permeability[294,295]. VEGF binding leads to receptor dimerization followed by autophosphorylation of the cytosolic domains of these receptors which results in the stimulation of several intracellular kinases[296]. This leads to the activation of a number of downstream signals, including the PI3K/Akt, phospholipase-C (PLC)/PKC, protein kinase Src, and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)-1/2 kinase pathways, which in turn have numerous downstream signals[297]. VEGF-induced Src-dependent processes are shown to result in increased vascular permeability associated with cerebral edema following ischemic stroke[298]. In isolated rat braincapillaries after embolism model of stroke, an increase in tyrosine phosphorylation of occludin was found to coincide with increased Src activity, in association with a

decreased occludin and ZO-1 expression[215]. A PKC-dependent model of VEGF-mediated TJ disassembly and vascular permeability has also been proposed. In this model, VEGF activation of flk-1 stimulates PLC-γ activation through Src, with subsequent production of inositol 1, 4, 5-triphosphate (IP3) and activation of conventional and novel PKC isoforms, directly mediating the TJ protein disassembly[299]. Furthermore, NO has been shown to mediate the ischemia-induced VEGF response, through an eNOS mediated mechanism[300]. The flk-1 mediated increases in eNOS expression have been identified to be downstream of the PKC signaling[301].

Enzymatic activity

tPA is a highly specific serine proteinase and is found predominantly in the blood, where its primary function is as a thrombolytic enzyme that convert inactive plasminogen to active plasmin[300]. Plasmin is a fibrinolytic enzyme capable of rapidly degrading fibrin-based blood clots. In the brain, tPA has been identified mainly in the endothelial cells of the BBB and in the endothelium of the small vessels, where it may regulate BBB permeability and vascular tone[302]. tPA can degrade components of the basal lamina, thus contributing to the BBB disruption in cerebral ischemia[303]. Moreover, MMP-9 may play a central role in tPA-induced BBB breakdown. Induction of MMP-9 via tPA is found to be mediated by low-density lipoprotein receptor-related protein[304]. In rodent stroke models, endogenous tPA activity in the brain is induced as early as 1 hour (preceding changes in MMP-9 and BBB integrity) after focal cerebral ischemia[305,306]. Increased endogenous tPA is required for the initial opening of the BBB after transient MCAO[306]. Genetic deficiency of tPA or inhibition of its activity by neuroserpin has been shown to decrease BBB disruption, edema, neuronal death, and brain infarction[305,307].

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that digest almost all ECM component proteins. MMPs have been implicated in the pathophysiology of cerebral ischemia, partly because they mediate the proteolytic degradation of a broad range of extracellular substrates, including some ECM proteins critically required for brain homeostasis[308]. Clinical and experimental studies have demonstrated that several MMPs such as MMP-2, MMP-3, MMP-7, or MMP-9 are upregulated and activated after ischemic stroke[309,310]. MMPs are synthesized and are secreted as

inactive proenzymes that subsequently become proteolytically cleaved and activated[311]. During cerebral ischemia, proMMP-2 can be activated by membrane-type MMP (MT1-MMP), and the latter is activated by furin[312]. ProMMP-9 can be activated by MMP-3 (stromelysin-1), as well as other mechanisms such as proinflammatory factors (e.g., IL-1β, TNF-α, CD40L, and many others) and ROS[313]. tPA has been shown to activate MMPs through plasmin-dependent and -independent mechanisms[312].

Among MMPs, MMP-2 and MMP-9 are two of the most widely studied enzymes that have been shown to be critical in regulating BBB permeability during cerebral ischemia. Experimental studies provide evidence that MMP-2 plays a key role in the initial opening of the BBB after cerebral ischemia. Increased expression of MMP-2 may contribute to the initial opening of BBB by degrading the basal lamina[314]. In a rat model of transient MCAO, the initial opening of the BBB occurred as early as 3 hours after reperfusion and increased activation of MMP-2 correlated with the early opening of the BBB[230]. Correspondingly, the mRNA expression of claudin-5 and occludin decreased in both hemispheres, and both proteins were degraded or fragmented in ischemic hemispheres after 2–3 hours of reperfusion. Treatment with the MMP inhibitor BB-1101 reversed the degradation of the TJ proteins[315]. Thus, the early degradation of the TJ proteins seems to be associated with a marked increase in MMP-2 in the early phase after cerebral ischemia.

Recent studies suggest that MMP-9 plays a critical role in mediating the second, delayed opening of BBB after ischemic stroke[230]. Emerging data indicate that MMP-9 is associated with severe BBB disruption by further degrading the TJs and basal lamina proteins, substantially contributing to brain infarction, edema, and hemorrhagic transformation in both animal models[316,317]and in human stroke patients[318,319]. MMP-9$^{-/-}$ mice displayed a significant reduction in BBB disruption and brain edema, which was associated with reduced degradation of intracellular ZO-1 as compared to wild-type mice after transientMCAO[320]. MMP-9 has been shown to degrade TJ proteins (claudin-5, occludin, and ZO-1) in cultured brain endothelial cells[321] and in animal models of focal cerebral ischemia[322-324]. MMP-9 may play a more prominent role in the BBB disruption during ischemic stroke under clinical relevant conditions linked to elevated systemic inflammation. Experimental data have shown that systemic inflammation

exacerbates neutrophil infiltration into the ischemic brain. The enhanced neutrophil-derived MMP-9 mediates the sustained disruption of the TJ protein (claudin-5) and the cerebrovascular basal lamina protein (collagen-IV) in ischemic brain injury in the presence of IL-1β-induced systemic inflammation[325].

All these factors contribute directly and indirectly in BBB damage,compromising BBB function, thus abolishing the protective role of the BBB in controlling the microenvironment at the site of infarction. The increased permeability and loss of function of the BBB causes the secondary progression of brain injury by increasing cerebral edema, promoting the recruitment and migration of macrophages and neutrophils into the infracted region, and increasing the inflammatory responses.

1.2.6 Vascular effects of diabetes associated with ischemic stroke

According to the International Diabetes Federation, diabetes affects at least 285 million people worldwide in 2010, and that number is expected to reach 438 million by the year 2030[326]. In the U.S., 25.8 million people, or 8.3% of the population are affected by diabetes. The vascular damage sustained during the course of diabetes will increase likelihood that these affected individuals will develop micro- and macrovascular complications.

Diabetes constitutes one of the major risk factors for stroke. It has been well documented that diabetes is associated with an increased risk of ischemic stroke with relative risks ranging from 2 to 6[327]. Population risk of stroke attributable to diabetes (proportion of cases which potentially could be prevented by eliminating diabetes) was 18% in men and 22% in women[328]. Beyond its paramount effect on stroke risk, diabetes also worsens stroke outcome. In both retrospective and prospective human studies, patients with diabetes have a higher mortality rate, more severe disability and slower recovery from stroke[329].

Vascular health is not only important for maintenance of cerebral blood flow to provide nutrients and remove metabolites from this highly metabolically active organ but also for structural and functional stability of the BBB. Therefore, micro- and macrovascular disease of the brain can have profound effects on neurologic function in diabetes especially when a secondary injury like ischemia is superimposed on this existing pathology. Diabetes or chronic hyperglycemia has been shown to contribute to thickening of the cerebral microvascular basement membrane[330]. The widening of the basement membrane

compromises the integrity of adjacent pericytes and astrocytic end feet that sit on the basement membrane and serve as a functional bridge between the vasculature and neuronal cells of the brain[331]. Diabetes also leads to degeneration of endothelium[332] and significant vascular remodeling which is characterized by increased tortuosity[333]. Changes in vessel structure in diabetes ultimately affect BBB permeability. Studies reported increased BBB permeability in both type I and type II diabetic animal models[333-335]. The increased permeability is associated with concurrent decrease in TJ protein occludin and/or ZO-1 and increases in MMP-2 and 9. Clinical studies also found a BBB permeability increase in diabetic patients[336]. Besides the structural changes in cerebrovasculature, diabetes also induces alterations in vascular dilator pathways that lead to impaired reactivity and contribute to the pathogenesis of stroke. Endothelium-dependent relaxation is attenuated in patients as well as experimental animal models of diabetes. All these changes in cerebral vascular function induced by diabetes/hyperglycemia may be coordinated by multiple mechanisms including increased oxidative stress, disturbances in NO synthesis and production, impairment of vascular smooth muscle ion channels, and inhibition of Rho-kinase activity, most of which also contribute to ischemia/reperfusion injury[330].

Approximately 30-40% of patients admitted with acute ischemic stroke are hyperglycemic[337]. While a proportion of this group are known to be diabetic, a further 25-50% have previously unrecognized abnormalities of glucose intolerance. The remaining is the result of acute stress response, defined as stress hyperglycemia[338]. Stress hyperglycemia usually resolves spontaneously after dissipation of the acute illness. The stress reaction that results in hyperglycemia is initiated by activation of the hypothalamic-pituitary-adrenal axis, which leads to raised amounts of glucocorticoids, including cortisol, and activation of the sympathetic autonomic nervous system, resulting in increased catecholamine release. Increased levels of stress hormones stimulate glucose production by glycogenolysis, gluconeogenesis, proteolysis, and lipolysis. Augmented epinephrine also can result in insulin resistance and hyperinsulinaemia[339]. Hyperglycemia is an independent predictor of poor clinical outcome in stroke patients. Numerous studies in experimental animal models have shown that hyperglycemia is associated with increased infarct size, edema, and hemorrhage[340]. An increasing number of studies demonstrate that

admission hyperglycemia has been linked to increased risk of hemorrhagic complications in patients treated with thrombolytic therapy using rtPA[341].

Hyperglycemia has been shown to aggravate cerebral damage in ischemic stroke by impairing recanalization, decreasing reperfusion, and increasing reperfusion injury[341]. Impaired recanalization has been attributed to disturbances in coagulation and in fibrinolytic pathways[342-344]. Hyperglycemia has been shown to stimulate coagulation by increasing the production of thrombin-antithrombin complexes and by stimulating the tissue factor pathway, whereas hyperinsulinemia decreases fibrinolytic activity by increasing the production of plasminogen activator inhibitor. Hyperglycemia is also associated with decreased reperfusion to the ischemic tissue. Inhibition of vasodilatation is an important mechanism by which hyperglycemia seems to reduce CBF. Vasodilatation is predominantly mediated by endothelium-derived nitric oxide, which is synthesized by eNOS. Hyperglycemia not only decreases the expression of eNOS[345], it also reduces the bioavailability of nitric oxide by increasing the production of superoxide which neutralizes nitric oxide by forming peroxynitrite[346]. During reperfusion, the oxidative stress and inflammation contribute to the vascular and neuronal injury. Hyperglycemia aggravates reperfusion injury through both pathways. It is well established that hyperglycemia causes a robust increase in mitochondrial free radical generation which then triggers downstream mediators such as NADPH oxidase to cause further increases in the formation of ROS, which ultimately lead to BBB breakdown and neuronal death[347,348]. Hyperglycemia also increases expression of several proinflammatory transcription factors, such as NF-κB. These factors have key roles in the regulation of the inflammatory responses by increasing the production of proinflammatory cytokines and promoting the adhesion of inflammatory cells to the vascular endothelium[349]. The inflammatory response leads to BBB opening, which results in further diapedesis of inflammatory cells out of the circulation and into the brain, and edema formation, resulting in tissue injury and increased infarct size. In addition, studies have suggested that hyperglycemia increases cerebral lactate production and exacerbates tissue acidosis by increasing the available glucose for anaerobic respiration and by inhibiting the mitochondrial respiration, leading to reduced penumbral salvage after infarction [350].

In summary, the brain is a site of both macro- and microvascular complications of diabetes. Microvessel structural and functional changes due to acute and chronic hyperglycemia lead to increased incidence and worsened outcomes from stroke in diabetic patients. Acute hyperglycemia also reduces both the safety and efficacy of reperfusion therapy in acute ischemic stroke patients. Current guidelines advocate optimal glycemic control in diabetic patients, in addition to aggressive blood pressure control and strict management of dyslipidemia for primary and secondary prevention of stroke. More investigations need to be performed to identify novel targets for therapeutic intervention so that the consequences of cerebrovascular complications can be mitigated in patients with diabetes.

1.3 Hypoxia-inducible factor-1 (HIF-1)

HIF-1 is a master regulator of hypoxia-responsive genes. It regulates the expression of a broad range of genes that facilitate adaptation to low oxygen conditions. Its targets include genes that code for molecules that participate in vasomotor control, angiogenesis, erythropoiesis, cell proliferation, and energy metabolism. All of these genes may potentially contribute to survival and recovery of neuronal cells following ischemic stroke. Because of the potentially important roles of genes regulated by HIF-1 following ischemia, it has been suggested that regulating HIF-1 induction and accumulation is a highly promising therapeutic approach for stroke. Nevertheless, HIF-1 may also contribute to ischemic tissue damage by promoting BBB disruption. Both neuroprotective and detrimental effects of HIF-1 have been observed in experimental animal models of ischemic stroke. Understanding the regulation and function of HIF-1 will provide new insight into the design of new therapeutic strategies. The following sections provide information about the accumulation mechanism of HIF-1 and its roles in ischemic stroke.

1.3.1 The structure of HIF-1

HIF-1 is one of the most essential molecules implicated in the response to hypoxia[351]. It was initially identified as a hypoxia-inducible DNA-binding protein capable of interacting with a hypoxia response element (HRE; 5′-RCGTG-3′) in the 3’ region of the erythropoietin gene[352]. It is a heterodimeric transcriptional factor consisting of two subunits: an oxygen-regulated α subunit and an oxygen independent β subunit[353,354]. HIF-1 β subunit is also known as aryl hydrocarbon receptor nuclear transporter (ARNT)[355]. Both HIF-1 subunits are members of the basic helix-loop-helix (bHLH)-containing PER-ARNT-SIM (PAS)-domain family of transcription factor[356]. Interactions between bHLH-PAS domains from the two subunits mediate their dimerization, and individual basic regions of the two subunits then make contact with their corresponding DNA sequences, namely HRE[357]. Based on the available data, HIF-1α is the major factor regulating the response to hypoxia. In response to decreased oxygen tension, dimerization of α and β subunits occur in the nucleus and this active heterodimer binds to the *cis*-acting HRE in target genes with transcriptional co-activator p300/CBP (CREB-binding protein) and DNA polymerase II. The active transcription complex induces a set of genes responsible for

angiogenesis, erythropoiesis, vascular tone, glycolysis, mitochondrial function, and cell survival[358-361]. HIF-1α contains two transactivation domains (TAD): N-terminal TAD (N-TAD) and C-terminal TAD (C-TAD)[362]. The main function of the TADs is to recruit and interact with co-activators, which are crucial for the transcriptional activation of target genes[363]. These domains are also important because HIF-1α undergoes post-translational regulation mediated through hydroxylation, phosphorylation, acetylation, and/or redox modifications of these two TAD domains[364]. HIF-1α also contains an oxygen-dependent degradation domain (ODD domain) that allows regulation of protein stability as a function of the O_2 concentration[365] (see **Fig. 1-5**).

In addition to HIF-1α and -β, two other proteins have been identified. These are additional α isoforms termed HIF-2α and HIF-3α. HIF-2α is closely related to HIF-1α and both are able to interact with HREs to upregulate transcriptional activity. Although HIF-2α shares 48% amino acid sequence identity with HIF-1α and accordingly shares a number of structural and biochemical similarities with HIF-1α, they each regulate both common and unique target genes and may be differentially regulated depending on the duration and severity of hypoxia exposure [366]. In contrast to ubiquitously expressed HIF-1α, though, HIF-2α is primarily expressed in the lung, carotid body, and endothelium[367-369]. Little is known about the HIF-3α isoform. Several splice variants of HIF-3α have been shown to be a dominant-negative regulator of the other two alpha isoforms and has a limited expression pattern in the eye and the cerebellum. It also dimerizes with HIF-1β and binds to HREs[370371].

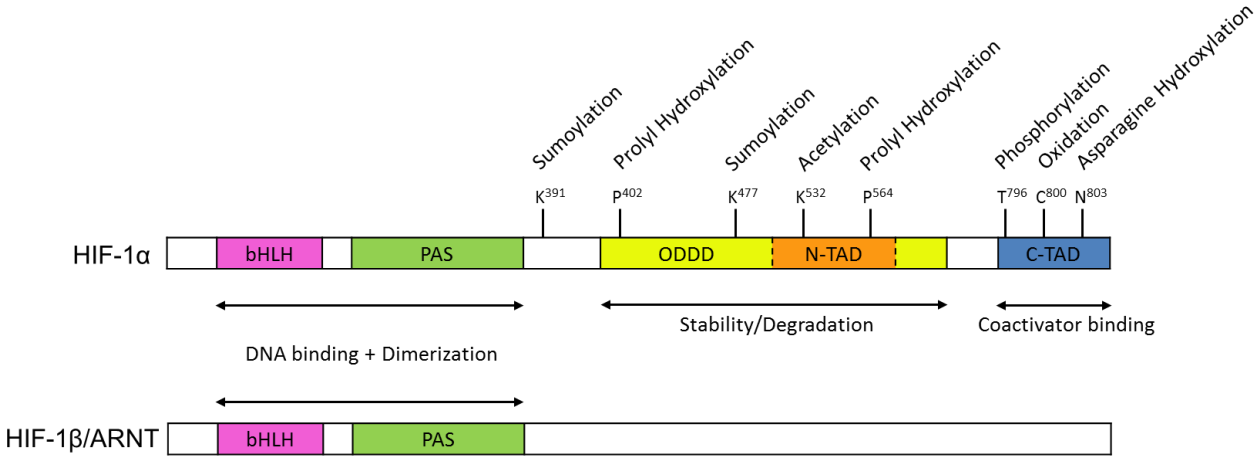

Fig. 1-5 Domain structure of HIF-1α and HIF-1β.bHLH: basic helix-loop-helix; PAS: Per/Arnt/Sim; N-TAD: N-terminal transactivation domain; ODDD: oxygen dependent degradation domain; C-TAD: C-terminal transactivation domain. Major modification sites are approximately shown within the domains of HIF-1α.

1.3.2 The regulation of HIF-1α

The dominant regulation mechanism of HIF-1α occurs through oxygen-dependent enzymatic hydroxylation and subsequent proteolysis (summarized in **Fig 1-6**). HIF-1α is hydroxylated on the prolyl residues 402 and 564 within the ODD domain[372-374]. Both sites of modification contain a conserved LXXLAP motif and the hydroxylation occurs at their 4-position[375]. The hydroxylation is mediated by a family of prolyl hydroxylases, namely prolyl-4-hydroxylase-domain proteins (PHD1, PHD2, PHD3)[375,376]. PHD1 is specifically localized in the nucleus, PHD2 is mainly localized in the cytoplasm and PHD3 seems to have no preference[377]. These three homologs were originally designated EGLN 2,1,3, respectively, on the basis of protein sequence homology to EGL-9, the HIF-1 prolyl hydroxylase of *Caenorhabditis elegans*[378]. All three PHDs have the potential to hydroxylate HIF-1α*in vitro* with their relative activities as PHD2 ≫ PHD3 > PHD1, and PHD2 is shown to be the key limiting enzyme that controls the HIF-1α turnover *in vivo*[376,379]. These iron-dependent enzymes convert proline into hydroxyproline, a reaction that requires oxygen, 2-oxoglutarate (2-OG), Fe^{2+} and ascorbate[380]. The hydroxylation process splits O_2; one oxygen atom is transferred to the proline residue and the other reacts with 2-OG to generate succinate and CO_2[374]. The activity of these enzymes is governed by the O_2 concentration within the cell, which defines these proteins as oxygen sensors. The requirement for iron and 2-OG as cofactors explains the observed hypoxia-mimetic effects of iron chelators such as desferrioxamine (DFO), iron competitive inhibitors such as cobalt chloride ($CoCl_2$) and 2-OG analogues such as dimethyloxaloylglycine (DMOG) [381]. DFO, $CoCl_2$, and DMOG are routinely used both *in vitro* and *in vivo* to inhibit PHD enzyme activity and thus stabilize HIF-1α.

In the presence of oxygen, PHDs are active and hydroxylate the prolines of HIF-1α, leading to a recognition signal for binding of pVHL to the HIF-1α ODD domain[382]. The pVHL associates with the proteins elongin C, elongin B, cullin-2, and Rbx1 to form the VCB-Cul2 E3 ligase complex[382]. Binding of HIF-1α to this multiprotein E3 complex causes polyubiquitination of HIF-1α, ultimately leading to its degradation by the 26S proteasome[383]. The half-life of HIF-1α is < 5 min in normoxia[384]. Under hypoxic conditions (< 6% O2), PHDs activity is inhibited, resulting in HIF-1α stabilization.

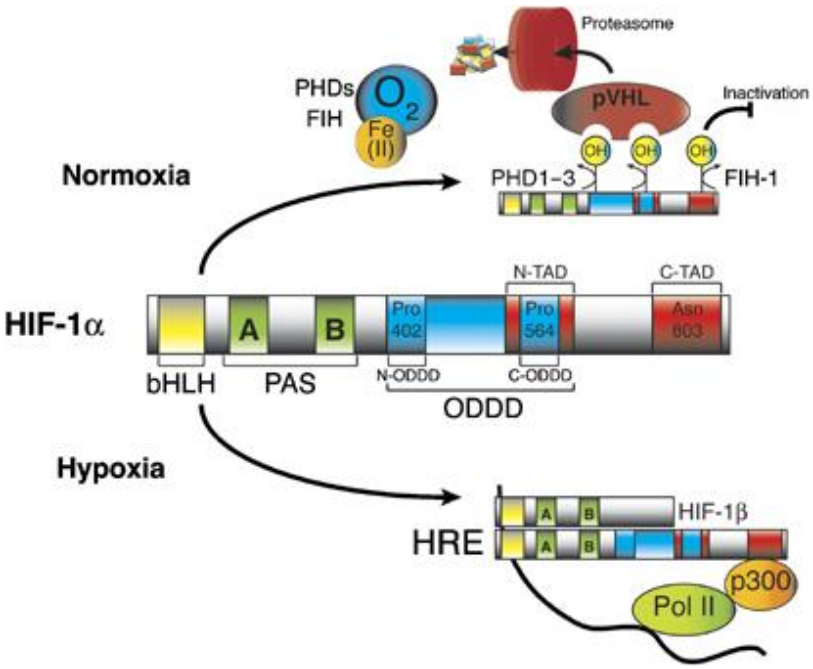

Fig. 1-6 Regulation of HIF-1α protein by prolyl hydroxylation and proteasomal degradation. In the presence of oxygen, prolyl hydroxylation is catalyzed by the Fe^{2+}-, oxygen- and 2-OG-dependent PHDs. The hydroxylated prolyl residues allow capture of HIF-1α by the pVHL, leading to ubiquitination and subsequent proteasomal degradation. Asparaginyl hydroxylation is catalyzed by an enzyme termed as factor-inhibiting HIF (FIH) at a single site in the C-TAD. This hydroxylation prevents cofactor recruitment. In the absence of hydroxylation due to hypoxia or PHD inhibition, HIF-1α translocates to the nucleus, heterodimerizes with HIF-1β and binds to HREs in the regulatory regions of target genes. This schematic drawing was cited from Weidemann A and Johnson RS (Weidemann A and Johnson RS, 2008).

This leads to an accumulation of HIF-1α subunits in the cytoplasm. HIF-1α then translocates into the cell nucleus, where it dimerizes with HIF-1β and forms the active transcription factor HIF-1. HIF-1 binds to HREs within the promoter regions of its target genes, promoting the adaptation to low oxygen concentrations on cellular and systemic levels.

Additionally, regulation of HIF-1α transcriptional activity is governed by the factor inhibiting HIF (FIH) present in the nucleus as well as cytoplasm[377]. FIH is an asparginyl hydroxylase that catalyzes the hydroxylation of a conserved asparagine residue Asn803 within the C-TAD under normoxic conditions. The hydroxylation of Asn803 leads to a steric inhibition of the interaction between HIF-1α and its co-activator CBP/p300[385,386], interfering with its recruitment. Eventually, this results in the prevention of the formation a transcriptional active HIF-1 complex. Hypoxia abrogates asparagine hydroxylation, which allows the C-TAD of HIF-1α to efficiently interact with CBP/p300 therein, activating the transcription of the respective target genes[387].

Although O_2-sensitive prolyl and asparaginyl hydroxylation events are two principal mechanisms regulating the HIF-1α, several additional regulatory pathways have also been uncovered. HIF-1α can be acetylated on lysine residue 532 in the ODDD domain[388]. Acetylation may affect HIF-1α hydroxylation and ubiquitination. K532 acetylation by the murine acetyltransferase arrest-defective-1 (ARD-1) enhances the interaction between HIF-1α and pVHL and, thus, leads to increased ubiquitination and concomitant proteasomal degradation[388]. This indicates that acetylation of HIF-1α is critical for accelerated degradation. Direct phosphorylation of HIF-1α on threonine 796 (T796) and serine 641 and 643 (S641, S643) by MAPK has been reported[389]. Phosphorylation does not affect its stability or binding affinity to DNA but it is able to increase HIF-1 transcriptional activity[390]. One mechanism to explain the increased activity is that HIF-1β binds preferentially to the phosphorylated form of HIF-1α[391]. Moreover, it has been reported that phosphorylation at T796 in HIF-1α increases the affinity of the interaction between HIF-1α and the transcriptional co-activator CBP/p300[392]. Consistent with the above, it has also been reported that phosphorylation of T796 prevents the hydroxylation of N803 by FIH. In addition, phosphorylation of HIF-1α by glycogen synthase kinase (GSK)-3β may target HIF-1α for proteasomal degradation [393].

Hydroxyl radical and hydrogen peroxide can destabilize HIF-1α protein in both normoxic and hypoxic conditions[394,395]. The oxidized HIF-1α protein might be recognized and degraded by the ubiquitin-independent 20S proteasomal pathway, which primarily degrades cellular oxidized proteins under oxidative stress conditions[396]. Therefore, the 20S proteasomal pathway may play an important role in the degradation of HIF-1α in ischemic conditions. Moreover, studies have revealed that *S*-nitrosation stabilizes HIF-1α protein and *S*-nitrosation of cysteine 800 of HIF-1α promotes its interaction with CBP/p300, eventually stimulating transactivation of the HIF-1 complex[397]. Hypoxia induces small ubiquitin-like modifier (SUMO)-1 expression and increases HIF-1α SUMOylation[398]. Conflicting results have been reported leading to an increase or decrease of HIF-1α. SUMOylation of HIF-1α promotes hydroxyproline-independent HIF-1α–pVHL E3 ligase complex binding, thus leading to HIF-1α ubiquitylation and proteasomal degradation[399]. By contrast, the RWD-containing SUMOylation enhancer (RSUME), which increases overall SUMO conjugation by interacting with the SUMO E2 enzyme Ubc9, increases HIF-1α SUMOylation, resulting in increased HIF-1α protein levels and transactivation[400]. Hence, hypoxia-induced HIF-1α SUMOylation can promote either its stabilization or pVHL-dependent degradation.

The oxygen pVHL independent regulation of HIF-1α is also mediated by the molecular chaperone heat shock protein 90 (Hsp90) and receptor of activated protein kinase (RACK1)[401]. Hsp90 is a molecular chaperone that protects client proteins from misfolding and degradation through its ATPase activity[402,403]. Hsp90 binds to the PAS domain of HIF-1α and increases its stability. Hsp90 inhibitors geldanamycin and 17-allyaminogeldanamycin (17-AAG) compete with ATP for binding to Hsp90 and disrupt the interaction of Hsp90 with HIF-1α[404,405]. Hsp90 inhibition results in proteasomal degradation of HIF-1α even in cells without functional pVHL[405,406]. Stimuli that promote interaction of Hsp90 and HIF-1α may stabilize HIF-1α and increase HIF-1 activity. RACK1 competes with Hsp90 for binding with HIF-1α. It binds HIF-1α and recruits the elongin C and other components of the E3 ligase complex, leading to HIF-1α ubiquitination and degradation[401]. Thus, by competing Hsp90 for binding to HIF-1α, RACK1 mediates a degradation pathway that is mechanistically similar to pVHL pathway with the

difference being that it is oxygen independent. Hypoxia-associated factor (HAF) also mediates oxygen independent HIF-1α degradation that is complementary to oxygen dependent pathways mediated by pVHL, thus providing an additional level of control that allows for HIF regulation under diverse conditions[407]. HAF has a dual nature on one side it inhibits HIF-1α signaling by degradation and on the other side it has DNA binding activity and promotes the transcription of some of the HIF-1α key downstream targets[408].

Together with the mentioned modifications, HIF-1α transcription and translation have been shown to be regulated by signaling pathways like PI3K, PKB/Akt, and mammalian target of rapamycin (mTOR) in a tissue-specific manner[409-412]. A battery of growth factors and cytokines, such as epidermal growth factor (EGF), transforming growth factor-α,insulin-like growth factor-1 and -2, heregulin, and IL-1β, can stimulate HIF-1α synthesis via activation of PI3K or MAPK pathways[381,413]. These pathways can be activated by signaling via receptor tyrosine kinases, non-receptor tyrosine kinases or G-protein-coupled receptors. PI3K activates the downstream serine/threonine kinases Akt and mTOR. mTOR phosphorylates the translation initiation factors, which increases the rate at which a subset of mRNAs (including HIF-1α mRNA) are translated into protein[414].

1.3.3 Neuroprotective effect of HIF-1

HIF-1 as a transcription factor in response to changes in oxygen levels has been found to be expressed in brain after ischemic insults. It was first reported that both mRNA of HIF-1α and β subunits were induced in the brain of rats and mice subjected to hypoxia for 30 to 60 min[415]. Systemic hypoxia for 1, 3, or 6 h rapidly increased the nuclear content of HIF-1α in mouse brain[416]. Focal cerebral ischemia induced a robust upregulation of mRNAs encoding HIF-1α in the peri-infarct penumbra by 7.5 h after ischemia and increased further at 19 and 24 h[417]. Another study reported a 1.8–2.5-fold increase in both HIF-1α and HIF-1β proteins by 20 h after reperfusion[418]. A study examining the temporal and spatial expression of HIF-1α in the penumbra after transient MCAO showed that HIF-1α began to be induced 1 h after ischemia, peaked at 12 h and then decreased within 48 h[419]. In the global ischemic model, HIF-1α expression was increased 8–24 h after ischemia and persisted for 96 h after reperfusion[420,421]. Baranova *et*

al. demonstrated a biphasic activation of HIF-1α in neurons. The expression of HIF-1α began to increase 1 h after MCAO, then reacheda maximum by 6 h. After a transient decline at 1 day, HIF-1α protein increased again by the second day of recovery and remained elevated for at least 8 days[422].

In response to lower oxygen concentration, HIF-1α accumulates and triggers the expression of several genes which initiate angiogenesis, erythropoiesis, vascular tone maintenance, energy metabolism, and cell survival following ischemic/hypoxic injury. There is growing evidence which shows that activation of HIF-1α offers protection in various cerebral ischemic models. Preconditioning involves exposing brain to moderate and brief stimuli that induce autoprotective pathways that better equip the cell and tissue to deal with subsequent episodes of ischemia[423]. HIF-1 has been implicated in the neuroprotection induced by hypoxic/chemical preconditioning in brains. *In vivo* studies in both neonatal and adult rats have shown that preconditioning with hypoxia or PHD inhibitor DFO or $CoCl_2$ is protective in pre-clinical models of cerebral ischemia[424-426]. It has been reported that elevation of HIF-1α levelsby inhibiting PHDs prevents neuronal damage againstoxidative stress *in vitro* and permanent focal ischemic injury *in vivo*[427]. Administration of DFO reduced brain damage and promoted functional recovery after transient MCAO[428-431]. Moreover, DMOG has been reported to enhance HIF-1α activation and attenuate ischemic brain injury in both permanent and transient ischemia models[432,433]. A novel HIF-PHD specific inhibitor exhibited neuroprotective properties after global cerebral ischemia in the gerbil[434]. Transgenic mice with neuron-specific PHD2 ablation exhibited a strong reduction in infarct size along with increased stability of HIF-1α after focal ischemia[435]. Injection of a novel protease inhibitor induced long-term neuroprotection and enhanced angioneurogenesis in a mouse MCAO model, which was dependent on HIF-1α accumulation[436]. Conversely, it was observed that HIF-1α inhibitor 2-methoxyestradiol(2ME2) treatment lowered neuronal cell survival, which suggested that HIF-1 suppression worsened outcomes after global ischemia in rats[437]. Baranova et al. demonstrated that the pharmacologic HIF-1 activators significantly reduced ischemic injury in wild-type mice, whereas the effectiveness of these compounds was attenuated in mice with neuron-specific HIF-1α knockdown[422].

Some evidence also exists to support a role for HIF and HIF target genes in aspects of neural cell progenitor biology (e.g., maintenance of the neural stem cell niche, neural stem cell proliferation, mobilization of neural stem cells (NSCs) to areas of ischemic insult, and differentiation of neural progenitors). Interestingly, recent studies investigating stem cell transplantation as a therapy for ischemic damage have demonstrated that hypoxic preconditioning of the stem cells prior to transplantation results in improved outcome[438,439]. In a rodent stroke model, less cell death and increased motor function were observed in rodents transplanted with hypoxic preconditioned stem cells, compared to those transplanted with untreated stem cells[438]. Chu *et al.* showed that treatment with DFO stabilized HIF-1α and activated HIF-1 target genes involved in compensation for ischemia in human NSCs. Furthermore, rats transplanted with DFO-treated NSCs, which were subsequently subjected to focal ischemia, displayed further reduction of infarct volume compared to the animals transplanted with naïve NSCs[440]. In consistent, transplantation of NSCs which overexpressed HIF-1α into rat brain after cerebral ischemia induced a greater neurological improvement than those treated with control NSCs[441]. Taken together, the evidence above suggests that HIF-1 activation within the brain may play a beneficial role in supporting endogenous neuroregeneration following ischemia.

The evidence above reveals that induction of HIF-1α produces neuroprotection against cerebral ischemia.HIF-1α accumulates in ischemic brain and triggers the expression of several genes which have been proposed to account for the neuroprotective effects of HIF-1. The following section discusses a limited selection of genes known to be activated by HIF-1, highlighting some of the evidence which supports their role in neuroprotection.

HIF-Regulated Tools for Neuroprotection

EPO

EPO is a circulating glycoprotein hormone that is predominantly synthesized in the kidney and liver; its primary role is in the stimulation of erythrocyte production. In fact, EPO is the prototypic HIF-1α responsive gene, since it was investigation of the mechanism underlying increased expression of EPO in response to low oxygen that led to the identification of HIF[442]. The brain expresses both EPO and the

EPO receptor, and preclinical and preliminary clinical studies indicate that EPO may play a crucial role in protecting cells against hypoxic/ischemic injury[443-445]. Studies of human and rat brain show that cerebral EPO is produced by both neuronal and glial cells and that neurons, glia, and cerebral endothelial cells all express the EPO receptor[446,447]. Adding EPO to the hippocampal slice *in vitro* has shown to provide neuroprotection of cortical neurons from oxygen and glucose deprivation (OGD)[448]. Numerous *in vivo* studies have reported that directly EPO administration into the brain reduced neurologic dysfunction in rodent models of cerebral ischemia[449-454]. Furthermore, neutralization of endogenous brain EPO potentiates ischemic brain injury, confirming a pivotal role for the endogenous EPO system in neuronal survival after ischemia[455]. EPO is implicated in the ischemic tolerance in preconditioning, since experiments in which endogenous EPO was neutralized showed that EPO inhibition was sufficient to abrogate, at least in part, the neuroprotective effects of hypoxia preconditioning[450]. A clinical trial conducted in 13 patients that received recombinant human EPO intravenously once daily for the first 3 days after stroke showed a reduction in the infarct size when compared with controls, this effect being associated with an improvement in clinical outcome[456]. The neuroprotectant role of EPO may be multimodal, with anti-apoptotic, neurotrophic, angiogenic, and anti-inflammatory effects[457,458]. Marti *et al.* have suggested that EPO might lead to the formation of new blood vessels, which may further increase the blood flow and oxygen availability that neutralizes the damaging effect of hypoxia on neuronal cells[459]. Further, the programmed neuronal apoptosis was inhibited by increased EPO expression in the ischemic penumbra following MCAO in rats[460]. The signaling pathways involved in anti-apoptosis are not fully elucidated, but it has been found that EPO downregulates the proapoptotic genes BAX and DP5 and induces the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL in the ischemic brain[461,462]. EPO may also limit cerebral damage through anti-inflammatory effects. Recombinant EPO administered in a rat model of cerebral ischemia reduced both the activation of glial cells and the number of infiltrating leukocytes[463]. Levels of the pro-inflammatory factors TNFα, IL-6, and MCP1 were also decreased. EPO could also enhance repair of damaged areas by promoting neurogenesis and angiogenesis through stimulation of neural and erythroid progenitor proliferation and differentiation[464,465].

VEGF

VEGF is induced following ischemic stroke and shows neuroprotective effects in several animal models of cerebral ischemia[466-470]. VEGF promotes neurorestoration after ischemia either directly as a neuroprotective agent or indirectly by inducing angiogenesis[471-473]. Local(i.e., ICV or brain) application of VEGF, VEGF-secreting cells, or vectors on structural histological injury consistently revealed a reduction of ischemic brain damage[292,474-477]. For example, topical administration of VEGF to the surface of rat brains reduces infarct size[471,474]. Intracerebroventricular VEGF treatment following transient cerebral ischemia reduced infarct volume and attenuated sensorimotor and cognitive deficits[478]. Conversely, administration of neutralizing anti-VEGF antibodies or antisense knock-down of VEGF increased neuronal damage after ischemia[479-481]. VEGF's ability to promote cerebral angiogenesis may be beneficial following stroke by improving oxygen and nutrient delivery to the ischemic area. In rodent models of stroke, an increase in angiogenesis by VEGF is associated with reduced infarct volume and neurological deficits after focal cerebral ischemia[289,471]. Neuron-specific overexpression of VEGF induces higher brain microvessel density in the cortical ischemia penumbra[482]. Also, it has been postulated that VEGF protects ischemic brain via direct neurotrophic effects. Direct neuroprotection by VEGF may be related to activation of flk-1 receptors, modulation of the PI3K/Akt/NF-κB signaling pathway[483], and inhibition of caspase-3 activity[484].

VEGF can also stimulate neurogenesis *in vitro* in cortical neuronal cell cultures and *in vivo* in the rostral subventricular zone (SVZ) and in the subgranular zone (SGZ)[471,485,486]. In VEGF-transgenic mice, brains examined 1–4 weeks after ischemia showed markedly increased SVZ neurogenesis, with chains of neuroblasts extending from the SVZ to the peri-infarct cortex, along with an increase in the number of newly generated cortical neurons at 2–4 weeks after ischemia[485]. Delivering VEGF via an adeno-associated viral vector system reduced the size of the infarct and promoted neurological recovery by mechanisms involving enhanced SVZ neurogenesis and neural precursor cell migration in the direction of the evolving brain infarct[487]. Additionally, VEGF promotes the outgrowth of axons from existing neurons in the contralesional hemisphere, leading to functional improvements of motor function and

coordination[488,489]. In summary, VEGF is involved in angiogenesis, neuronal survival, neurogenesis, and brain plasticity that result in functional neurological improvements after stroke.

Glycolytic pathway

The brain consumes a large amount of ATP, and blood flow to the brain is critical for maintaining the supply of glucose and oxygen required for mitochondrial ATP production. Ischemic disturbance in CBF results in high oxygen and glucose starvation. During the neuronal activity induced by ischemia, astrocytes respond to glutamatergic activation by increasing glucose utilization, enhancing glycolysis and lactate release. Neurons use lactate released from astrocytes as their primary energy substrate. Glucose needs to be supplied to meet its required amount. Hence, to overcome the lack of glucose availability, the neurons and astrocytes have to fulfill the glucose demand by increasing the activity of glycolytic enzymes and glucose transporters (GLUTs)[490], which enhance the concentration of glucose and thereby inducing neuroprotection. HIF-1 activation is known to trigger a switch from oxidative to glycolytic metabolism by inducing GLUT1, GLUT3 and genes involved in the glycolytic pathway (e.g., glucosephosphate isomerase, aldolase A and C, phosphoglycerate kinase 1, and lactate dehydrogenase A)[491,492]. Also, HIF-1 is known to upregulate pyruvate dehydrogenase kinase, which inhibits the key mitrochondrial enzyme pyruvate dehydrogenase, in turn preventing the flow of pyruvate into the tricarboxylic acid (TCA) cycle, instead shunting it to lactate[493,494]. Lactate is used as an energy substrate in active neurons for ATP generation by TCA cycle. In addition to maintaining the cellular energy supply, this also results in decreased cellular oxidative stress during hypoxia by reducing mitochondrial ROS leakage. Glucose transport and glycolytic flow as a result of HIF-1α activation by hypoxia has been linked to the ability to maintain energy homeostasis as oxygen levels are lowered. However, the relevance of changes in glycolysis to survival in ischemia is likely to be limited due to severely limited availability of glucose and the accumulation of lactate because of the lack of oxygen in neurons.

Other pathways

Heme oxygenase (HO) is an antioxidant enzyme and its activation is regulated by HIF-1α. HO-1 cleaves the heme molecule, producing free iron, carbon monoxide (acts as anti-apoptotic agent), and bilirubin.

Bilirubin is a potent free radical scavenger and can therefore limit ischemic reperfusion injury by preventing free radical mediated damage[495,496]. Neuronal overexpression of HO-1 in mice has been shown to reduce lipid peroxidation, infarct volume and cerebral edema compared to wild-type 24 h after ischemia[497]. Furthermore, a recent study has reported that ischemic preconditioning conveyed partial protection (reduced infarct volume and neurological deficits) in wild-type, but not HO-1 knockout mice in experimental models of both transient and permanent cerebral ischemia, suggesting that the neuroprotective properties of ischemic preconditioning are at least in part dependent on HO-1[498]. HO-1 has also been demonstrated to play an anti-inflammatory role by inhibiting the expression of proinflammatory cell adhesion molecules such as ICAM [499,500].

Adrenomedullin (AM) is a vasodilating hormone which is induced in the ischemic and hypoxic preconditioning brain in a HIF-1 dependent manner[501]. AM protects murine primary cortical neurons against OGD*in vitro*[502]. Transgenic mice overexpressing AM displayed enhanced vascular regeneration and subsequent neurogenesis[503]. The vasculo-neuro-regenerative actions observed in transgenic mice in combination with neuroprotection resulted in improved recovery of motor function. Furthermore, administration of exogenous AM has been reported to be neuroprotective in numerous experimental models of ischemic brain injury[504].

Brain-derived neurotrophic factor (BDNF) shows neuroprotection in case of cerebral focal and global ischemia[505,506]. A recent study shows that HRE regulates the expression of adenovirus-mediated BDNF, constructed by adenoviral vector with five copies of HRE found in VEGF gene responsible for the regulation of BDNF in hypoxia[507]. BDNF is beneficial for the survival of ischemic neurons though direct anti-apoptotic and anti-inflammatory effects, whereas it also promotes neural regeneration and contributes to cognitive functions and memory acquisition after ischemia[508].

Necrosis and apoptosis are the main pathways of cell death in cerebral ischemia. There is also evidence showing that HIF-1α exertsanti-apoptotic effect by the inhibition of cytochrome C release, caspase activation or poly ADP-rebose polymerasecleavage[509,510]. Inhibition of cytochrome C release, caspase activity and activation of Akt play an important role in preventing the DNA fragmentation and

cell injury[511]. In some cases, HIF-1α restricts the apoptosis by the suppression of p53 too [512]but this is dependent upon the severity or duration of hypoxia/ischemia.

1.3.4 Detrimental role of HIF-1

Although HIF-1 plays a significant role in neuroprotective mechanisms, it also behaves as an apoptotic inducer in cerebral stroke. Jiang *et al.*have reported that inhibiting HIF-1 activity by a dominant negative form of HIF reduces neuronal damage in response to OGD. This is further supported by the observation that HIF-1α is responsible for hypoxia-induced apoptosis in embryonic stem (ES) cells[513]. The study indicates that HIF-1α binds and stabilizes p53, and HIF-1α-dependent induction of p53 and p21 induce apoptosis in the hypoxic ES cells. Similarly, it has been observed that HIF-1α signaling elicits delayed death involving the participation of p53 in ischemic primary cortical neurons[514]. Suppressing HIF-1α with hyperbaric oxygen treatment remarkably attenuates HIF-1α and p53 interactions with reduced apoptosis after neonatal hypoxia-ischemia[515,516]. HIF-1α also governs the expression of a number of proapoptotic family members including the Bcl-2/adenovirus EIB 19kDa-interacting protein 3 (BNIP3), Nip-like protein X (NIX), and NOXA[517]. Early Inhibition of HIF-1α significantly attenuates neuronal injury and down-regulates NIX expression in both *in vitro* neuron culture and rat MCAO models, whereas late inhibition aggravates neuronal damage[518]. Treatment withHIF-1α siRNA protects the brain from ischemic damage by inhibiting HIF-1α-induced apoptotic pathway such as p53 and caspase-3 [519]. It has also been reported that HIF-1α inhibitors, 2ME2 and tricyclodecan-9-yl-xanthogenate (D609), protect brain from ischemic injury by suppressing neuronal apoptotic via BNIP3 pathway[520]. It has been suggested that BNIP3 contains HRE in its promoter site [521] which confirms that it is a direct target of HIF-1 and its activation leads to ischemic cell death by mitochondrial dysfunction, membrane depolarization, and mitochondrial permeability transition pore (MPTP) opening [522]. Furthermore, HIF-1 upregulates the expression of iNOS [523], inflammatory cytokines such as IL-20 and IL-1β [524] and lactate dehydrogenase [491], resulting in increased nitric oxide, inflammation and acidosis, which might also cause potentially detrimental effects on ischemic brain tissue. HIF-1 activation may play a role in brain vascular endothelium disruption and edema formation after stroke, which is reviewed in section 1.3.5.

Using the same neuron-specific HIF-1α knock-out mice, Baranova *et al.* and Helton *et al.* have reported distinct effects of HIF-1 on neuronal injuries following ischemia. Helton et al. first reported an advantageous effect of neuronal HIF-1α knock-out in the bilateral common carotid artery occlusion (BCCAO) model of global ischemia [525]. This was attributed to suppression of pro-apoptotic HIF-1 target genes, such as BNIP3. In contrast, Baranova et al. observed that HIF-1α knock-out was deleterious in the MCAO stroke model [422]. The cause of the discrepancies between these reports remains unclear; but may be a reflection of the stroke models used global (BCCAO) vs. focal (MCAO) ischemia and the duration of the ischemic insult (75 vs. 30 min). The pro-survival and pro-death effects of HIF-1α may depend on the severity of the insults, the timing of HIF-1α induction, and cell types that express HIF-1α.

In summary, HIF-1 plays an important role in the fate of ischemic neurons. Activation of HIF-1α is likely to mediate both beneficial and detrimental effects. Understanding the mechanism of HIF-1 accumulation will undoubtedly shed new light on its role in cerebral ischemia and provide potential approaches to regulate its expression.

1.3.5 HIF-1 in BBB disruption

Hypoxia is an important pathogenic factor for the alteration of TJ proteins and induction of vascular leakage in the brain. The role of HIF-1 and its target genes in major cellular adaptions in response to low oxygen availability suggests they may be instrumental modulators of BBB integrity. Indeed, current evidence supports that HIF-1 is a likely mediator of BBB disruption.

A study by Witt *et al.* first suggested that transcription factors such as HIF-1 and NF-κB are upstream mediators of TJ protein alterations under the conditions of hypoxia and post-hypoxia/reoxygenation, which may involve VEGF induction and expression. VEGF is a strong inducer of vascular permeability, and increased VEGF levels positively correlate with changes in TJ redistribution of ZO-1 and occludin, as well as the alterations on the actin cytoskeleton[526]. Engelhardt *et al.*recently found that hypoxic exposure rapidly induced stabilization of HIF-1α concomitantly with BBB impairment and TJ disruption mainly through delocalization and increased tyrosine phosphorylation of TJ proteins in the rat brain endothelial cell line RBE4. Similar observations were obtained by normoxic stabilization of

HIF-1α using $CoCl_2$, DFO, and DMOG underlining the involvement of HIF-1 in barrier dysfunction particularly via TJ alterations. In agreement, inhibition of HIF-1 stabilization by HIF-1α inhibitors 2ME2and YC-1 improved barrier function in hypoxic cells[527]. Additionally, Yeh *et al.* demonstrated that YC-1 prevented chemical hypoxia and H/R-induced hyperpermeablity and ZO-1 decrease and disassembly in adult rat brain endothelial culture, which was likely via the inhibition of VEGF expression. Pretreatment of YC-1 also protected BBB *in vivo* against ischemia/reperfusion-induced injury[528]. Post-ischemic administration of two other HIF-1α inhibitors, 2ME2 andD609, protected brain from cerebral ischemic injury by inhibiting HIF-1α expression, attenuating superfluous VEGF to avoid BBB disruption[520]. Elevation of HIF-1α also seemed to be detrimental in neonatal brain injury in a rat pup hypoxic-ischemic model[529]. Acute inhibition of HIF-1α by 2ME2 exhibited neuroprotection by preserving BBB integrity and reducing brain edema. On the contrary, Upregulation of HIF-1α by DMOG increased the permeability of BBB and brain edema. However, most of the studies focusing on HIF-1's effect on BBB have not demonstrated convincingly that the crucial HIF-1 modulation takes place in brain endothelial cells. Recently, a study in our lab showed that YC-1 ameliorated ischemia-induced BBB permeability increase (determined by EB extravasation) although it did not affect brain edema formation and remarkably enlarged infarct volume evaluated by MRI and histological staining[530]. The data also indicate that HIF-1-induced VEGF increases BBB permeability while certain other proteins coded by HIF-1's down-stream genes such as EPO and GLUT provide neuroprotection in ischemic stroke. The study implies that HIF-1 may function differently in different cell types depending on the functions of its down-stream factors in the specific type of cells, and HIF-1 modulation may have different effects on ischemic outcome and BBB permeability.

Notably, deleterious effects of HIF-1 on BBB may not only be limited to ischemic stroke. It has been reported that HIF-1 plays an important role in brain edema formation and BBB disruption via a molecular pathway cascade involving aquaporin-4 (AQP-4) and MMP-9in a model of traumatic brain injury[531]. HIF-1α activation in the brain of dystrophic mouse was also suggested to be partly responsible for both BBB opening and increased angiogenesis through down-regulation and phosphorylation of ZO-1

via VEGF signaling[532]. In subarachnoid hemorrhage models, inhibition of HIF-1 significantly suppressed the level of AQP-4 and MMP-9 and partially repressed brain edema and BBB impairment[533,534]. Our lab previously has demonstrated that HIF-1 is highly involved in high glucose-induced paracellular permeability increase in *in vitro* BBB model[535]. Upregulating HIF-1 activity by $CoCl_2$ increased the permeability of endothelial monolayer exposed to normal glucose, whereas suppressing HIF-1 activity by pharmacological inhibitors and genetic depletion ameliorated the permeability changes by restoring expression and localization of ZO-1 and occludin in high glucose-treated cells.

1.4 Aims of the study

1.4.1 Part I: The role of HIF-1 in antioxidant-induced neuroprotection in ischemic stroke

Stroke is a devastating disease, which annually affects 17 million people worldwide, and is the third leading cause of death in the U.S. Today the only FDA-approved agent in clinical use for the thrombolytic treatment of acute ischemic stroke is tPA, which is currently used in less than 5% of cases because of its narrow therapeutic window. Consequently, developing therapeutics for neuroprotection in ischemic stroke remains one of the major challenges in clinical medicine. Ischemic stroke compromises a complex cascade of pathophysiological mediators among which ROS play a pivotal role. Ischemia/reperfusion causes overproduction of ROS, which trigger many cellular and molecular events including protein oxidation, lipid peroxidation and DNA damage, leading to neuronal death. Although the involvement of ROS in ischemia/reperfusion injury is not under debate and beneficial effects due to antioxidants in experimental stroke models are evident, the clinical efficacy of antioxidative agents has not yet convincingly proven. The translational disappointment of antioxidants likely arises from the failure to understand the drug candidate's mechanism of action in relationship to stroke. Research progress in cellular redox signaling suggests that antioxidants may exert their biological functions through specific signaling pathways other than reducing ROS level.A greater understanding of the molecular targets of antioxidants will improve the chances of identifying promising therapeutic approaches.

Our previous study has demonstrated that HIF-1α can be stabilized by a reducing redox environment in primary cortical neurons exposed to hypoxia.<u>The specific aim of the current study is to characterize HIF-1's role in the antioxidant-mediated neuroprotection in ischemic stroke animal model.</u> In this study, rats and mice were subjected to 90 min focal cerebral ischemia and 24 h reperfusion to dissect the key molecular mechanisms involved in the antioxidant N-acetylcysteine (NAC)-mediated neuroprotection. We were interested in whether pretreatment with NAC upregulatesthe expression of HIF-1α and its target genes in the neurons of ischemic brain. Also, we aimed to determine whether the HIF-1 pathway mediates NAC's protective effect. As such, we examined how HIF-1 inhibitors and neuron specific HIF-1α knock-out influenced NAC's neuroprotection.

This study evaluated a new pathway through which NAC exerts its neuroprotection in a clinically relevant stroke model (presented in Chapter 2). We expect this finding can help design therapeutic approaches for ischemic stroke.

1.4.2 Part II: The role of HIF-1 in hyperglycemia-induced BBB disruption in ischemic stroke

Diabetes mellitus, which results in chronic hyperglycemia, has been linked with accelerated development of various macro- and microvascular diseases throughout the body. Diabetes holds a 2 to 6 fold increased risk for cerebrovascular disease and stroke. Moreover, hyperglycemia is associated with worsened outcomes in acute ischemic stroke. Accumulating evidence indicates that the worsened outcomes may be due to diabetes-induced cerebral vascular complications, especially disruption of the BBB. Increased BBB permeability has been found in both diabetic patients and experimental animal models after stroke. Understanding the mechanism of diabetes-induced BBB disruption may offer insights for the prevention and treatment of vascular dysfunction and disruption following ischemic stroke in diabetic patients.

As reviewed above, cerebral ischemia leads to brain vascular hyperpermeability and vasogenic edema, which contribute to the development of neurological damage. HIF-1 is an important regulator of vascular permeability. HIF-1α induction can alter the structure of vasculature and cause vascular remodeling through activation MMPs and VEGF. Furthermore, previous study in our lab suggested that HIF-1 activity is upregulated in BMECs exposed to high glucose. Therefore, <u>the specific aim was to investigate the involvement of HIF-1 in hyperglycemia-aggravated BBB disruption in ischemic stroke.</u>

To address this specific aim, first we used an *in vitro* BBB model, hCMEC/D3 cell culture. Cells were pretreated with normal glucose and different concentrations of high glucose before they were subjected to OGD, followed by reoxygenation with the same glucose concentration in pretreatment. We examined the effect of high glucose on HIF-1α and VEGF protein level, MMPs secretion, paracellular permeability of cell monolayer, and TJ proteins expression. Since we were interested in the role of HIF-1 on BBB permeability, we inhibited HIF-1α to test if *in vitro* BBB disruption in response to high glucose is related to HIF-1 activity. This study is presented in Chapter 3.

The next step was to determine if HIF-1 mediates the worsened BBB function after cerebral ischemia in a diabetic animal model. In the *in vitro* experiments, the endothelial cell culture is not able to fully mimic the NVU *in vivo* since BBB is formed by the interaction of several cell types. Therefore, we performed *in vivo* experiments on streptozotocin (STZ)-induced type I diabetic mice models. The diabetic mice were subjected to MCAO/reperfusion. We detected the expression of HIF-1α, VEGF, and TJ proteins on isolated cerebral blood vessels, and we also measured the BBB permeability and brain infarction. To further validate the role of HIF-1 in causing BBB damage, endothelial specific HIF-1α knock-out mice were used to determine whether genetic HIF-1 inhibition is able to ameliorate BBB disruption and brain damage in diabetic mice after stroke. This study is presented in Chapter 4.

The results of the studies will serve as a fundamental platform elucidating the pathogenesis of hyperglycemia-aggravated BBB disorder in focal ischemia, and will indicate important target for preventing BBB disruption in stroke.

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CHAPTER 2: Hypoxia-Inducible Factor 1 Contributes to N-acetylcysteine's Protection in Stroke

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Abstract

Stroke is a leading cause of adult morbidity and mortality with very limited treatment options. Evidence from pre-clinical models of ischemic stroke has demonstrated that the antioxidant N-acetylcysteine (NAC) effectively protects the brain from ischemic injury. Here, we evaluated a new pathway through which NAC exerted its neuroprotection in a transient cerebral ischemia animal model. Our results demonstrated that pre-treatment of NAC increased protein levels of hypoxia-inducible factor-1α (HIF-1α), the regulatable subunit of HIF-1, and its target proteins erythropoietin (EPO) and glucose transporter (GLUT)-3 in the ipsilateral hemispheres of rodents subjected to 90 min middle cerebral artery occlusion (MCAO) and 24 h reperfusion. Suppressing HIF-1 activity by two widely used pharmacological inhibitors, YC-1 and 2ME2, and specific knock-out of neuronal HIF-1α abolished NAC's neuroprotective effects. The results also showed that YC-1 and 2ME2 massively enlarged infarcts, indicating their toxic effect was larger than just abolishing NAC's neuroprotective effects. Furthermore, we determined the mechanism of NAC-mediated HIF-1α induction. We observed that NAC pre-treatments upregulated heat-shock protein 90 (Hsp90) expression and increased the interaction of Hsp90 with HIF-1α in ischemic brains. The enhanced association of Hsp90 with HIF-1α increased HIF-1α stability. Moreover, Hsp90 inhibition attenuated NAC-induced HIF-1α protein accumulation and diminished NAC-induced neuroprotection in the MCAO model. These results strongly indicate that HIF-1 plays an important role in NAC-mediated neuroprotection and provide a new molecular mechanism involved in the antioxidant's neuroprotection in ischemic stroke.

2.1 Introduction

Stroke is a leading cause of death in the United States and worldwide[1,2]. Reduced supply of oxygen and nutrients results in devastating loss of neurons and leads to defect in brain function in stroke patients [3]. Developing stroke therapeutics remains one of the major challenges in clinical medicine. There has been a considerable body of evidence suggests that oxidative stress associated with excessive production of reactive oxygen species (ROS) is a fundamental mechanism of brain damage in stroke[4]. Antioxidants prevent oxidative stress by counter balancing the harmful effects of ROS, and therefore it is logical to assume that they are useful in ischemic stroke. However, despite promising results conferred by antioxidants in experimental stroke studies[5,6], clinical trials of antioxidant therapeutics in human have shown very little benefit[7]. Among many factors contributing to the translational disappointment of antioxidants is the incomplete understanding of the drug candidates' mechanism of action in relationship to stroke [8]. A further exploration of the mechanism of antioxidants in stroke is crucial to the design and implementation of human trials.

Pharmacological effects of N-acetylcysteine (NAC)have been studied in stroke models by several research groups. Rodents treated with NAC before ischemia showed reduction in brain infarct volume[9,10], reduced neuronal cell death[11-13], and improvement in neurological function[9]. In addition, NAC is protective in other organs subjected to ischemia, such as heart, liver, lung, and kidney [14-18], and is beneficial in other types of brain diseases such as Parkinson's disease [19], Alzheimer's disease [20], and amyotrophic lateral sclerosis [21], by slowing down aging and increasing life span [22].

The neuroprotective effects of NAC in ischemia have generally been accredited to its ability to reduce ROS levels and to inhibit oxidation of lipids, proteins, and DNA. Over the last decades, research progress in cellular redox signaling suggests that antioxidants may exert their biological functions through specific signaling pathways. Studies on pathways that contribute to NAC's neuroprotective effects in ischemia are scarce although NAC has been suggest to mediate cell survival signaling pathways in other pathological conditions including cardiovascular, respiratory, and hepatic diseases[23]. One study has suggested that the neuroprotection of NAC is related to its anti-inflammatory activity through suppressing

the activity of nuclear factor-κB (NF-κB)[24]. However, it has been reported that NAC's protective effect is retained even administrated after the time period of burst of NF-κB activation [25].This observation suggested that anti-inflammation might not critically contribute to NAC's neuroprotection. A greater understanding of NAC-mediated changes on key pathways in pathological conditions such as ischemia may provide insights for developing promising therapeutic approaches.

HIF-1 is a predominant mediator of adaptive responses to decreased oxygen availability, a characteristic of ischemic stroke.HIF-1 is a heterodimer of two subunits, the regulatable HIF-1α and constitutively expressed and stable HIF-1β [26].The activity of HIF-1 is primarily determined by the level of its α subunit[27,28].Our previous study has demonstrated that NAC is able to induce HIF-1α expression in primary cortical neurons exposed to hypoxia[29]. Our present study provided experimental evidence that NAC stabilized HIF-1α and increased its down-stream target genes expression in the brains of transient cerebral ischemia animal models. More importantly, we demonstrated for the first time that the protective effects of NAC against ischemic injury were abolished when HIF-1 activity was inhibited by either pharmacological inhibitors or genetic depletion. Furthermore, we revealed a novel mechanism by which NAC upregulated HIF-1α protein expression in ischemic brains.

2.2 Materials and Methods

Animals

All procedures using animals were approved by the Institutional Animal Care and Use Committees of University of Kansas and University of New Mexico and conformed to the National Institutes of HealthGuidelines for use of animals in research. Animals were maintained in a climate-controlled vivarium with a 12 h light-dark cycle with free access to food and water. Male Sprague-Dawley rats, 280-310g, were from Charles River Laboratory (Wilmington, MA). Mice (B6.129-*hif-1α*^{tm3Rsjo}/J) carrying homozygous HIF-1α floxed alleles (HIF-1α$^{F/F}$) were generated by engineering loxP sites flanking exon 2 of the HIF-1α gene as described previously [30] and bought from the Jackson Laboratory (stock number: 007561, Bar Harbor, Maine, USA). Mice (B6.Cg-Tg (*CaMk2a-cre*) T29-1Stl/J) expressing *cre* recombinase under the control of the calcium/calmodulin-dependent kinase (CaMKII) promoter were generated as described previously [31] and also bought from the Jackson Laboratory (stock number: 005359). All mouse strains were maintained on a C57BL/6J background. All animals were acclimated to the environment for 7 days before the experiments. The mouse strain B6.Cg-Tg *(CaMk2a-cre)* was crossed with homozygous HIF-1α$^{F/F}$ mice to generate Cre$^{+/-}$: HIF-1α$^{F/Wt}$, which were then crossed with homozygous HIF-1α$^{F/F}$ mice to generate HIF-1α mutants Cre$^{+/-}$: HIF-1α$^{F/F}$, designated as neuron specific HIF-1α knock-out HIF-1α$^{\Delta/\Delta}$ as described previously [32]. Littermates with the Cre$^{-/-}$: HIF-1α$^{F/F}$ genotypes were used as controls for each group of experiments.

Genotyping

Genomic DNA was isolated from tail biopsies collected at 21 d of age using the DNeasy genomic DNA isolation kit (Qiagen, Valencia, CA, USA). HIF-1α^{F} and wild-type alleles were detected using the following primers: 5′-CGT GTG AGA AAA CTT CTG GAT G- 3′ and 5′-AAA AGT ATT GTG TTG GGG CAG T-3′. Transgenic mice expressing Cre recombinase were identified using primers: 5′-GCG GTC TGG CAG TAA AAA CTA TC-3′ and 5′-GTG AAA CAG CAT TGC TGT CAC TT-3′. The PCR

reactions were performed with the Omni Clenttaq polymerase (DNA Polymerase Company, St. Louis, Missouri, USA). The products were run on a 3% agrose gel for HIF-1α or Cre.

Middle cerebral artery occlusion (MCAO)

For the surgical procedures of rats, 4.0% isoflurane in N_2O:O_2 (70%:30%) was used for anesthesia induction, and 2.0% for anesthesia maintenance. Duration of anesthetic exposure was kept the same for each animal. MCAO followed by reperfusion was conducted using an intraluminal model as previously described [33]. Briefly, external carotid artery (ECA), internal carotid artery (ICA), and pterygopalatine artery of ICA were exposed. A silicone rubber-coated monofilament nylon suture (Doccol Corporation, Sharon, MA) with a diameter of 0.37mm was inserted into the ICA via a slit on the ECA. The suture was advanced along the ICA to the extent of 18 to 19 mm from the bifurcation of rats. Reperfusion was produced by gently withdrawing the suture until the suture tip reached the bifurcation and the incision closed 90 min after the onset of ischemia. After surgery, the animals were allowed to recover from anesthesia while being given food and water ad libitum. Buprenorphine was administrated at 0.1mg/kg subcutaneously as post-operative analgesia. For mouse anesthesia, 2.0% isoflurane in N_2O:O_2 (70%:30%) was used for induction, and 1.0% for maintenance. Similar surgical procedure was performed on mice with smaller size of suture with a diameter of 0.23 mm (Doccol Corporation, Sharon, MA). The suture was advanced along the ICA to the extent of 9 to 10 mm from the bifurcation of mice. For all animals used in this study, successful MCAO was confirmed by laser Doppler flowmetry (LDF) (Moor Instruments, Wilmington, DE) as described in the literature[34]. During ischemia, LDF regional cerebral blood flow dropped to 16.9 ± 3.6% (mice) and 15.4 ± 1.8% (rats) of the pre-ischemic level; and after reperfusion the blood flow was restored to 87.6 ± 4.7% (mice) and 90.5 ± 3.6% (rats) of pre-ischemic level. In addition, animals were placed on a heating pad during surgery. Body temperature was monitored through the surgery process. There was no significant difference in body temperature, which was in the range of 37.0 ± 0.3^{o}C. Animal that did not show any neurological deficits or had intracranial bleeding during the surgical process were excluded.

Experimental groups

SD male rats were randomly assigned to the following 9 groups: (1) 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) (without MCAO, n=5); (2) NAC (without MCAO, n=5); (3) 2-methoxyestradiol (2ME2) (without MCAO, n=5); (4) MCAO (n=25); (5) MCAO pretreated with NAC (n=25); (6) MCAO pretreated with YC-1 (n=30); (7) MCAO pretreated with YC-1 + NAC (n=30); (8) MCAO pretreated with 2ME2 (n=30); and (9) MCAO pretreated with 2ME2+ NAC (n=30). In all the six groups subjected to MCAO, 5 rats were used for Western blotting of whole contralateral and ipsilateral hemispheres; 5 rats were used for Western blotting of peri-infarct tissue; 5 rats were used for immunostaining; and 5 to 8 rats were used for infarct size measurement by magnetic resonance imaging (MRI)and 2,3,5-triphenyltetrazolium chlorid(TTC) staining **(Table 2-1)**. NAC (A7250, Sigma, St. Louis, MO) dissolved in saline with 1% dimethyl sulfoxide (DMSO) was administered at 150 mg/kg body weight intraperitoneally (i.p) at 30 min prior to the onset of ischemia according to a previous publication [13]. Growing evidence suggests that YC-1 and 2ME2 exert an inhibitory effect on the accumulation of HIF-1α induced by hypoxia, iron chelation, and proteasomal inhibition [35-39]. YC-1 has widely been used as a HIF-1 blocker in research. It has been demonstrated that YC-1 effectively inhibits HIF-1 expression in heart [40], kidney [41], and brain [42]. YC-1 may directly degrade HIF-1α protein by inducing the degradation of C-terminal of HIF-1α protein [43]. It can also suppress the translation of HIF-1α through PI3K/Akt/mTOR/4E-BP pathway [44]. YC-1 has been reported to inhibit the expression of HIF-1 down-stream genes such as EPO and vascular endothelial growth factor[37]. 2ME2 inhibits HIF-1α protein synthesis by disrupting microtubules [45]. Both YC-1 and 2ME2 were used as a complementary approach to knock-out in determining the effect of HIF-1. YC-1 (Cayman Chemical Company, Ann Arbor, MI) and 2ME2 (Enzo Life Science, Farmingdale, NY) were both dissolved in a solution of 1% DMSO in saline. YC-1 was administered at 2 mg/kg body weight through femoral vein at 24 h and 30 min prior to the onset of ischemia. 2ME2 was administered at 5 mg/kg body weight (i.p.) at 1 h prior to the onset of ischemia. Rats in control group received equal volume injections (i.p.) of the DMSO solution. We chose the dose of the HIF-1 inhibitors based on previous publications [35,36] and our own analysis of HIF-1α expression.

The HIF-1α knock-out (HIF-1$\alpha^{\Delta/\Delta}$) male mice were randomly assigned to two groups: MCAO group (n=6) and MCAO pretreated with NAC (n=6). Twelve male littermates (HIF-1$\alpha^{F/F}$) were used as controls and randomly assigned to two groups: MCAO group (n=6) and MCAO pretreated with NAC (n=6). In all the four groups, 3 mice were used for Western blotting analysis; and 3 mice were used for infarct size measurement by TTC. In mouse models of MCAO/reperfusion, NAC was administered at 240 mg/kg (dissolved in saline) body weight (i.p.) at 30 min prior to the onset of ischemia. The NAC dose for mice was calculated from rat dose using body surface area normalization method [46]. Mice in control and HIF-1α knock-out groups received equal volume of saline injections (i.p.).

Effect of 17-allylamino-17-demethoxygeldanamycin (17-AAG) (AG Scientific, San Diego, CA) was tested in the following groups of mice: NAC (without MCAO, n=3), 17-AAG (without MCAO, n=3), MCAO (n=3), MCAO pretreated with NAC (n=3), MCAO pretreated with 17-AAG (n=3), and MCAO pretreated with 17-AAG+ NAC (n=3). 17-AAG is a derivative of the antibiotic geldanamycin and is widely used as an Hsp90 inhibitor. 17-AAG was dissolved in a solution of 1% DMSO in saline and administrated at 25 mg/kg (i.p.) at 1 h prior to the onset of ischemia. Mice in control and NAC groups received equal volume of the DMSO solution (i.p.).

Usually four to five animals were randomly selected for surgery on a given day. We used a randomized block design.Each animal was subjected at random to one of the available treatments within each day.The following final analyses were performed by researchers who were blinded to the treatments and animals groups.

Table 2-1.HIF-1α inhibitor-induced mortality of MCAO rats and groups of rats for final analyses.

Groups	Analyses	Initial Group Size	Failed MCAO	Death after successful MCAO	Group Size for Final Analyses
MCAO	MRI+TTC	7	0	0	7
	HIF	18	1	2	15
	Total	25	1[a]	2 (8.3%)[b]	
NAC	MRI+TTC	10	1	1	8
	HIF	15	0	0	15
	Total	25	1[a]	1 (4.2%)[b]	
YC-1	MRI+TTC	10	1	4	5
	HIF	20	1	4	15
	Total	30	2[a]	8 (28.6%)[b]	
YC-1+NAC	MRI+TTC	10	1	3	6
	HIF	20	1	4	15
	Total	30	2[a]	7 (25.0%)[b]	
2ME2	MRI+TTC	11	0	5	6
	HIF	19	1	3	15
	Total	30	1[a]	8 (27.6%)[b]	
2ME2+NAC	MRI+TTC	10	0	3	7
	HIF	20	1	4	15
	Total	30	1[a]	7 (24.1%)[b]	

[a]Animals were excluded due to a lack of obvious neurological deficits or due to intracranial bleeding during the procedure. The intracranial bleeds happen when the monofilament suture is inserted too far and perforated the anterior cerebral artery. When the bleed occurred, the animals died immediately due to bleeding within the first 2 hours post-reperfusion.

[b]Death rate after successfully completed MCAO.

Western blotting and co-immunoprecipitation (Co-IP)

At 24 h reperfusion, the animals were anesthetized and euthanized by decapitation.The ipsilateral and contralateral hemispheres were isolated and homogenized separately for Western blotting assay. We also collected peri-infarct brain tissues and the corresponding region in the contralateral (non-ischemic) sides. The peri-infarct region was identified using TTC staining in control MCAO rat model (90 min ischemia and 24 h reperfusion, no pharmacological treatments) as described in our previous publication [47]. As marked in **Fig. 2-4A**, the ischemic peri-infarct was selected on the 1 o'clock line in the ipsilateral cortex. Tissues between the dashed lines (about 1.5mm in width) **(Fig. 2-4A)** were isolated from the ipsilateral and contralateral hemispheres and homogenized separately for Western blotting. The fixed location, as marked in **Fig. 2-4A**, was used for tissue isolation in animals received different pharmacological treatments. However,the location of peri-infarct varies from animal to animal, especially in animals from different groups. The selected region of treated animals may represent different pathological state from that of control MCAO rats. Standard Western blotting and Co-IP procedures were followed as described previously [35]. The primary antibodies were rabbit anti-HIF-1α (Millipore, Billerica, MA), mouse anti-Hsp90 (SPA-830, Enzo Life Science, Farmingdale, NY), rabbit anti-GLUT-3 (ab53095, Abcam, Cambridge, MA), and rabbit anti-EPO (sc-7956, Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody for HIF-1α, GLUT-3, and EPO was goat anti-rabbit IgG-HRP (sc-2030, Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody for Hsp90 was goat anti-mouse IgG-HRP (sc-2030, Santa Cruz Biotechnology, Santa Cruz, CA). β-actin was used as an internal control.

Measurement of infarct size by magnetic resonance imaging and TTC staining

The rats were transported to the MRI room next to the surgery room at 24 h reperfusion and placed in the isocenter of the magnet before the imaging session. MRI was performed on a 4.7 T Biospecs MR scanner (Bruker Biospin, Billerica, MA). An actively shielded gradient coil with a 120-cm inner diameter was used. The animals were kept in the same position throughout imaging. For each animal, we performed T2-weighted MRI by using a rapid acquisition with refocused echos sequence. Image data were then

transferred to a workstation running Linux for further processing. From the T2-weighted magnetic resonance images, we calculated the volume of infarction using ImageJ.

After MRI imaging, the brains were removed and sectioned into 2 mm slices. The slices were incubated in a 2% solution of TTC in 0.1 M PBS (pH 7.4) at 37°C for 30 min and fixed in 10% formalin. TTC staining has been widely used to reflect accurately the extent of irreversible ischemic damage in cerebral tissues in rats [48]. TTC-stained brain sections were photographed using a digital camera (Powershot 400 digital camera, Canon). The infarct size was calculated; and the percentage of the infarct area with respect to the total area was digitally quantified by ImageJ. To compensate for the effect of brain edema, the corrected infarct area was calculated as previously described [49].

Mortality and neurological deficits

Mortality was calculated at 24 h after MCAO/reperfusion. The neurological scores were performed in a blinded fashion at 24 h, based on amodified scoring standard of Rogers *et al.*[50].We excluded the dead animals in the scoring scale.

Immunohistochemical staining

After 24 h reperfusion, rats/mice were transcardially perfused with ice-cold PBS under anesthesia and then with 4% paraformaldehyde. After decapitation, brains were isolated and fixed overnight in 4% paraformaldehyde. The brains were then embedded in O.C.T. compound (Sakura Finetek USA, Torrance, CA) and sectioned coronally at 10 μm thickness using a vibrating microtome (Leica Microsystems, Bannockburn, IL). Brain sections were washed and the nonspecific binding sites were blocked with PBS containing 0.05 % triton-X100 and 0.25 % BSA for 1 h. Primary antibodies were diluted in blocking buffer and incubated with sections overnight at 4°C [35]. Primary antibodies were rabbit anti-HIF-1α (04-1006, Millipore, Billerica, MA), rabbit anti-GLUT-3 (ab53095, Abcam, Cambridge, MA, USA), rabbit anti-EPO (sc-7956, Santa Cruz), and mouse anti-NeuN (MAB377, Millipore). Secondary antibodies were donkey anti-rabbit Alexa 488 and goat anti-mouse Alexa 488 (Molecular Probes, Carlsbad, CA). The

process for selecting area of interests for imaging was detained in our previous publication [47]. The imaging location was selected at per-infarct cortex based on TTC staining. As marked in Fig. 2-2A, the squares indicated the selected area for imaging.Images were routinely captured with a Leica DMI 4000B fluorescent microscope. The original images were converted to 8-bit RBG and the immunoreactivity of each image was analyzed with Image-Pro Plus 5 (Media Cybernetics, Bethesda, MD). We chose pixel intensity to reflect changes in protein expression levels in neurons over cell counts. The rationale was that HIF-1α is already extensively expressed in the neurons in ischemic brain without NAC treatment [32], so the numbers of the HIF-1α-positive neurons may not be able to reflect the change of neuronal HIF-1α in NAC-treated group.The optical density approach can reflect the increase of HIF-1α level in HIF-1α positive neurons. An approximate threshold for pixel intensity was set and applied to images to discriminate positive staining from background signal.Total immunoreactivity was calculated by the area occupied by immunopositive pixels multipled the optical density of those pixels. Five consecutive sections per animal were used for analysis. Average values for each animal were used to generate mean values for each treatment. All immunohistochemical staining data were obtained and analyzed in a blinded manner.

Statistical analysis

Neurological score results were presented as median with range. The other results were presented as mean with a standard error of mean. Comparisons of Western blotting, immunoreactivity, and infarct volumes were carried out by ANOVA test, followed by Tukey's correction (R 3.0.1). Neurological scores were compared using Kruskal-Wallis analysis followed by Bonferroni correction. A $p<0.05$ was considered statistically significant. For the statistical analysis of mortality, the Fisher exact test was used.

2.3 Results

NAC enhanced global HIF-1α expression in ischemic brains

Previous reports have demonstrated that pretreatment of NAC significantly increased neuronal survival and reduced brain infarct [9,12,13,51]. To investigate the mechanism of NAC's neuroprotective effects during ischemia and reperfusion, NAC was administrated (i.p.) to rats before the onset of MCAO in this study. Fig. 1 shows Western blotting results of the overall levels of HIF-1α and its down-stream proteins in contralateral and ipsilateral hemispheres.Ischemic exposure increased HIF-1α protein expression in the ipsilateral hemisphere, compared to that of the contralateral one **(Fig. 2-1A-B)**. NAC further increased HIF-1α expression in the ipsilateral brain tissues. NAC also upregulated the protein expression of EPO and GLUT-3, two down-stream factors of HIF-1, in the ipsilateral hemisphere **(Fig. 2-1A-D)**. The results indicated that NAC effectively enhanced HIF-1α protein accumulation and HIF-1 functional activity in the ischemic brains. In addition, it is of interest to note that NAC also increased the HIF-1α expression in the contralateral side, which usually used as control in stroke studies. To confirm the effect of NAC on contralateral brain tissue, we also administered NAC to rats without receiving MCAO (naïve animals). As shown in **Fig. 2-2**, NAC promoted HIF-1α expression in the naïve rats' brains.

The above results were obtained using whole hemisphere homogenates and reflected the effect of NAC on the overall levels of HIF-1α, EPO, and GLUT-3 in both hemispheres. We also determined changes in the protein level in selected peri-infarct area as marked in **Fig. 2-4A**. Results demonstrated that NAC increased the protein level of HIF-1α, EPO, and GLUT-3 in the selected region **(Fig. 2-3)**. However, it needs to be pointed out that we isolated the peri-infarct tissue of all the rats from the fixed location which was determined based on the animals received no pharmacological treatments.Because the drug treatments altered the brain infarction, the selected region in thedrug-treated animals might represent different pathological state from that of control rats. We do not exclude the possibility that changes in HIF-1α and its target proteins level might be a result of sampling tissues of different viability. Nevertheless, these results from selected brain region provided additional insights into the effect of NAC on the expression of HIF-1α, EPO, and GLUT-3 in the ischemic brain.

One of the aims in this study was to determine the role of HIF-1 in NAC-mediated neuroprotection. Inhibiting HIF-1 activity would confirm specifically whether HIF-1 contributes to NAC's effects. YC-1 and 2ME2 are commonly used HIF-1α inhibitors. To inhibit HIF-1, we chose the double injections of YC-1 at 2 mg/kg based on our previous report of HIF-1α inhibition [35]. It has been shown that 2ME2 (5 mg/kg) (i.p.) could successfully decrease HIF-1α level in rat [36]. To successfully inhibit HIF-1α expression in NAC group, the inhibitors were administrated prior to the injection of NAC. Both inhibitors at the tested doses effectively suppressed HIF-1α, EPO, and GLUT-3 expression in ischemic brains **(Fig. 2-1A-D)**. It's noteworthy that YC-1 and 2ME2 were able to inhibit HIF-1α expression in the presence of NAC in the contralateral sides **(Fig. 2-1)** and in the brains of naïve rats **(Fig. 2-2)**.

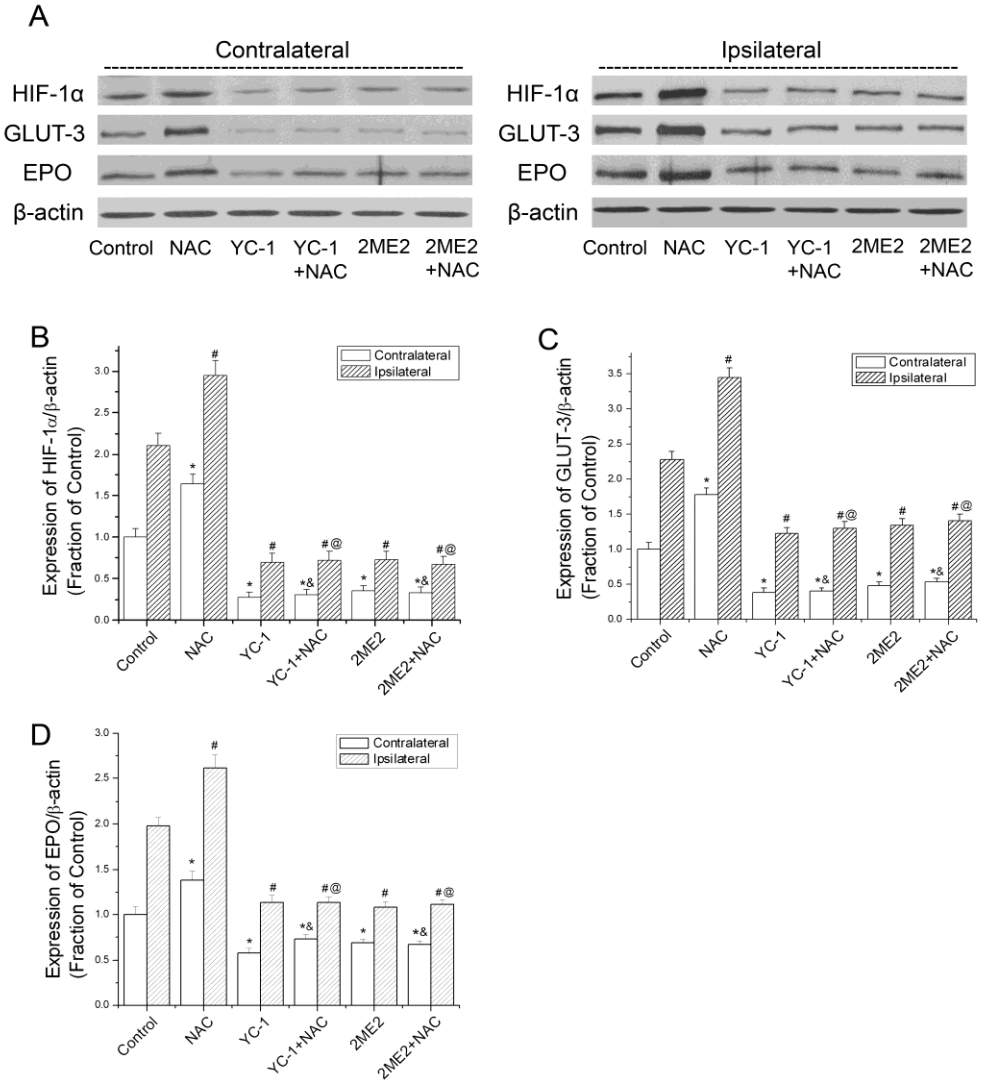


Fig. 2-1 Effect of NAC on HIF-1α and its target gene expression in ischemic brains of rats. The protein level of HIF-1α, GLUT-3, and EPO was analyzed by Western blotting in brain hemispheres of rats subjected to 90 min ischemia and 24 h reperfusion. Rats received NAC (150 mg/kg, i.p.) at 30 min prior to the onset of ischemia. YC-1 (2mg/kg, i.v.) was administrated at 24 h and 30 min prior to the onset of ischemia. 2ME2 (5mg/kg, i.p.) was administrated at 1 h prior to the onset of ischemia. **(A)** Representative Western blots of HIF-1α and its down-stream proteins in contralateral and ipsilateral hemisphere. **(B)** Quantification of the HIF-1α protein level in contralateral and ipsilateral hemispheres. **(C)** Quantification

of the GLUT-3 protein level in contralateral and ipsilateral hemispheres. **(D)** Quantification of the EPO protein level in contralateral and ipsilateral hemispheres. Values were normalized to β-actin and corresponding hemispheres of control animals. Values are means ± SEM, n = 5. $^{*}p < 0.05$ vs. contralateral hemispheres from control animals. $^{\#}p < 0.05$ vs. ipsilateral hemispheres from control animals. $^{\&}p < 0.05$ vs. contralateral hemispheres from NAC animals. $^{@}p < 0.05$ vs. ipsilateral hemispheres from NAC animals.

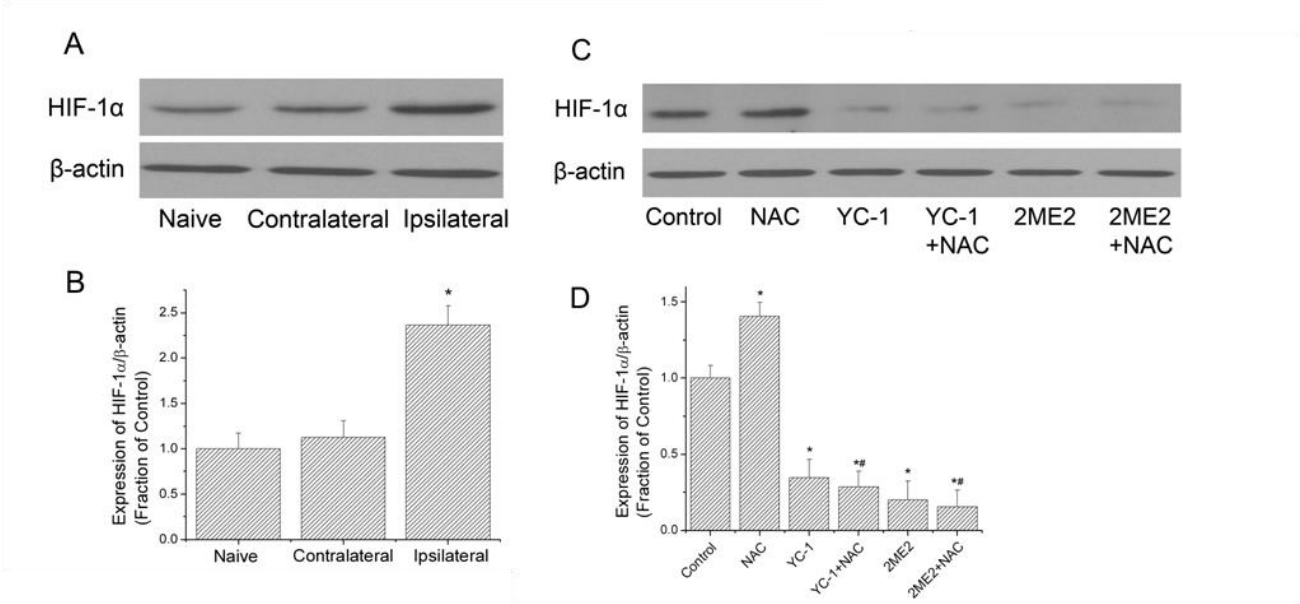


Fig. 2-2 Effect of NAC, YC-1 and 2ME2 on HIF-1α expression in naïve rat brains.The protein level of HIF-1α was analyzed by Western blotting in the brains of naïve rats and rats subjected to 90 min ischemia and 24 h reperfusion. Naïve rats received NAC (150 mg/kg, i.p.), YC-1 (2mg/kg, i.v.) or 2ME2 (5mg/kg, i.p.) injections as indicated in the figure. **(A)** Representative Western blots of HIF-1α in the brain samples from naïve rats and MCAO rats. **(B)** Quantification of the HIF-1α protein level in naïve rat brains, contralateral and ipsilateral hemispheres in MCAO rats (n = 3). $^{*}p < 0.05$ vs. naïve rat brains.**(C)** Representative western blots of HIF-1α in the naïve rat brain samples. **(D)** Quantification of the HIF-1α protein level in naïve rat brains. Values were normalized to β-actin. Values are means ± SEM, n = 3. $^{*}p < 0.05$ vs. control animals.$^{\#}p < 0.05$ vs. NAC animals.

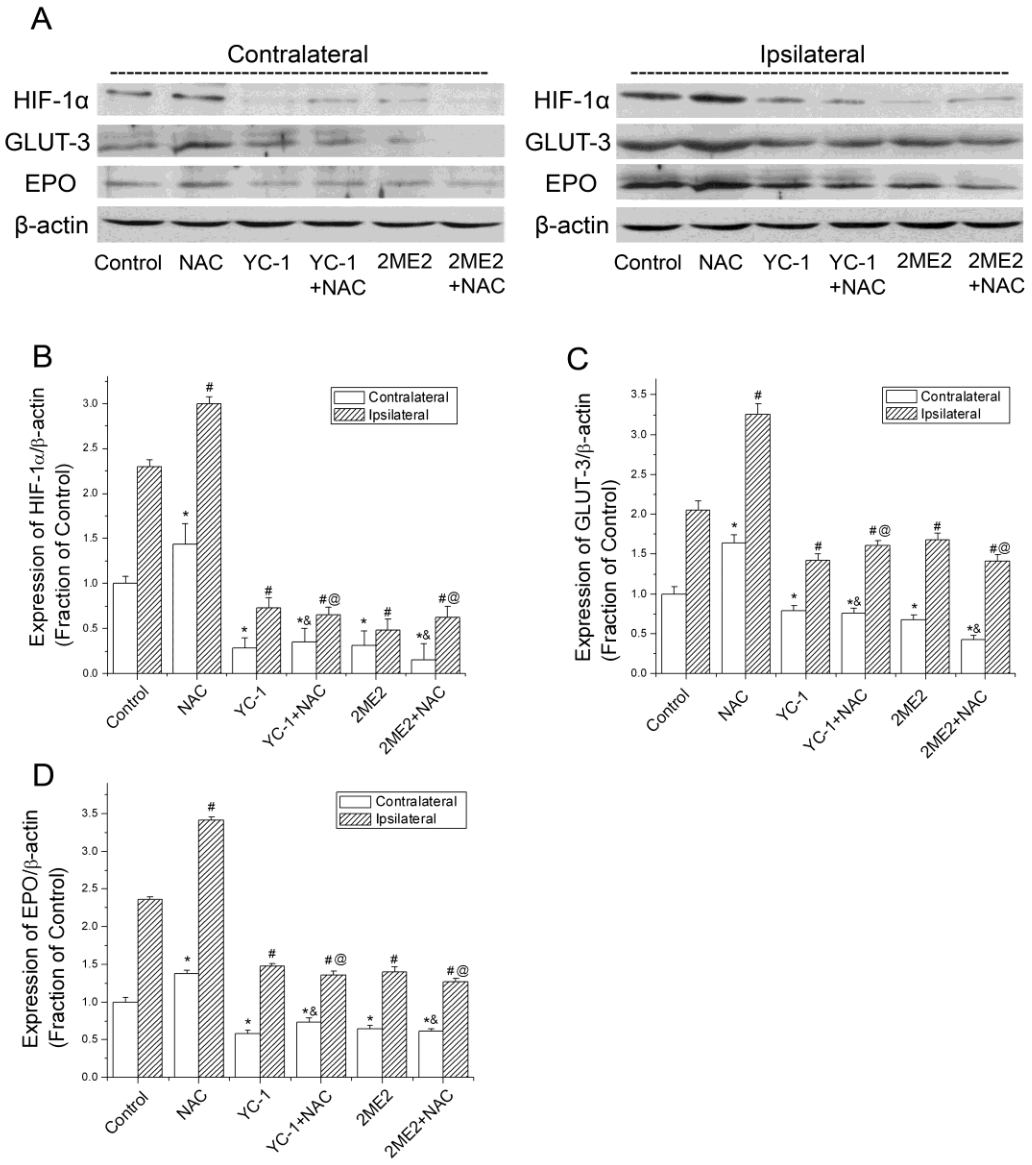


Fig. 2-3 Effect of NAC on HIF-1α and its target gene expression in peri-infarct brain tissue of rats. **(A)** Representative Western blots of HIF-1α and its down-stream proteins. The protein level of HIF-1α, GLUT-3, and EPO was analyzed by Western blotting in the peri-infarct tissue of rats subjected to 90 min ischemia and 24 h reperfusion. **(B)** Quantification of the HIF-1α protein level in contralateral and ipsilateral hemispheres. **(C)** Quantification of the GLUT-3 protein level in contralateral and ipsilateral hemispheres. **(D)** Quantification of the EPO protein level in contralateral and ipsilateral hemispheres. Values were normalized to β-actin and corresponding hemispheres of control animals. Values are means

± SEM, n = 5. $^{*}p < 0.05$ vs. contralateral hemispheres from control animals. $^{\#}p < 0.05$ vs. ipsilateral hemispheres from control animals. $^{\&}p < 0.05$ vs. contralateral hemispheres from NAC animals. $^{@}p < 0.05$ vs. ipsilateral hemispheres from NAC animals.

NAC elevated neuronal HIF-1α expression in ischemic brains

As ischemia causes devastating neuronal death in stroke, it was of great interest to determine if NAC enhanced the expression of HIF-1α in neurons. As illustrated in **Fig. 2-4 B**, nuclear immunostaining of HIF-1α was observed in the selected area of the ipsilateral hemispheres.HIF-1α protein accumulation primarily occurred in neurons, as indicatd by colocalization with the neuronal specific marker NeuN. The intensity of HIF-1α immunostaining was further increased in ipsilateral side of the brains of NAC-treated rats. Moreover, the neuronal expression of EPO and GLUT-3 significantly increased in the ipsilateral neurons of NAC-treated rats, compared to control rats **(Fig. 2-5A-B)**. The ipsilateral expression of all the three proteins (HIF-1α, EPO, and GLUT-3) was remarkably reduced by the HIF-1α inhibitors, YC-1 and 2ME2, in either NAC-treated or control rats. The above results demonstrated that NAC remarkably enhanced HIF-1α and its down-stream genes expression in neurons in ipsilateral hemisphere of an ischemic brain.

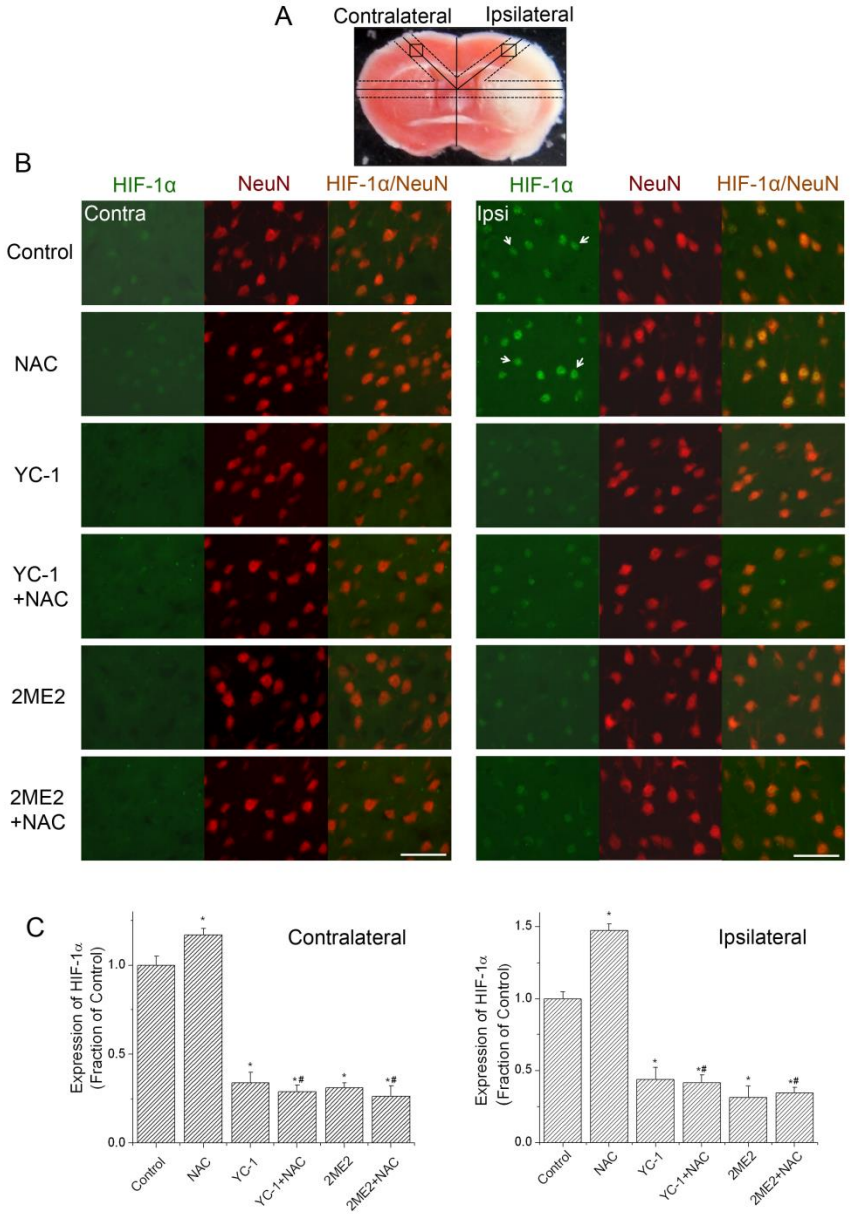
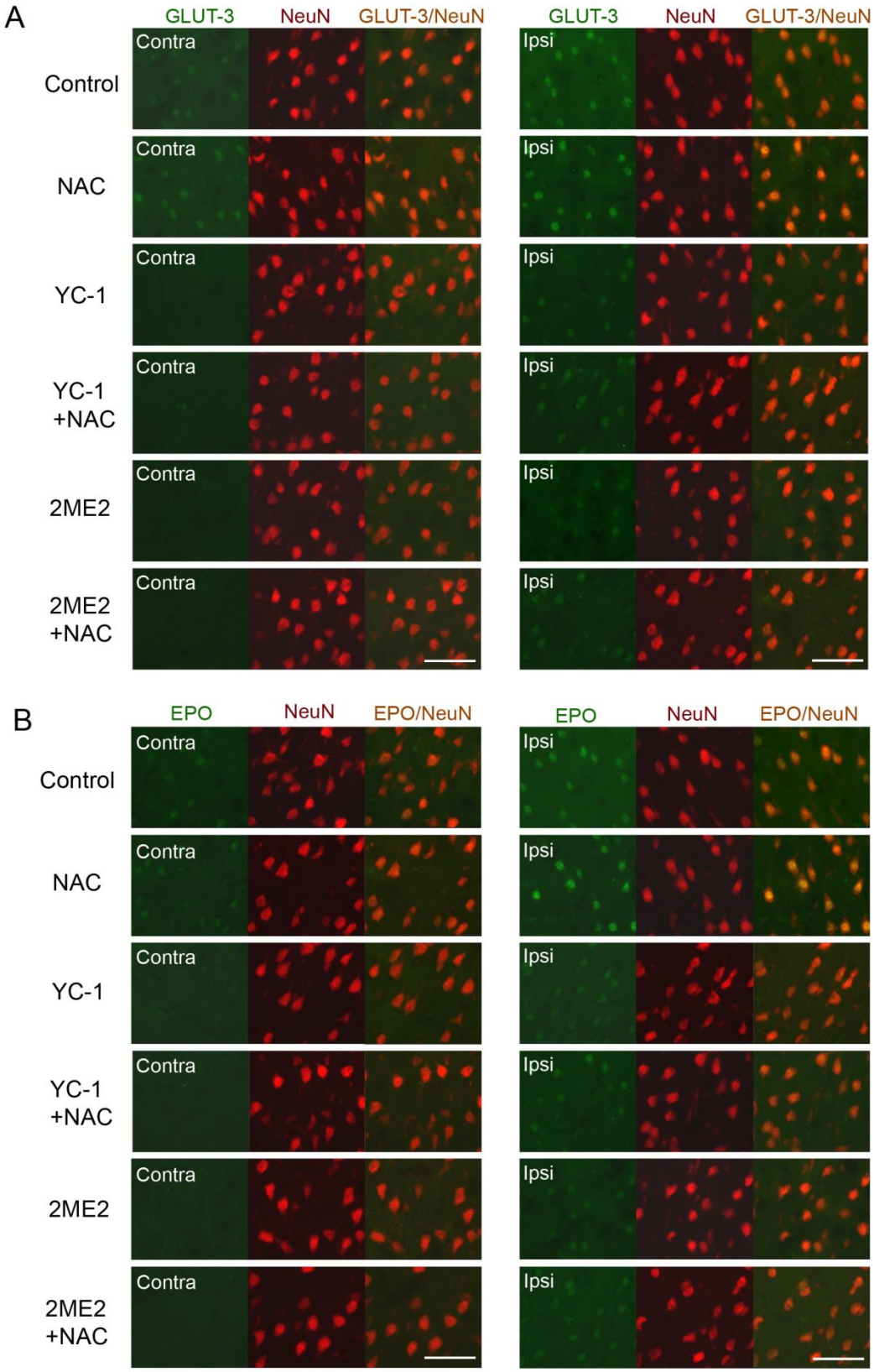


Fig. 2-4 Effect of NAC on HIF-1 expression in neurons in ischemic brains of rats. The protein level of HIF-1α was analyzed by double immunostaining with the neuronal marker NeuN after rats were subjected to 90 min ischemia and 24 h reperfusion. **(A)** TTC-stained rat brain coronal section. Labeled square areas represent locations of immuno images (see ref 47 for details). **(B)** Double immunostaining of HIF-1α (green) and NeuN (red). The white arrows indicate positively stained neurons. Scale bar, 50 μm. **(C)** Quantification of the HIF-1α immunostaining intensity in contralateral and ipsilateral hemispheres. Values are means ± SEM, n = 5. $^{*}p < 0.05$ vs. control animals. $^{\#}p < 0.05$ vs. NAC animals.


A
GLUT-3
NeuN
GLUT-3/NeuN
GLUT-3
NeuN
GLUT-3/NeuN
Control
NAC
YC-1
YC-1
+NAC
2ME2
2ME2
+NAC
Contra
Ipsi
B
EPO
NeuN
EPO/NeuN
EPO
NeuN
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Control
NAC
YC-1
YC-1
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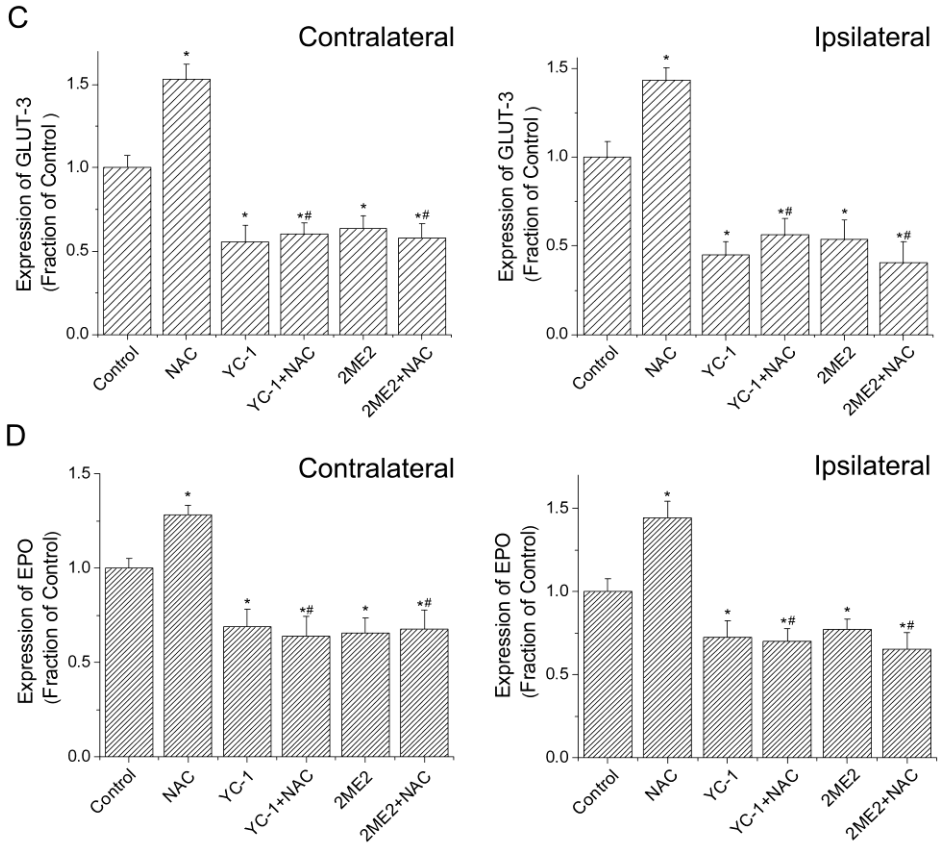

Fig. 2-5 Effect of NAC on HIF-1 target gene expression in neurons in ischemic brains of rats. GLUT-3 and EPO were analyzed by double immunostaining with the neuronal marker NeuN after rats were subjected to 90 min ischemia and 24 h reperfusion. Selection of areas for imaging was same as those shown in Fig. 2-4. **(A)** Double immunostaining of GLUT-3 (green) and NeuN (red). **(B)** Double immunostaining of EPO (green) and NeuN (red). Scale bar, 50 μm. **(C, D)**Quantification of the GLUT-3 and EPO immunostaning intensity in contralateral and ipsilateral hemispheres. Values are means ± SEM, n = 5. $^{*}p < 0.05$ vs. control animals. $^{\#}p < 0.05$ vs. NAC animals.

HIF-1 was responsible for NAC-mediated neuroprotection against ischemic brain injury

To determine the role of HIF-1 in NAC's neuroprotection in ischemic stroke, we first evaluated NAC's protective effects against ischemia/reperfusion-induced brain injury by T2-weighted MRI and TTC staining. **Fig. 2-6A** demonstrated a serial of brain sections of a rat subjected to 90 min MCAO and 24 h reperfusion. The brain infarct volume was calculated based on the area of hyperintensity of the MRI images. As shown in **Fig. 2-6A** and **B**, administration of NAC at 150 mg/kg significantly reduced the infarct volume (140.2 ± 18.8 mm^3), compared to the control group (226.0 ± 26.1 mm^3) (p=0.0135). Consistent with the T2-weighted MRI data, the infarct volume measured by TTC-staining also demonstrated that NAC significantly reduced the brain infarction (p=0.0007). These results were in line with previous reports that NAC is neuroprotective in ischemic stroke[9-12,24,52].

Fig. 2-6 also demonstrated that inhibiting HIF-1α remarkably augmented ischemia-induced brain damage. YC-1 and 2ME2 increased the infarct volume estimated by T2-weighted MRI from 226.0 ± 26.1 mm^3 to 481.6±27.7 mm^3 and 477.8 ± 24.1 mm^3, respectively (p<0.0001). No significant difference was observed among YC-1, YC-1+NAC, 2ME2, and 2ME2+NAC groups, indicating that NAC failed toprovide protection against cerebral ischemia in the presence of YC-1 or 2ME2. Results from TTC staining showed similar effects. It is noteworthy that YC-1 and 2ME2 massively enlarged the infarcts, indicating their toxic effect was beyond just abolishing NAC's neuroprotective effects.

We further determined the functional recovery of rats subjected to NAC and HIF-1 inhibitor treatments by behavioral assessment according to Rogers *et al*.[50]NAC treatment significantly decreased the median neurological scores from 3 (range 2-4) in control rats to 2 (range 1-3) (control, n=22; NAC, n=23; p=0.0097) **(Fig. 2-6D)**. However, NAC was not able to alleviate neurological abnormalities when HIF-1 activity was inhibited by YC-1 or 2ME2.In addition, two animals were dead in the control group while one dead was observed in NAC-treated groups**(Table 1)**. The treatment of NAC, YC-1 or 2ME2 alone did not cause death in negative control animals (without MCAO, data not shown).

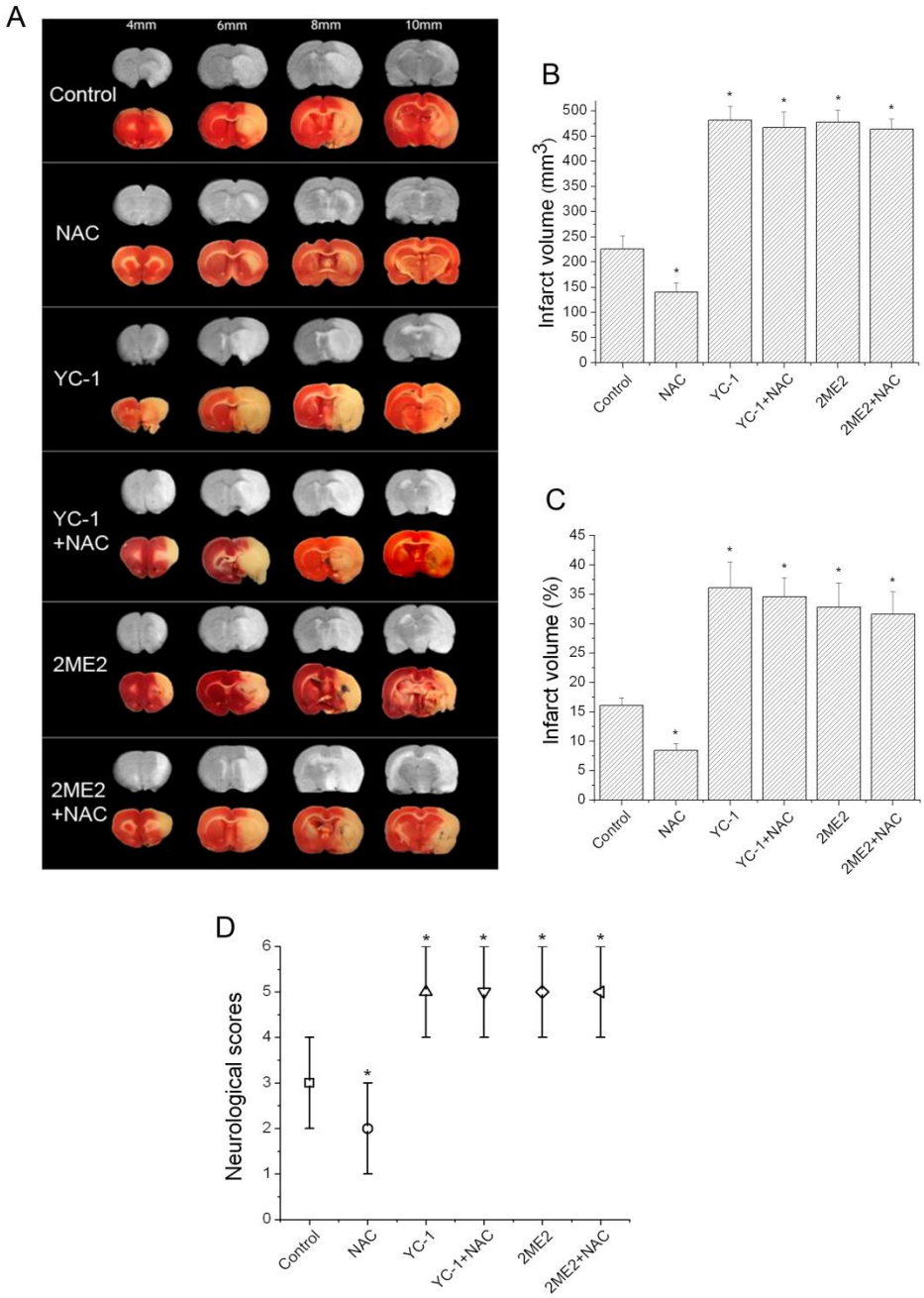

Fig. 2-6 Effect of NAC on ischemia/reperfusion-induced brain damage and neurological deficit. Brain damage was estimated by MRI and TTC staining after rats were subjected to 90 min ischemia and 24 h reperfusion. **(A)** Representative TTC staining (lower panel) and T2-weighted MRI (upper panel) images of MCAO brain sections of rats. The brains were sectioned beginning at the 4 mm position from the frontal pole and continued in 2-mm interval to 10 mm. **(B)** Quantification of infarct volume with T2-

weighted MRI images of rat brain (n= 7 (control), 8 (NAC), 5 (YC-1), 6 (YC-1+NAC), 6 (2ME2), and 7 (2ME2+NAC)). **(C)** Quantification of infarct volume estimated by TTC stained sections (n= 7 (control), 8 (NAC), 5 (YC-1), 6 (YC-1+NAC), 6 (2ME2), and 7 (2ME2+NAC)). Values are means ± SEM, $^{*}p<$ 0.05 vs. control animals. **(D)**Quantification of neurological deficit scores rate(n= 22 (control), 23 (NAC), 20 (YC-1), 21 (YC-1+NAC), 21 (2ME2), and 22 (2ME2+NAC)). Values are medians with ranges, $^{*}p<$ 0.05 vs. control animals.

Neuron-specific HIF-1α knock-out abolished NAC-mediated neuroprotection in ischemic brains

The above results demonstrated that the pharmacological HIF-1α inhibitors YC-1 and 2ME2 abolished NAC's neuroprotection in the ischemic brains. We further studied the specific role of HIF-1 in NAC-mediated neuroprotection with neuron-specific HIF-1α knock-out mice (HIF-1$\alpha^{\Delta/\Delta}$). CAMKII- dependent Cre expression removed the lox-HIF-1α-lox cassette in genome of neurons of HIF-1$\alpha^{\Delta/\Delta}$ mice **(Fig. 2-7A)**. To evaluate the efficiency of HIF-1α ablation in neurons of the HIF-1$\alpha^{\Delta/\Delta}$ mice, we used immunostaining to visualize HIF-1α expression in NeuN-positive cells in the ipsilateral hemisphere of a mouse brain after 90 min MCAO and 24 h reperfusion. HIF-1α expression was diminished in majority of neurons in HIF-1$\alpha^{\Delta/\Delta}$ mice**(Fig. 2-8)**. Post-ischemic accumulation of HIF-1α,GLUT-3, and EPO were significantly attenuated in the HIF-1$\alpha^{\Delta/\Delta}$ mice compared with the controls, HIF-1$\alpha^{F/F}$**(Fig. 2-7 B-C)**. Moreover, NAC administration failed to increase the expression of HIF-1α and its target genes in ischemic brains of HIF-1$\alpha^{\Delta/\Delta}$ mice. We observed increases in the infarction-induced brain damage in HIF-1$\alpha^{\Delta/\Delta}$ mice compared with wild type controls **(Fig. 2-7 D-E)**. Pretreatment with NAC significantly reduced the infarct volume in control mice (50% decrease from 63.0 ± 5.6 mm^3 to33.3 ± 6.7 mm^3,p=0.0316), but not in HIF-1$\alpha^{\Delta/\Delta}$ mice (HIF-1$\alpha^{\Delta/\Delta}$, 83.7 ± 7.5 mm^3; HIF-1$\alpha^{\Delta/\Delta}$ +NAC,76.3 ± 5.5 mm^3, p=0.8421). The results further confirmed that NAC's neuroprotective effects were suppressed by HIF-1α inhibition, indicating that HIF-1α is involved in NAC's protection against ischemic brain injury. It is noteworthy that when compared to the rat MCAO model with NAC treatment which led to infarct damage mainly in the striatum, in the mouse model the damage was restricted largely inthe cortex.

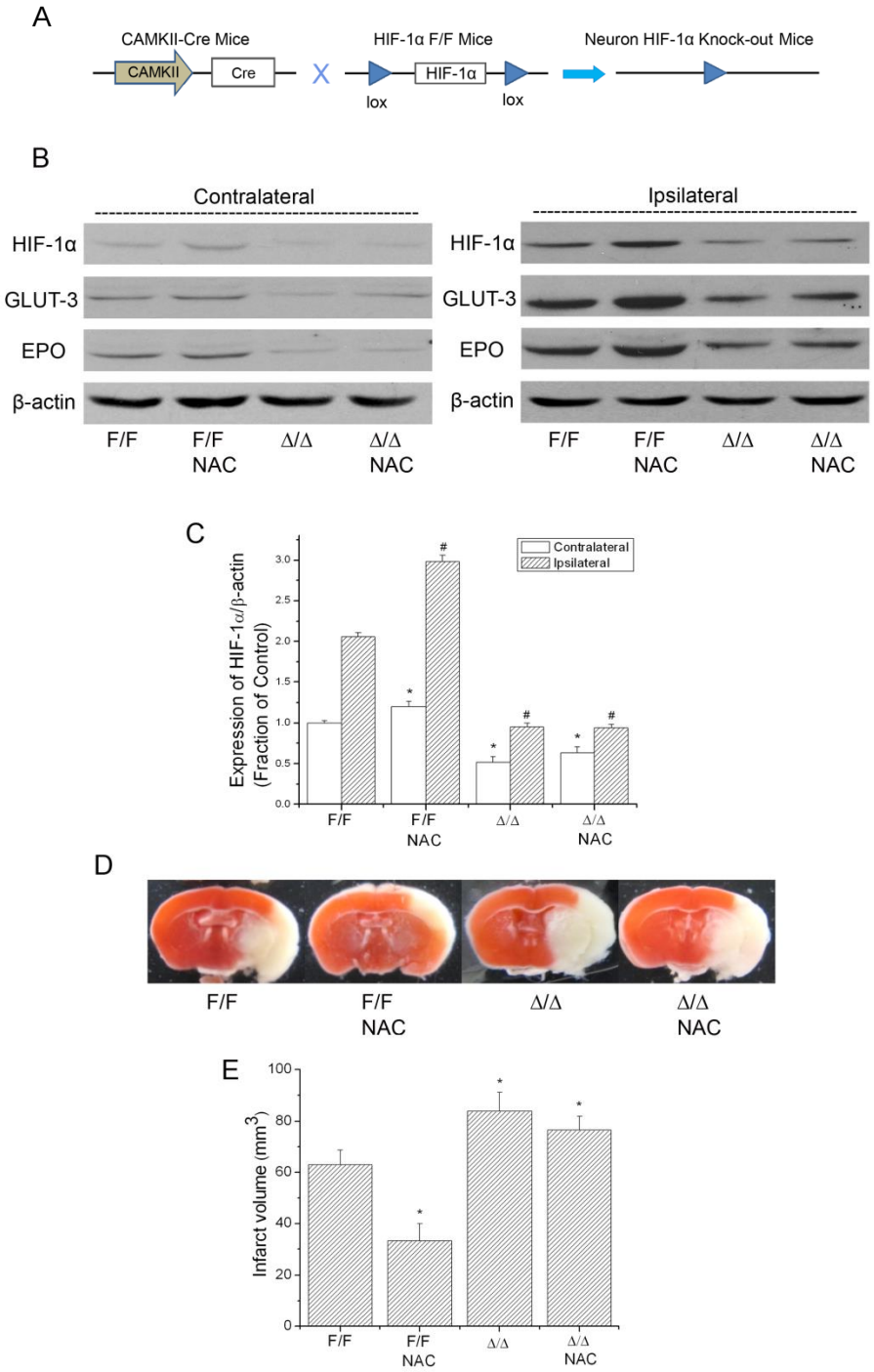

Fig. 2-7 Effect of NAC on HIF-1 expression and ischemia/reperfusion-induced brain damage in wild type (HIF-1$\alpha^{F/F}$) and neuronal HIF-1α deficient (HIF-1$\alpha^{\Delta/\Delta}$) mice. The protein level of HIF-1α, GLUT-3, and EPO were analyzed by Western blotting in the brains of mice subjected to 90 min ischemia and 24 h reperfusion. Mice received NAC (240 mg/kg, i.p.) at 30 min prior to the onset of ischemia. **(A)** Schematic

of the neuron-specific HIF-1α knock-out mouse model. **(B)** Representative Western blots of HIF-1α and its down-stream proteins GLUT-3 and EPO. **(C)** Quantification of the HIF-1α protein level in contralateral and ipsilateral hemispheres (n=3). $^{*}p < 0.05$ vs. contralateral hemispheres from control (HIF-1$\alpha^{F/F}$) mice. $^{\#}p < 0.05$ vs. ipsilateral hemispheres from control (HIF-1$\alpha^{F/F}$) mice. **(D)** Representative TTC staining images of brain sections of MCAO mice. The brains were sectioned from the 6 mm position from the frontal pole. **(E)** Quantification of infarct volume estimated by TTC stained sections (n= 3). Values are means ± SEM, $^{*}p < 0.05$ vs. control (HIF-1$\alpha^{F/F}$) mice.

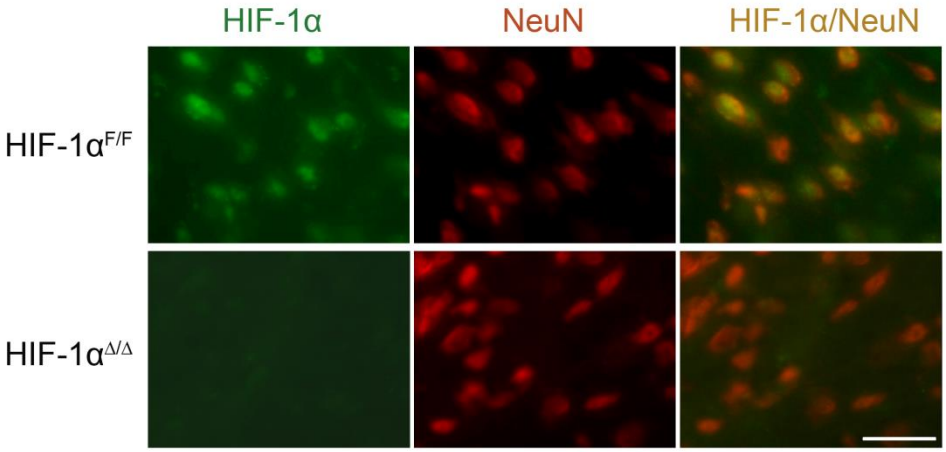

Fig. 2-8 HIF-1α expression in neurons in ischemic brains of wild type (HIF-1α$^{F/F}$) and neuronal HIF-1α deficient (HIF-1α$^{\Delta/\Delta}$) mice. The protein level of HIF-1α (green) was analyzed by double immunostaining with the neuronal marker NeuN (red) after mice were subjected to 90 min ischemia and 24 h reperfusion. Scale bar, 50 μm.

Hsp90 was involved in NAC-mediated HIF-1α upregulation in ischemic brains

We then investigated the mechanism underlying NAC-induced HIF-1α upregulation in ischemic brains. Previous studies have shown that Hsp90 is able to bind and stabilize HIF-1α[53]. We postulated that NAC might increase HIF-1α protein levels by promoting the expression and chaperone activities of Hsp90. We therefore determined the Hsp90 protein level and Hsp90-HIF-1α interaction in NAC-treated MCAO models. As we expected, NAC administration significantly upregulated Hsp90 expression in the ipsilateral hemispheres of MCAO mice **(Fig. 2-9A and D)**. Meanwhile, NAC also increased the amount of Hsp90 pulled down by anti-HIF-1α antibody **(Fig. 2-9 A and B)**, indicating that NAC enhanced the association between Hsp90 and HIF-1α. The results strongly supported the concept that NAC-induced HIF-1α upregulation was accompanied by increased Hsp90 expression and strengthened Hsp90-HIF-1α interaction.

To better evaluate the contribution of Hsp90 to NAC-mediated HIF-1α upregulation, we inhibited Hsp90 activity with 17-AAG, which is blood-brain barrier permeable and specifically binds to Hsp90[54]. As shown in **Fig. 2-9 A**, 17-AAG markedly reduced the interaction of Hsp90 with HIF-1α and suppressed HIF-1α protein expression. It completely abolished the HIF-1α upregulation in NAC-treated ischemic mice brains, suggesting that Hsp90 was involved in NAC-induced HIF-1α accumulation. Furthermore, 17-AAG significantly increased the infarct volume in control and NAC-treated animals subjected to 90 min MCAO and 24 h reperfusion **(Fig. 2-10)**. In conclusion, our results suggested that NAC promoted HIF-1α stabilization by increasing Hsp90 protein expression and the Hsp90-HIF-1α interaction.

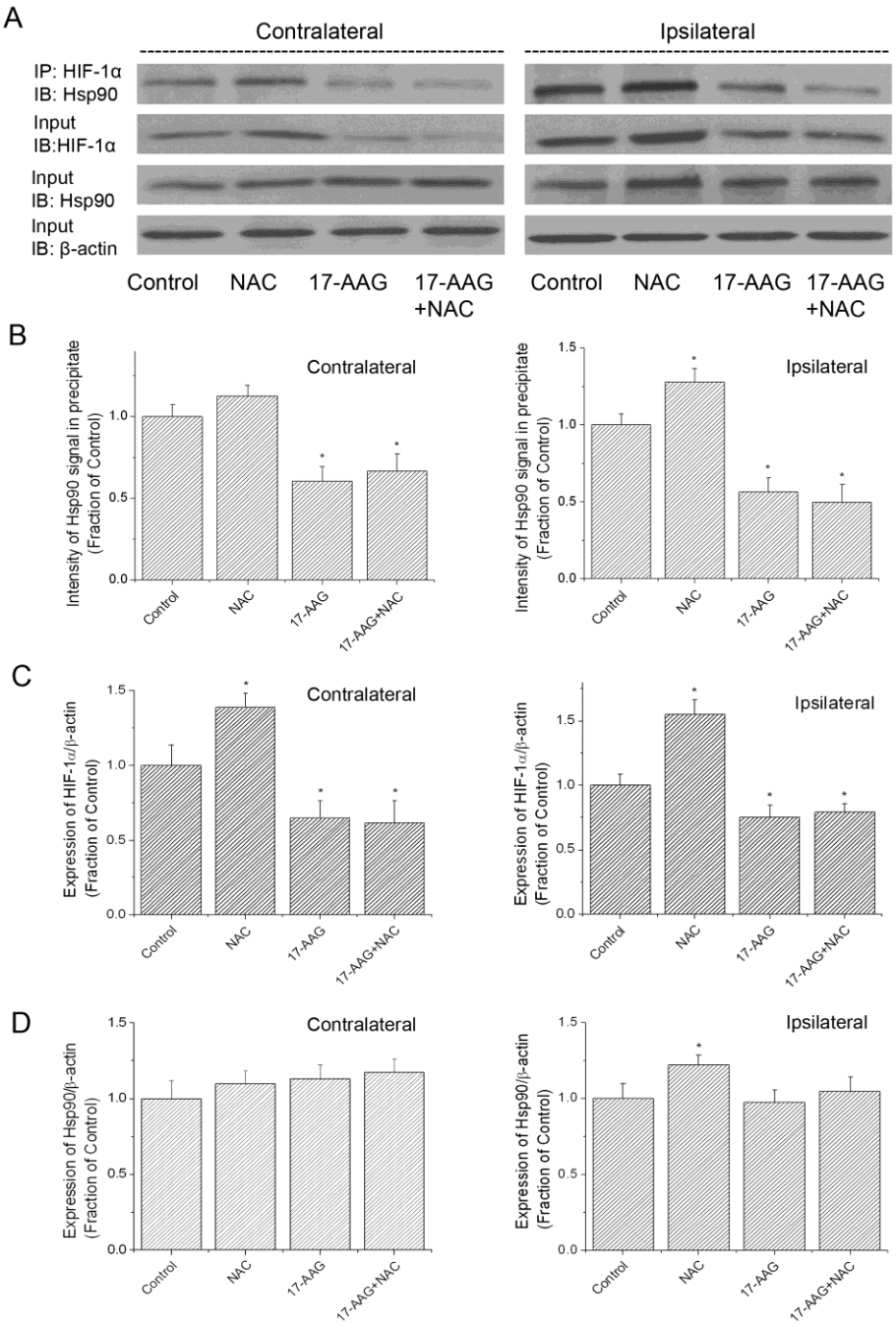

Fig. 2-9. Effect of NAC on HIF-1α and Hsp90 interaction in ischemic brains of mice. The protein level of HIF-1α and Hsp90 was analyzed by Western blotting in the brains of mice subjected to 90 min ischemia and 24 h reperfusion. Mice received NAC (240 mg/kg, i.p.) at 30 min prior to the onset of ischemia. 17-AAG (25 mg/kg, i.p.) was administrated at 1 h prior to the onset of ischemia. **(A)** Representative immunoblots of HIF-1α and Hsp90 (IB: HIF-1α or Hsp90) in immunoprecipitates of HIF-1α (IP: HIF-

1α). **(B)** Quantification of the Hsp90 protein level in immunoprecipitates of HIF-1α in contralateral and ipsilateral hemispheres. **(C)** Quantification of the HIF-1α protein level in contralateral and ipsilateral hemispheres. **(D)** Quantification of the Hsp90 protein level in contralateral and ipsilateral hemispheres. Values were normalized to β-actin and corresponding hemispheres of control animals. Values are means ± SEM, n = 3. $^{*}p < 0.05$ vs. control animals.

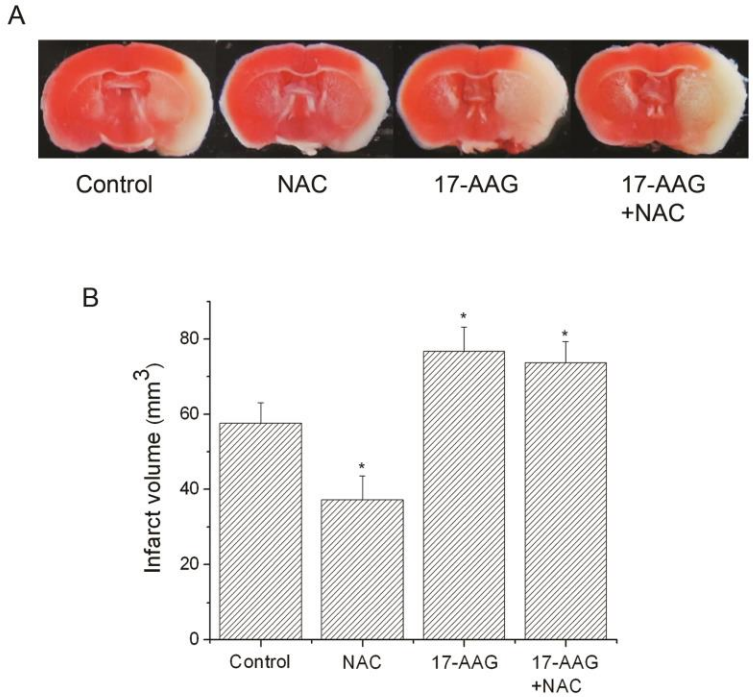

Fig. 2-10 Effect of 17-AAG on ischemia/reperfusion-induced brain infarction in control and NAC-treated mice.**(A)**Representative TTC staining images of brain sections of MCAO mice. The brains were sectioned from the 6 mm position from the frontal pole. **(B)** Quantification of infarct volume estimated by TTC stained sections (n= 3). Values are means ± SEM, $^{*}p < 0.05$ vs. control mice.

2.4 Discussion

Many previous studies have demonstrated that NAC protects cellular injury from various stresses including ischemia. The beneficial effect of NAC has been largely attributed to its antioxidant properties, specifically speaking, to its ability to reduce ROS and lipid peroxidation. In this study, we defined a new mechanism (i.e., upregulating the protein level of HIF-1α) by which NAC exerts its protective effect against ischemic insults.

As an antioxidant, NAC alters cellular redox environment that plays a critical role in normal cellular functions. HIF-1α, the unstable subunit of HIF-1, can be strongly regulated by cellular redox environment. For example, excessive ROS disrupt HIF-1α accumulation such as in Hela cells [27] and renal medullary interstitial cells [55]. In contrast, antioxidants such as NAC have been reported to stabilize HIF-1α in epithelial cells [56] and in primary culture cortical neurons as demonstrated in our previous study [29]. However, it was not known whether NAC stabilizes HIF-1α in ischemic brain. In line with these observations, the present study, for the first time, showed that NAC administration increased the protein expression of HIF-1α and its target genes EPO and GLUT-3 [57] in ipsilateral side of an ischemic brain. More importantly, HIF-1 inhibition diminished the neuroprotection of NAC, substantiating the involvement of HIF-1 in NAC's protective effects. These results clearly present that upregulating HIF-1 activity is a new function of NAC in ischemic brain.

We also determined HIF-1α change in specific region of an ischemic brain, the peri-infarct region defined with control MCAO rats (90 min ischemia and 24 h reperfusion). Theperi-infarct region is at risk for delayed neuronal death due to the deleterious metabolic processes propagated from the ischemic core to the neighboring tissue, including excitotoxicity, oxidative stress and inflammation [58].The surviving neurons in the peri-infarct region directly contribute to behavioral recovery after stroke [59,60]. Previous studies have demonstrated that HIF-1α induction reduced cell death of neurons located in the peri-infarct region [61]. Thus, the peri-infarct tissue is a crucial neuroprotection target, and it is ideal to define how NAC alters HIF-1α expression in this region. Our result showed that NAC upregulated the protein level of HIF-1α, GLUT-3, and EPO in the peri-infarct brain region. However, results from the selected peri-

infarct brain tissue have limitation. The fixed brain region was identified based on control MCAO animals. We isolated the samples from the same location as peri-infarct in animals received different treatments. Actually, the location of peri-infarct area varies between animals, and it moves especially when the treatment induces neuroprotection. As the result, the selected brain region which represents peri-infarct region in control MCAO animals does not necessarily represent peri-infarct region in animals received different treatments. In this study, the selected brain tissue in NAC-treated rats may be more normal (healthier) than that in control rats. The measurement of protein level in whole hemisphere homogenates avoided the issue of peri-infarct shifting in different groups. The Western blotting results of whole hemisphere reflected the effect of NAC on the overall levels of HIF-1α, EPO, and GLUT-3 in whole ischemic hemisphere including infarct core, peri-infarct regions and non-ischemic remote areas. Many previous studies have used whole hemisphere homogenate to determine protein expression in contra- and ipsilateral brains[32,62-65]. Our results demonstrated NAC enhanced HIF-1α expression in ischemic brain. It is noteworthy that NAC also enhanced HIF-1α and its target genes expression in contralateral brain in the absence of any infarction. Furthermore, our study showed that NAC failed to reduce the brain infarction in HIF-1α knock-out mice, which indicates that NAC's neuroprotective effect depends on increasing HIF-1α expression. Both aspects of the experimental evidence support that HIF-1α upergulation is the mediator of NAC's neuroprotection, rather than the consequence.

Although it is known that redox regulates HIF-1α expression in many cells as discussed above, the mechanism responsible for the regulation is far from completely understood. Hsp90 is among the most abundant proteins in the cytosol of eukaryotic cells. As chaperone, Hsp90 prevents the aggregation of unfolded proteins induced by stresses such as heat shock and ischemia. It has been shown that Hsp90 interacts with transcription factors with bHLH-PAS domain, such as Sim and Ahrhr. HIF-1α is a transcription factor containing the bHLH-PAS domain, suggesting Hsp90 may interact with HIF-1α. In fact, Isaacs *et al.* have shown that Hsp90 antagonists inhibit HIF-1α expression and reduce its target genes mRNA[66]. Therefore, we postulated that NAC might stabilize HIF-1α by promoting its interaction with Hsp90. Indeed, we found that NAC administration upregulated the Hsp90 protein level by 27.3% and

enhanced the interaction between Hsp90 and HIF-1α in the ipsilateral hemisphere of an ischemic brain. Given the fact that Hsp90's chaperoning capacity vastly exceeds demand for more than 200 client targets under normal conditions[67,68], this moderate increase in Hsp90 protein level and interaction may sufficiently account for the 54% increase in the HIF-1α protein level (Fig. 2-9). The Hsp90 inhibitor 17-AAG attenuated the interaction between Hsp90 and HIF-1α although it did not reduce Hsp90 protein expression in ischemic brains. The fact that 17-AAG inhibited HIF-1α upregulation in the presence of NAC indicated that Hsp90 activity was required for NAC-induced HIF-1α accumulation. However, the mechanism of NAC-mediated Hsp90 upregulation is not known. Suppressing oxidation or other properties of NAC may contribute to the upregulation, which warrants further investigation.

In this study, we demonstrated that the neuroprotective effect by NAC was largely mediated by HIF-1α induction and HIF-1 activation. Although we have provided data to show that Hsp90 is involved in the stabilization of HIF-1α by NAC, it is not known how NAC exactly promotes the interaction between Hsp90 and HIF-1α and stabilizes HIF-1α. We postulate that NAC, an antioxidant, may do so by maintaining redox homeostasis in ischemic brains. In a previous study, we found that NAC, which increased the GSH/GSSG ratio, induced significant expression of HIF-1α in peri-infarct. L-buthionine sulfoxide (BSO), which decreased the GSH/GSSG ratio, decreased HIF-1α expression in the peri-infarct(unpublished data). Moreover, NAC increased HIF-1α expression in the contralateral side (Fig. 2-1) as well as in the brain of naïve animals (Fig. 2-2). BSO decreased the level of HIF-1α in the contralateral side although no significance was observed due to low basal level of HIF-1α in normal brain tissue (unpublished data). The opposite effects of NAC and BSO indicate that NAC might up-regulateHIF-1α by reducing oxidants. These results are in line with our previous observation on primary cultured neurons [29]. In the previous studies, we investigated the relationships between HIF-1α expression, ROS, and redox status in neurons. We observed low levels of HIF-1α protein expression in the neurons exposed to *in vitro* ischemic conditions that had high levels of ROS (oxidizing environments), and vice versa. NAC induced HIF-1α protein expression in hypoxic neurons while BSO inhibited the expression. Moreover, (-)-epicatechin gallate, an ROS scavenger, elevated HIF-1α expression in the neurons

subjected to *in vitro*ischemia. Taken together, these data indicate that NAC may promote HIF-1α stabilization through suppressing ROS and maintaining cellular redox homeostasis. Evaluation on oxidative stress levels in the experimental groups and effects of other antioxidants on HIF-1 would provide more evidence for the concept, which needs to be investigated in future studies.

There were several considerations for using YC-1 and 2ME2 in this study. First, the two compounds are the most specific inhibitors available. Second, they have been widely used to inhibit HIF-1 activity [31-41]. Third, as in previous publications, they provided effective HIF-1 inhibition. Fourth, they were included in this study to complement the HIF-1α knock-out model. The results demonstrated that the effect of these drugs was enormous on infarct volume, indicating they may act on other pathways that exacerbate brain injury following MCAO. The effects of YC-1 and 2ME2 were so enormous that the effects of NAC were completely saturated by the HIF-1 inhibitors. These are novel findings regarding the two commonly used HIF-1 inhibitors in ischemic brain. To some extent, the effects of the two HIF-1 inhibitors provided evidence to support the concept that NAC lost its protective effect in the MCAO models in the presence of either of the two drugs although other unidentified pathway were obviously involved. In addition,we found that genetic inactivation of HIF-1α resulted in lower infarct volume augmentation than pharmacological inhibition of HIF-1α (Fig. 2-6A and Fig. 2-7D). This might be due to the activation of compensatory pathways in response to HIF-1α deletion and alteration of non-HIF-1 pathways by the inhibitors. For example, a previous study reported higher basal protein level of HIF-2α and an enhanced upregulation of HIF-2α under hypoxia in HIF-1α knock-out brains [32]. The upregulation of HIF-2α may partially substitute for the loss of HIF-1α function and participate in neuroprotection in the ischemic brain. YC-1 and 2ME2 downregulated the α subunit of both HIF-1 and HIF-2 [45,69]. As the result, YC-1 and 2ME2 further augment infarct volume than HIF-1αgene inactivation approach.Overall, the data obtained from knock-out mice are evident that NAC mediates its neuroprotection through regulating HIF-1 activity with support from the results of YC-1 and 2ME2.

HIF-1's role in cerebral ischemia is still arguable. Accumulating evidence shows that induction of HIF-1 provides protection against cerebral ischemic damage in adult animals [61,70,71]as well as neonatal

models [72]. The protective function of HIF-1 mainly results from a broad range of genes that HIF-1 regulates. The gene expression facilitates the adaptation to low oxygen conditions[73]. As demonstrated in this study, enhanced expression of HIF-1 by NAC induced the expression of EPO and GLUT-3, which facilitate cell survival and oxygen and glucose transport. However, others have reported detrimental effects of HIF-1 in models of hypoxia and cerebral ischemia [74-76]. The discrepancy of these observations may be partly explained by distinctive effects of HIF-1 in different severity of ischemia. For example, HIF-1 is neuroprotective in a 30 min transient MCAO model [32] while it is harmful in a 75 min model of bilateral common carotid artery occlusion [74]. Given the effect of NAC on HIF-1 expression, NAC (or other antioxidants) may have different effects on neurons in different ischemic conditions. Future studies on this aspect may help design therapeutic approaches for specific conditions of ischemia.

In a translational aspect, this study provides novel insight into the mechanism of NAC's prophylactic effect on ischemic brain injury. NAC is a commercially available supplement. It is of interest to know its effects when taken prophylactically. The pretreatment dose of NAC used in this study is comparable to previous studies. For example, Niu *et al.* reported that pretreatment of NAC at 150 mg/kg could prevent death-associated protein from trafficking and increase the number of the surviving CA1 pyramidal cells of hippocampus at 5 days of reperfusion [12]. Pretreatment of NAC 30 min before transient forebrain ischemia successfully increased the neuronal survival in rats [13]. Results by Zhang *et al.* showed that at a dose of 100 mg/kg, pretreatment of NAC distinctly inhibited the association of postsynaptic density protein 95 with kainate receptor glutamate receptor 6 and ameliorated brain injury induced by ischemia [51]. Sekhon *et al.* reported that animals pre-treated with NAC at 150 mg/kg produced a 49.7% reduction in brain infarct volume and 50% reduction in the neurological evaluation score as compared to the untreated animals [9]. Meanwhile, post-treatment of NAC has also been shown to be neuroprotective in ischemic brains at a similar dose range [10,24,77]. For instance, a single dose of NAC given 15 min after trauma might be effective on lipid peroxidation, antioxidant enzyme activity and neuronal protection in cerebral injury following closed head trauma [77]. However, the present study with pretreatment only has its limitation and caveats and provides no direct evidence to reveal mechanism of

NAC's neuroprotective effect in post-injury treatment. It is of interest and clinically relevant in future studies to determine the possible mechanism of neuroprotection of NAC treated after the occurrence of ischemia.

In summary, our data provide evidence that pre-treatment of NAC upregulates HIF-1 activity in ischemic brains and demonstrate for the first time that NAC-induced neuroprotection against ischemia is dependent on HIF-1 activity. Moreover, the results reveal a new pathway that NAC augments HIF-1 activity by enhancing Hsp90 expression and Hsp90-HIF-1α interaction. Our study suggests a new mechanism through which NAC protects stroke-induced brain injury, which may further improve the chances of identifying promising therapeutic approaches in future studies.

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CHAPTER 3: High Glucose Exacerbates *in vitro*Blood-Brain Barrier Disruption in Oxygen Glucose Deprivation Settings via Activation of HIF-1 Pathway

Abstract

Experimental evidence from human patients and animal models of diabetes has demonstrated that hyperglycemia exacerbates blood–brain barrier (BBB) disruption in ischemic stroke, which is associated with neurological worsening and poor outcome. However, the mechanism underlying high glucose-induced BBB disruption is not understood. Here we investigated the role of hypoxia-inducible factor-1 (HIF-1) in high glucose-induced endothelial permeability *in vitro* using an immortalized human cerebral microvascular endothelial cell line, hCMEC/D3. hCMEC/D3 cells were incubated in high glucose medium for 6 days and then subjected to 90 min oxygen glucose deprivation (OGD) and 24 h reoxygenation with high glucose medium. Our results demonstrated that high glucose (15 and 30 mM) upregulated the protein level of HIF-1α, the regulatable subunit of HIF-1 in the endothelial cells. Meanwhile, high glucose increased the paracellular permeability after OGD/reoxygenation, which was associated with diminished expression and disrupted continuity of tight junction (TJ) proteins occludin and zona occludens protein-1 (ZO-1) of hCMEC/D3 cells. Suppressing HIF-1 activity by HIF-1α inhibitors YC-1 and 2ME2 ameliorated the increased paracellular permeability and the alterations of expression and distribution pattern of occludin and ZO-1 induced in high glucose-treated cells. In addition, high glucose increased expression of vascular endothelial growth factor (VEGF) and the secretion of active matrix metalloproteinase (MMP)-2 and -9in endothelial cells. VEGF and MMP-2/9 areHIF-1 down-stream factors, both of which are implicated in vascular permeability. Inhibiting VEGF by VEGF-antibody attenuated the endothelial leakage. These results strongly indicate that HIF-1 plays an important role in high glucose-aggravated BBB dysfunction after OGD/reoxygenation. The study will help us understand the molecular mechanisms involved in hyperglycemia-induced BBB dysfunction and worsened neurological outcomes in ischemic stroke.

3.1 Introduction

Diabetes mellitus is a major health problem associated with both micro- and macrovascular diseases and leads to 2-6 fold higher risk for experiencing heart disease and stroke[1]. Accumulating evidence has shown that preexisting diabetes in conjunction with ischemia/reperfusion causes exacerbated cerebrovascular endothelial cell dysfunction and increased blood-brain barrier (BBB) permeability, which leads to augmented brain edema and hemorrhagic transformation in ischemic stroke[2-8]. Therefore, a better understanding of how diabetes aggravates vascular damage is critical in diabetic stroke research. Although several mechanisms like production of advanced glycation end products and superoxide have been implicated in diabetes-related peripheral vascular pathologies[9-12], the mechanism by which high glucose perturbs cerebrovasculature remains largely unexplored.

The BBB protects the brain from potentially neurotoxic substances and facilitates the exchange of nutrients and waste products between the brain and the blood, thus maintaining an optimal extracellular environment for neuronal function[13]. The restrictive properties of BBB is largely attributed to continuous presence of tight junctions (TJs) [14]. The transmembrane TJ proteins claudins and occludin are key molecules which seal the gaps between the adjacent endothelial cells and thus restrict paracellular permeability [15]. Stabilization of TJs involves a network of claudins and occludin-linked to the actin cytoskeleton via the zonular occluden protein-1 (ZO-1) [16]. Studies have shown that altered distribution or decreased expression of TJ proteins result in compromised BBB integrity and increased paracellular permeability[17,18].

Hypoxia-inducible factor 1 (HIF-1) is a transcriptional factor that activates genes involved in cellular adaptation to hypoxia by facilitating oxygen supply, glucose transport, angiogenesis, etc. Among the genes regulated by HIF-1, vascular endothelial growth factor (VEGF) is well defined in promoting new blood vessel formation, altering the structure of vasculature and causing vascular remodeling[19]. It is known that VEGF is a strong inducer of BBB permeability[20,21], and increased VEGF levels positively correlated with changes in TJ redistribution such as ZO-1[17,22] and occludin[22,23]. Moreoever, HIF-1 is well implicated in the control of matrix metalloproteinases (MMPs), including MMP-2 and MMP-9[24]. Both of

which have shown to critically contribute to BBB disruption by mediating the degradation of extracellular matrix and several TJ proteins including occludin, claudin-5 and ZO-1[25]. High glucose has been reported to upregulate HIF-1 activity in isolated hearts of rats [26], mouse kidney mesangial cells[27], human mesangial cells [27], mouse brain microvascular endothelial cells [28], etc. However, it is not known if HIF-1 is involved in permeability dysfunction of brain endothelial cells exposed to high glucose before and after OGD/reoxygenation.

Appropriate *in vitro* models that closely mimic the human BBB microenvironment are essential to understand the cellular/molecular mechanisms of brain microvessel endothelial disruption in diseased states[29,30]. The current study was carried out with an *in vitro* BBB model of an immortalized brain microvascular endothelial cell line, hCMEC/D3. This cell line was derived from isolated human primary BBB endothelial cells by lentiviral vector-mediated co-expression of human telomerase and SV40 T antigen[31]. This stable cell line exhibits robust proliferation while retaining the morphological and biochemical phenotype of differentiated human BBB endothelial cells over many passages[31,32]. This cell line has been extensively characterized for its utility as a model of human BBB for neurovascular research focusing on BBB disruption in various pathological conditions[33-37]. In this study, the hCMEC/D3 cells were subjected to oxygen glucose deprivation (OGD) which is taken to mimic the effect of ischemia associated with cessation of blood delivery. Cells were then returned to glucose containing medium in ambient air to mimic the restoration of oxygen and glucose associated with reperfusion[38].

Given the increased public attention to diabetes and its relevance to the pathogenesis of ischemic stroke, the main objective of this study is to investigate the mechanism of high glucose-exacerbated BBB disruption after ischemic insult. We hypothesized that HIF-1 was an important mediator in high glucose-induced increase in brain endothelial permeability. We expect that the present study will help us to identify new targets that may be utilized in clinical settings to prevent BBB damage in diabetic ischemic stroke.

3.2 Materials and Methods

Cell culture and treatments

An immortalized human cerebral microvascular endothelial cell line (hCMEC/D3) was kindly provided by Dr. Couraud (INSERM, Paris). The hCMEC/D3 cells were cultured in EBM-2 basal medium (Lonza, Walkersville, MD, 5.5 mM glucose) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT), chemically defined lipid concentrate (Life technologies, Carlsbad, CA), growth factors, penicillin/streptomycin (Invitrogen, Carlsbad, CA), and HEPES (10mM) and maintained at 37°C with 5% CO_2 exposure.Medium was changed every 2-3 days until the cells reached confluence.After growing the cells to confluency, the cell cultures were maintained in EBM-2 with various glucose concentrations (5.5, 15 or 30 mM) for 6 days. In the text, "glucose" refers to d-glucose unless otherwise noted. To determine whether the effect of high glucose (15 and 30 mM) resulted from osmotic changes of culture medium, effects of l-glucose at 30 mM were tested[39]. After 6 days pretreatment with different glucose concentrations (5.5, 15 or 30 mM), cells were exposed to OGD. The complete EBM-2 medium was replaced with a glucose-free solution of Dulbecco's modified Eagle medium (DMEM). The DMEM was thoroughly bubbled with a gas mixture of 95% $N_2$and 5% CO_2, before being added to the cell culture. The cell culture were placed in an humidified anaerobic chamber (Coy Laboratory Products Inc., Grass lake, MI) infused with 0.1% O_2, 95% $N_2$and 5% CO_2 maintained at 37 °C for 90 min. Following OGD, cells were removed from the anaerobic chamber and returned to an incubator (5% CO_2 in room air, 37°C) for 24 h of reoxygenation. No-glucose DMEM was replaced by complete EBM-2 medium with same glucose concentration as the pretreatment.Two HIF-1α inhibitors were used to examine the effect of HIF-1 on high glucose-induced endothelial permeability after OGD, 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1) (Cayman, Ann Arbor, MI) and 2-methoxyestradiol (2ME2) (ENZO, Plymouth Meeting, PA)[21,40-43]. For inhibitor experiments, 10 μM YC-1 or 10 μM 2ME2 was added to cell cultures at the onset ofreoxygenation. To inhibit the activity of VEGF, VEGF antibody (Ab-VEGF, sc-507, Santa Cruz, Santa Cruz, CA) at 100 ng/ml (final concentration) was added to the cell culture medium at the onset of reoxygenation based on a previous report[25]. The experimental protocol of was summarized in **Fig. 3-1**.

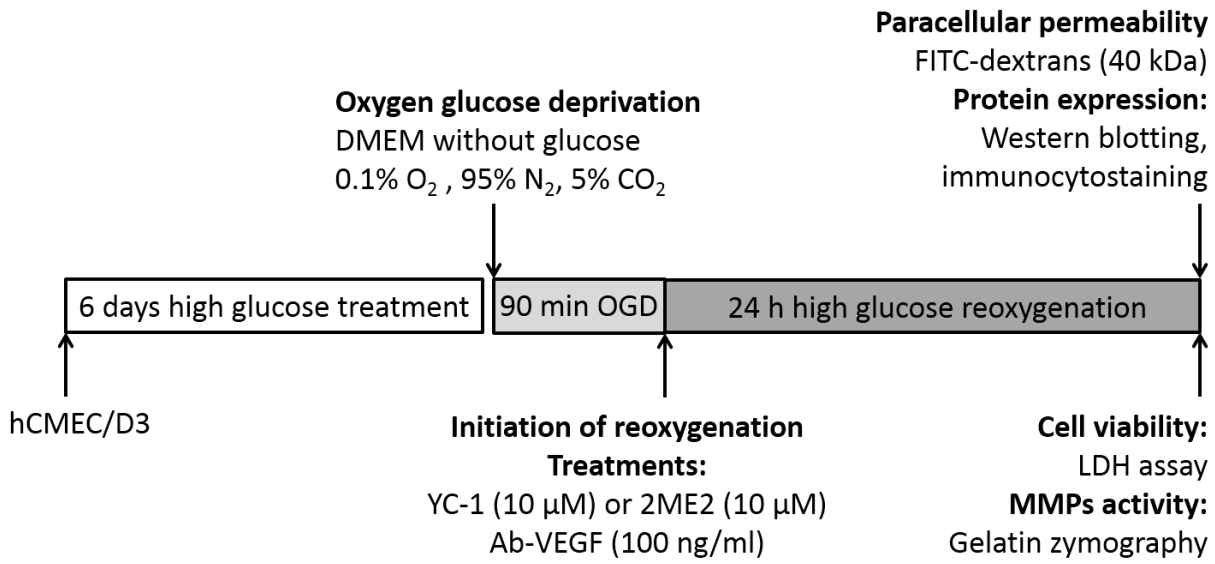

Fig. 3-1 Experimental protocol.hCMEC/D3 cells were pretreated with normal or high glucose concentration medium for 6 days. After 6 days, the cells were subjected to OGD by incubating in 0.1% O_2, glucose-free culture medium for 90 min, then reoxygenated with the media containing same glucose concentration as in pretreatment for 24 h in the absence or presence of YC-1 (10 µM), 2ME2 (10 µM) or Ab-VEGF (100 ng/ml). Paracellular permeability, cell viability, protein expression and MMPs activity were measured at 24 h reoxygenation.

Cell viability

Following 90 min OGD and 24 h reoxygenation, cell viability was determined by lactate dehydrogenase (LDH) measurements in the culture medium by a colorimetric enzymatic reaction (Cayman LDH cytotoxicity assay kit – Cayman Chemical Company, Ann Arbor, MI), according to the manufacturer guidelines. The result showed that high glucose did not significantly affect cell viability after OGD/reoxygenation **(Fig. 3-2)**.

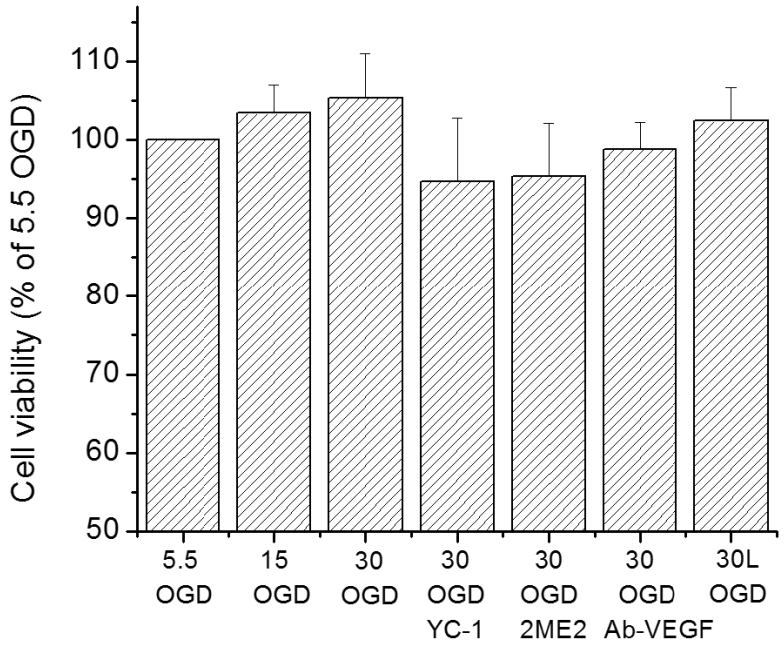

Fig. 3-2 Effects of high glucose on the cell viability of hCMEC/D3 cells. The cell viability was determined by the LDH assay. Cells were pretreated with glucose at 5.5, 15, and 30 mM. L-glucose at 30 mM in the presence of 5.5 mM D-glucose was used as osmotic control. After 6 days pretreatment, cells were subjected to 90 min OGD and 24 h reoxygenation. The glucose concentration during reoxygenation was the same as that in pretreatment. Values are shown as percentage of 5.5 OGD. Values are means ± SD, n = 3.

Paracellular permeability assay

The paracellular permeability was detected as described previously[21]. Briefly, cells were plated on the collagen-coated Transwell inserts (diameter, 10 mm; pore size, 0.4 μm; polycarbonate membrane, Nalge Nunc International, Rochester, NY). After reaching confluency, cells were treated with high glucose for 6 days and then subjected to 90 min OGD and 24 h reoxygenation. This was carried out in 24-well plates.Fluorescein isothiocyanate (FITC)-dextran (40 kDa, 1 mg/ml, 500 μl; Sigma, St. Louis, MO) was added to the cell culture medium inside the inserts 3 h before the end of the indicated time periods. After incubation for 3 h, 50 μl of medium from the outside of the insert was taken out and diluted to 500 μl with PBS. Fluorescence intensity of FITC-dextran was measured at the excitation wavelength of 492 nm and the emission wavelength of 520 nm by a fluorescent multi-mode microplate reader (Biotek, Winooski, VT). The permeability coefficient was calculated based on previous reports[44,45].

Western blotting assay

Cells were lysed in RIPA (RadioImmunoPrecipitation Assay) buffer with a cocktail of protease inhibitors (Thermo, Meridian, IL). The protein concentration was determined by the Bio-Rad DC protein assay reagent (Bio-Rad, Hercules, CA). Standard Western blotting procedures were conducted with primary antibodies rabbit anti-HIF-1α (Millipore, Billerica, MA), ZO-1 (40-2200, Invitrogen, Carlsbad, CA), occludin (33-1500, Invitrogen), claudin-5 (34-1600, Invitrogen), VEGF (sc-507, Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (sc-1616, Santa Cruz). The secondary antibody was goat anti-rabbit IgG-HRP (sc-2030, Santa Cruz Biotechnology, Santa Cruz, CA). β-actin was used as an internal control. The signal development was carried out with an enhanced chemiluminescence detection kit (Pierce, Rockford, IL). The intensity of immunoreactive bands was quantified using Image J. Results were normalized to β-actin.

Immunocytostaining

After 90 min OGD and 24 h reoxygenation, cells grown on collagen-coated coverslips were washed three times with PBS and fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. After being blocked with PBS containing 0.05% triton-X100 and 0.25% BSA for 45 min, cells were incubated with the primary antibodies against ZO-1, occludin or claudin-5 (Invitrogen) in the blocking solution at 4°C overnight. After three washes, cells were incubated with a fluorescent secondary antibody (goat anti-rabbit Alexa 488, Molecular Probes, Carlsbad, CA). After washing, the coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA). Images were routinely captured under a Leica DMI 4000B fluorescent microscope (Leica, Bannockburn, IL).All immunohistochemical staining data were obtained in a blinded manner.

Gelatin zymography

Cells were treated with high glucose in complete EBM-2 for 6 days and then subjected to 90 min OGD and 24 h reoxygenation in serum-free EBM-2 medium with same glucose concentration as the pretreatment. The supernatant medium was harvested after 24 h reoxygenation and clarified by centrifugation to remove cells and debris. The supernatants were then concentrated 50-fold using Amicon Ultra-15 centrifugal filter units with 30 kDa cut-off (EMD Millipore, Billerica, MA). The resulting sample was quantified for total protein using Bio-Rad DC protein assay reagent (Bio-Rad, Hercules, CA). Total protein (10 μg) per sample were loaded onto 10% Tris-Glycine gel containing 0.1% gelatin (Life Technologies, Grand Island, NY) in the presence of sodium dodecyl sulphate (SDS) under non-reducing conditions. Following electrophoresis, the gels were washed in the Renaturing Buffer (Life Technologies) to remove the SDS. Overnight incubation of the gel at 37 ºC in the Developing Buffer (Life Technologies) allowed the reactivated enzyme to degrade the copolymerized gelatin. Subsequently, the gels were stained with SimplyBlue™ SafeStain (Life Technologies) and the areas where the gelatin substrate has been degraded by gelatinases develop into white lines on a dark background. The molecular weight of the gelatinases was estimated by comparing the migration distance of the clear bands with the

distance migrated by markers of known molecular weight. Images of gels were captured by using a digital camera (Powershot 400 digital camera, Canon) and subsequently analyzed by Image J.

Statistical analysis

One-way ANOVA was used to determine overall significance of difference in various assays followed by post hoc Tukey's tests corrected for multiple comparisons. Data were presented as means ± SD. Differences were considered statistically significant at $p < 0.05$.

3.3 Results

High glucose increased HIF-1α expression in hCMEC/D3 cells after OGD and reoxygenation

HIF-1 is a heterodimer that is composed of α and β subunits. It is known that HIF-1α protein is continuously synthesized, but rapidly degraded in normoxic cells. HIF-1β is constitutively expressed in cells and relatively stable. The activity of HIF-1 is primarily determined by the level of its α subunit[46,47]. Therefore, we focused on the protein expression of HIF-1α in hCMEC/D3 cell culture. Cells were pretreated with high glucose medium for 6 days and then exposed to OGD by being placed in medium without glucose in an atmosphere of 95% N_2, 5% CO_2 at 37 °C. Cells were then removed to room air (reoxygenation) with complete culture medium containing the same glucose level as in pretreatment. In this study, 15 and 30 mM were used as high glucose with 5.5 mM as normal control. The normal glucose level in mouse blood is lower than 6 mM while in streptozotocin (STZ)-induced diabetic mouse, the blood glucose level is usually between 16 and 30 mM[48]. As the result, 15 and 30 mM glucose concentrations were selected for high glucose treatment. **Fig. 3-3** showed that high glucose enhanced HIF-1α accumulation after 24 h reoxygenation in adose-dependent manner as 30 mM glucose induced more HIF-1α expression than 15 mM. To determine if the increase in HIF-1α expression was due to osmotic changes, cells were treated with l-glucose at 30 mM in the presence of 5.5 mM d-glucose. The Western blotting results showed no significant difference in HIF-1α protein level compared to the control (5.5 mM d-glucose). High glucose also up-regulated the protein expression of HIF-1 down-stream factor VEGF. VEGF is transcriptionally activated by HIF-1 and is a potent inducer of BBB permeability[17,20,49-51]. These results indicated that high glucose effectively enhanced HIF-1α protein accumulation and HIF-1 functional activity after OGD/reoxygenation. It has been reported that HIF-1α is a key regulator for the induction of MMPs in hypoxic conditions. MMP-2/9 areassociated withdisruption of BBB integrity. Since MMP-2/9 are synthesized intracellular and secreted into the extracellular space to digest matrix components [52], we assessed the gelatinolytic activity of MMP-2 and -9 in the conditioned culture medium at 24 h reoxygenation by zymogram analysis. As shown in **Fig. 3-4**, MMP-2 and MMP-9 were detected in the gel at molecular weight of 72 and 95 kDa respectively. The gelatinase activity of MMP-2 was

significantly increased in the culture medium of both 15 mM and 30 mM high glucose groups, whereas MMP-9 activity was only significantly increased by 30 mM glucose treatment. The result clearly showed that high glucose induced higher MMP-2 and -9 activities in hCMEC/D3 after OGD/reoxygenation.

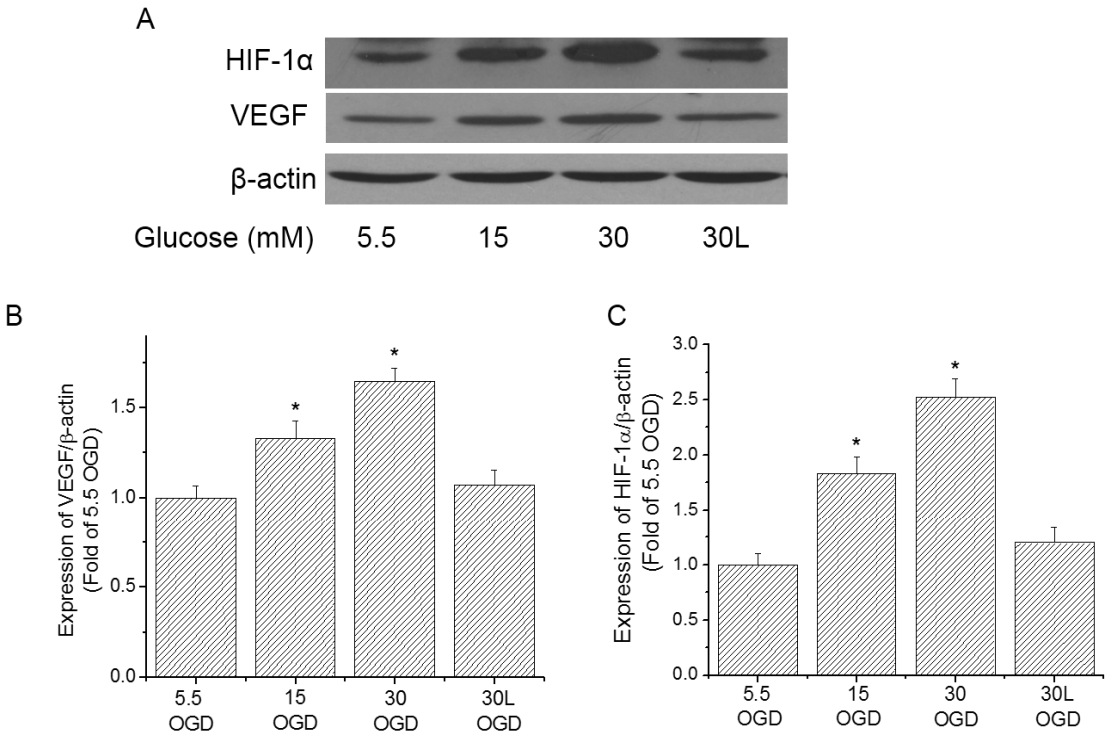

Fig. 3-3 Effect of high glucose on the expression of HIF-1α and VEGF in hCMEC/D3 cells. Cells were pretreated with glucose (D-glucose unless otherwise noted) at 5.5, 15, and 30 mM. L-glucose at 30 mM in the presence of 5.5 mM D-glucose was used as osmotic control (30 L). After 6 days pretreatment, cells were subjected to 90 min OGD and 24 h reoxygenation with the same glucose concentration as in pretreatment. **(A)** Representative Western blots of HIF-1α and VEGF with β-actin as a protein loading control. **(B)** Quantification of HIF-1α protein level from 4 independent experiments. **(C)** Quantification of VEGF protein level from 4 independent experiments. Values were normalized to β-actin and 5.5 mM OGD group. Values are means ± SD, n = 4. $^{*}p < 0.05$ vs. 5.5 mM OGD.

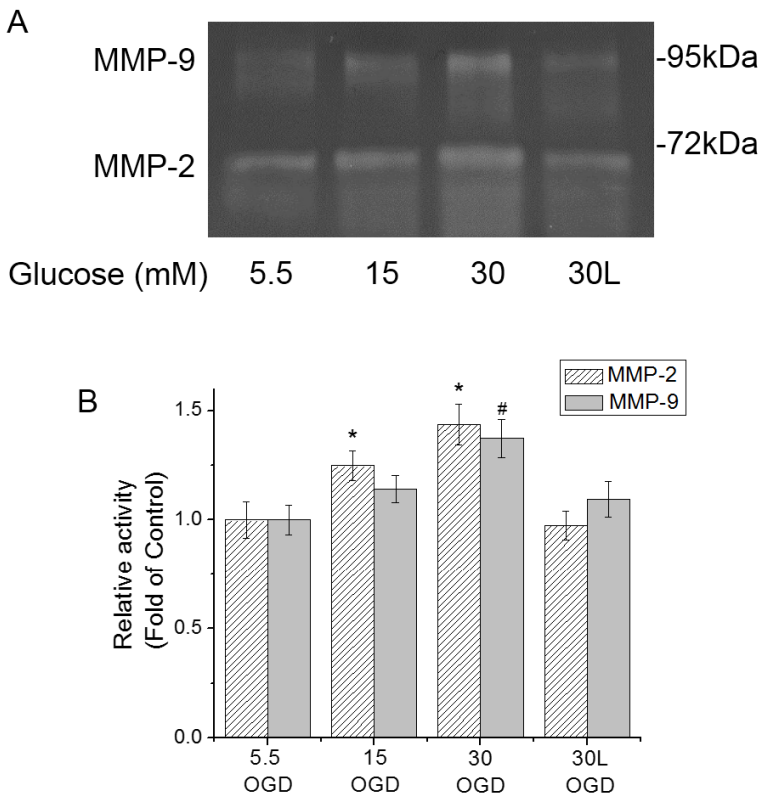

Fig. 3-4 Effect of high glucose on the gelatinolytic activity of MMP-2/9 in hCMEC/D3 conditioned medium. Cells were pretreated with glucose at 5.5, 15, and 30 mM. L-glucose at 30 mM in the presence of 5.5 mM D-glucose was used as osmotic control (30 L). After 6 days pretreatment, cells were subjected to 90 min OGD and 24 h reoxygenation with the serum-free media containing same glucose concentration as in pretreatment. **(A)** Representative gelatin zymography ofMMP-2 and MMP-9 in conditioned medium samples. Gelatinolytic bands of ~95 and 72 kDa correspond to MMP-9 and MMP-2, respectively. **(B)**Optical densitometry quantification of activated MMP-2/9. Values were normalized to 5.5 mM OGD group. Values are means ± SD, n = 3. $^{*}p < 0.05$ vs. 5.5 mM OGD (MMP-2), $^{\#}p < 0.05$ vs. 5.5 mM OGD (MMP-9).

High glucose increased paracellular permeability of hCMEC/D3 after OGD and reoxygenation

Next, we examined how high glucose affected the paracellular permeability change of hCMEC/D3 cells subjected to OGD with reoxygenation. Permeability was measured from the transfer of FITC-dextran (40 kDa molecular weight) across confluent monolayers of hCMEC/D3 seeded on the collage inserts based on a previous publication (see Materials and Methods) [35]. The permeability of cell monolayer cultured in normal glucose (5.5mM) was $0.86 \pm 0.07 \times 10^{-6}$ cm/s after 90 min OGD and 24 h reoxygenation. Similar to its effect on HIF-1α protein expression, high glucose caused a concentration-dependent increase in endothelial paracellular permeability (**Fig. 3-5**). The permeability was 1.13 ± 0.07 and $1.40 \pm 0.06 \times 10^{-6}$ cm/s for 15 and 30 mM glucose-treated cells, respectively. In addition, l-glucose at 30 mM had no effect on the paracellular permeability. To explore whether the increased permeability was due to reductions in cell number, we assessed cell viability by LDH release after OGD/reoxygenation. High glucose did not worsen the cell death compared with OGD 5.5 mM (**Fig. 3-2**), indicating that high glucose-induced permeability increase was a direct consequence of glucose concentration itself rather than a rise in cytotoxicity.

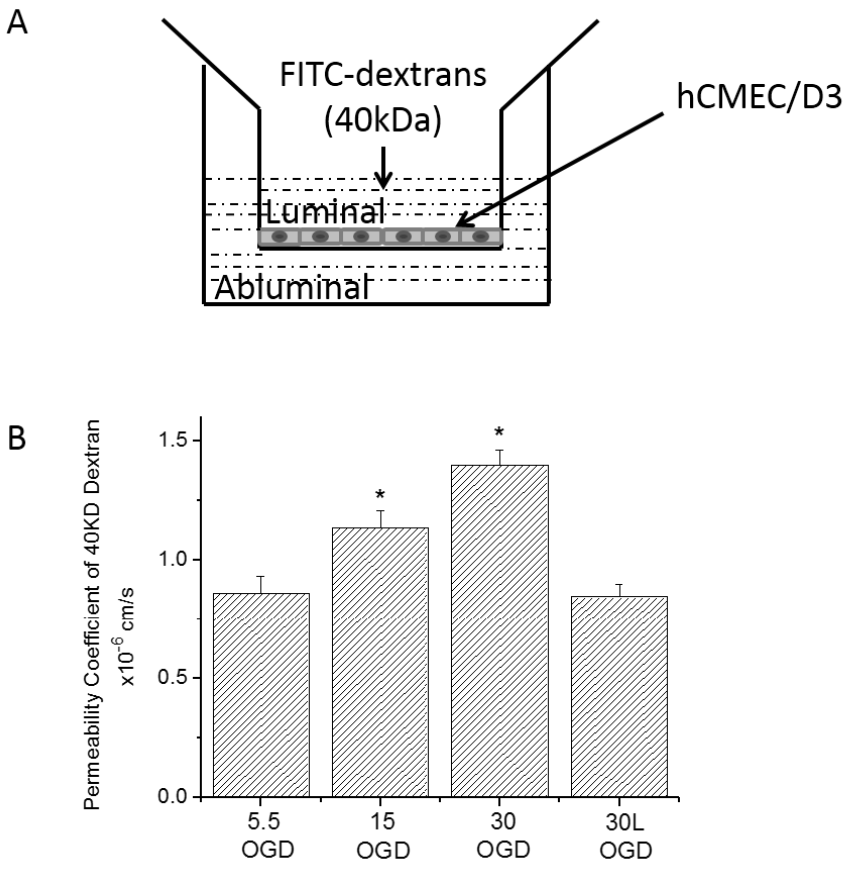

Fig. 3-5 Effect of high glucose on the paracellular permeability of hCMEC/D3 cells.The permeability was determined with 40 kDa FITC-dextran. Cells were pretreated with glucose at 5.5, 15, and 30 mM. L-glucose at 30 mM in the presence of 5.5 mM D-glucose was used as osmotic control. After 6 days pretreatment, cells were subjected to 90 min OGD and 24 h reoxygenation. The glucose concentration during reoxygenation was the same as that in pretreatment. **(A)** Schematic drawing of the hCMEC/D3 monolayer grown on an insert with FITC-dextran loaded in the luminal compartment. **(B)** The endothelial monolayer paracellular permeability was assessed by calculating the transfer rate of FITC-dextran from luminal compartment to abluminal compartment, and was described as absolute values of permeability coefficients (cm/s). Values are means ± SD, n = 4. $^{*}p<0.05$ vs. 5.5 mM OGD.

High glucose exacerbated TJ disruption on hCMEC/D3 after OGD and reoxygenation

The paracellular permeability of BBB is primarily mediated by the TJs between endothelial cells of the brain microvessels[53]. Decreased TJ proteins expression or variations in subcellular localization are associated with alterations in permeability and barrier dysfunction[13]. In this study, we evaluated ZO-1, occludin and claudin-5 protein expression levels and arrangement patterns by Western blotting and immunocytochemical labeling to further characterize high glucose-induced paracellular permeability. **Fig. 3-6A**demonstrated that high glucose decreased the protein levels of ZO-1 and occludin in hCEMC/D3 cells after 90 min OGD and subsequent reoxygenation. The degree of reduction of occludin was more severe than that of ZO-1. The ZO-1 level was reduced 31% and 50% by 15 and 30 mM glucose, respectively. The occludin level decreased 48% and 72% by 15 and 30 mM glucose, respectively**(Fig. 3-6B and C)**. The protein expression of claudin-5, however, was relatively stable in the cells exposed to high glucose **(Fig. 3-6A and D)**. It is noteworthy that l-glucose at 30 mM in the presence of 5.5 mM d-glucose did not induce significant changes of ZO-1, occludin or claudin-5 expression compared with control (5.5 mM glucose). Furthermore, results from immunocytostaining confirmed that high glucose altered the arrangement pattern of ZO-1 and occludin, but not claudin-5 after OGD/reoxygenation **(Fig. 3-6E)**. In other words, high glucose disrupted the immunoreactivity of the ZO-1 and occludin staining on the cell borders. These results support the observed permeability increase of the endothelial monolayer by high glucose and suggest that disruption of ZO-1 and occludin may be the potential reason for increased permeability in high glucose treated cells.

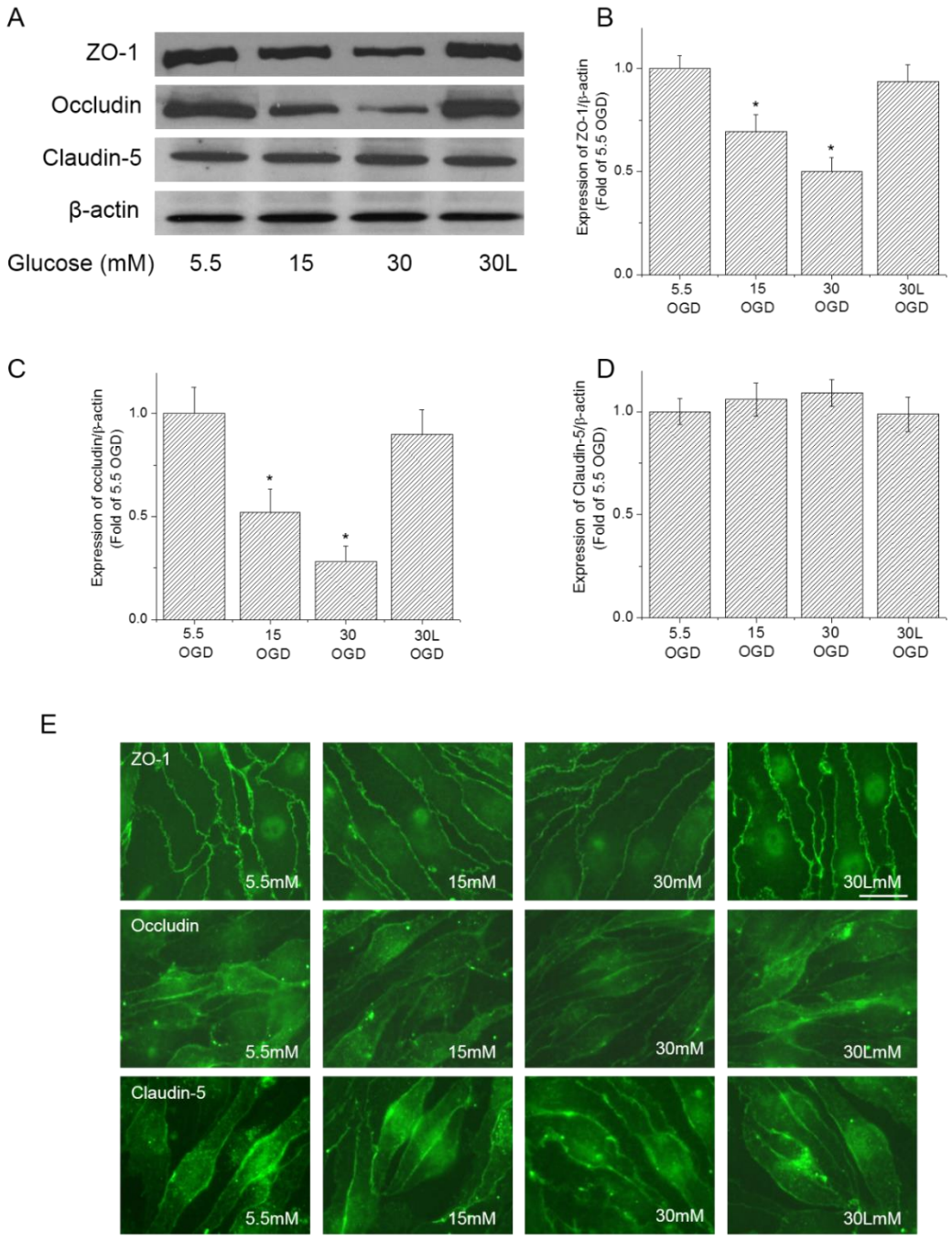

Fig. 3-6 Effect of high glucose on the expression and arrangement of ZO-1, occludin and claudin-5 in hCMEC/D3 cells. Cells were pretreated with glucose at 5.5, 15, and 30 mM. L-glucose at 30 mM in the presence of 5.5 mM D-glucose was used as osmotic control. After 6 days pretreatment, cells were subjected to 90 min OGD and 24 h reoxygenation with the same glucose concentration as in pretreatment. **(A)** Representative Western blots of ZO-1, occludin and claudin-5 after OGD/reoxygenation. β-actin

serves as a protein loading control. **(B)** Quantification of ZO-1 protein level from 3 independent experiments. **(C)** Quantification of occludin protein level from 3 independent experiments. **(D)** Quantification of claudin-5 protein level from 3 independent experiments. (E) Representative images of ZO-1, occludin and claudin-5 immunostaining. Scale bar 20 μm. Values in B, C and D were normalized to β-actin and 5.5 mM OGD. Data are means ± SD, n = 3. $^{*}p < 0.05$ vs. 5.5 mM OGD.

Inhibiting HIF-1α expression in reoxygenation attenuated paracellular permeability of hCMEC/D3

The above experiments indicated a positive correlation between HIF-1 expression and permeability increase of hCMEC/D3 cells exposed to high glucose pretreatment and reoxygenation. To further determine the role of HIF-1 in high glucose-induced paracellular permeability increase, we carried out experiments with inhibition of HIF-1 by two widely used HIF-1α inhibitors, YC-1 and 2Me2. YC-1 (10 μM) or 2Me2 (10 μM) were added to the culture medium at the onset of reoxygenation in 30 mM glucose groups. It is worth noting that the inhibitors did not influence the cell viability (**Fig. 3-2)**. As seen in **Fig. 3-7**, both inhibitors effectively suppressed the expression of HIF-1α and its target protein VEGF in high glucose treated hCMEC/D3 cells at 24 h reoxygenation. HIF-1α inhibition remarkably reduced the increase in paracellular permeability caused by high glucose **(Fig. 3-7D)**. It is noteworthy that the permeability in YC-1 and 2Me2 treated 30mM groups were still significantly higher than that in 5.5 mM group even though HIF-1α protein levels showed no significant difference between these groups. This indicated that other pathways independent of HIF-1α might be involved in permeability increase caused by high glucose. We also investigated the effect of HIF-1α inhibition on TJ proteins expression and localization. The western blotting results showed that downregulating HIF-1 activity alleviated the high glucose-induced loss of ZO-1 and occludin **(Fig. 3-8A)**. Moreover, the immunocytostaining showed that HIF-1α inhibitors partially restored ZO-1 and occludin expression at endothelial cell-cell contacts in 30 mM glucose groups**(Fig. 3-8E)**. The results demonstrated that high glucose-induced BBB permeability increase after OGD exposurewas suppressed by HIF-1α inhibition, indicating that HIF-1 is highly involved in high glucose-mediated BBB dysfunction.

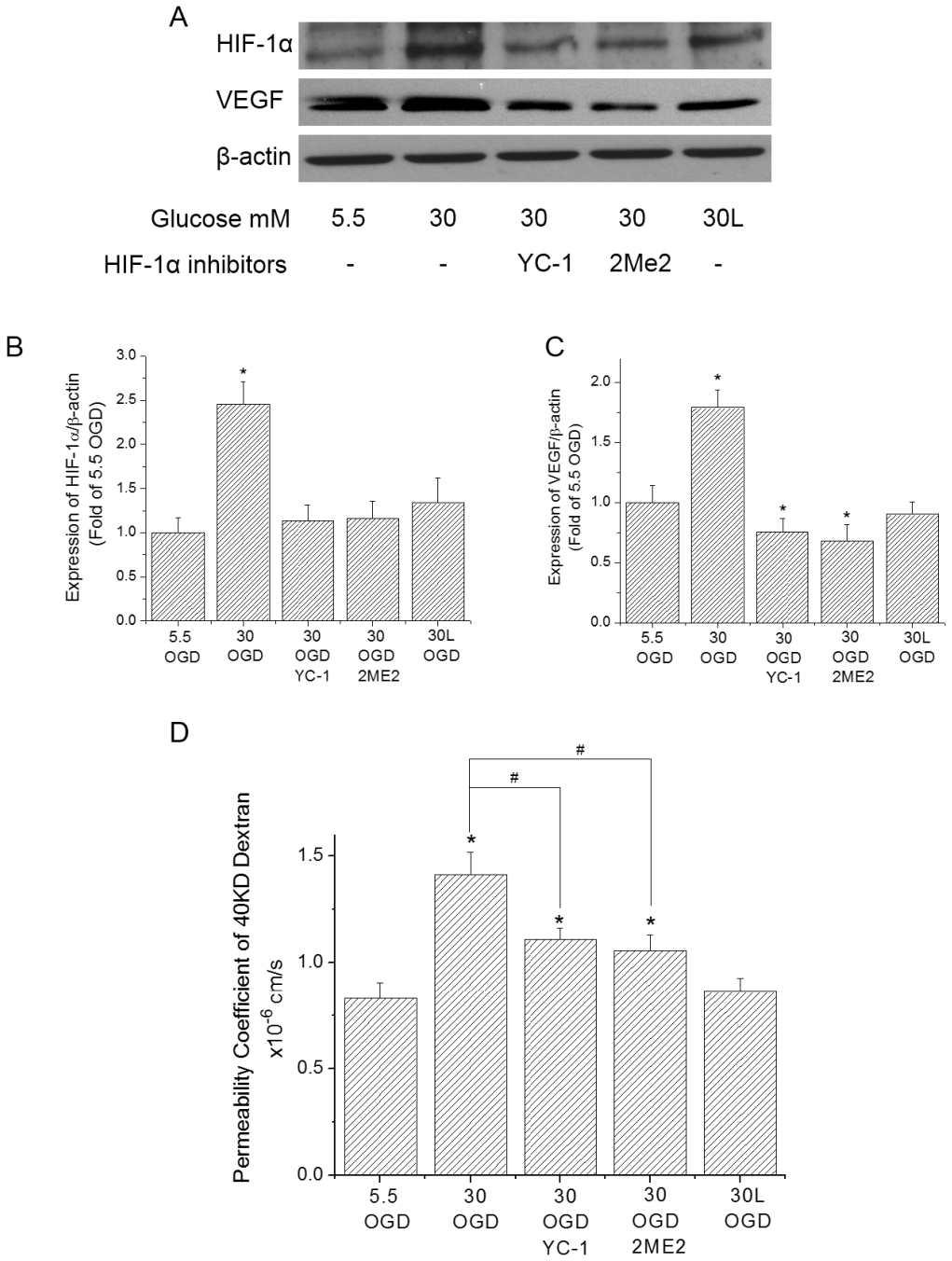

Fig. 3-7 Effect of HIF-1α inhibition on the paracellular permeability of hCMEC/D3 cells. HIF-1α inhibitors (10 μM YC-1, or 10 μM 2ME2) were added to the culture medium at the onset of reoxygenation in high glucose (30 mM) treated groups.Cells were incubated in high glucose medium with or without HIF-1α inhibitors for 24 h. Cells treated with 30 mM L-glucose (30 L) in the presence of 5.5 mM D-glucose serve as an osmotic control. **(A)** Representative Western blots of HIF-1α and VEGF

with β-actin as a protein-loading control. **(B)**Quantification of HIF-1α protein level from 3 independent experiments. **(C)** Quantification of VEGF protein level from 3 independent experiments. **(D)**Effects of HIF-1α inhibitors, YC-1 and2ME2, on the paracellular permeability of hCMEC/D3 cells. Values in B and C were normalized to β-actin and 5.5 mM OGD. Values are means ± SD, n = 3. $^{*}p < 0.05$ vs. 5.5 mM OGD. $^{\#}p < 0.05$ vs. 30 mM OGD.

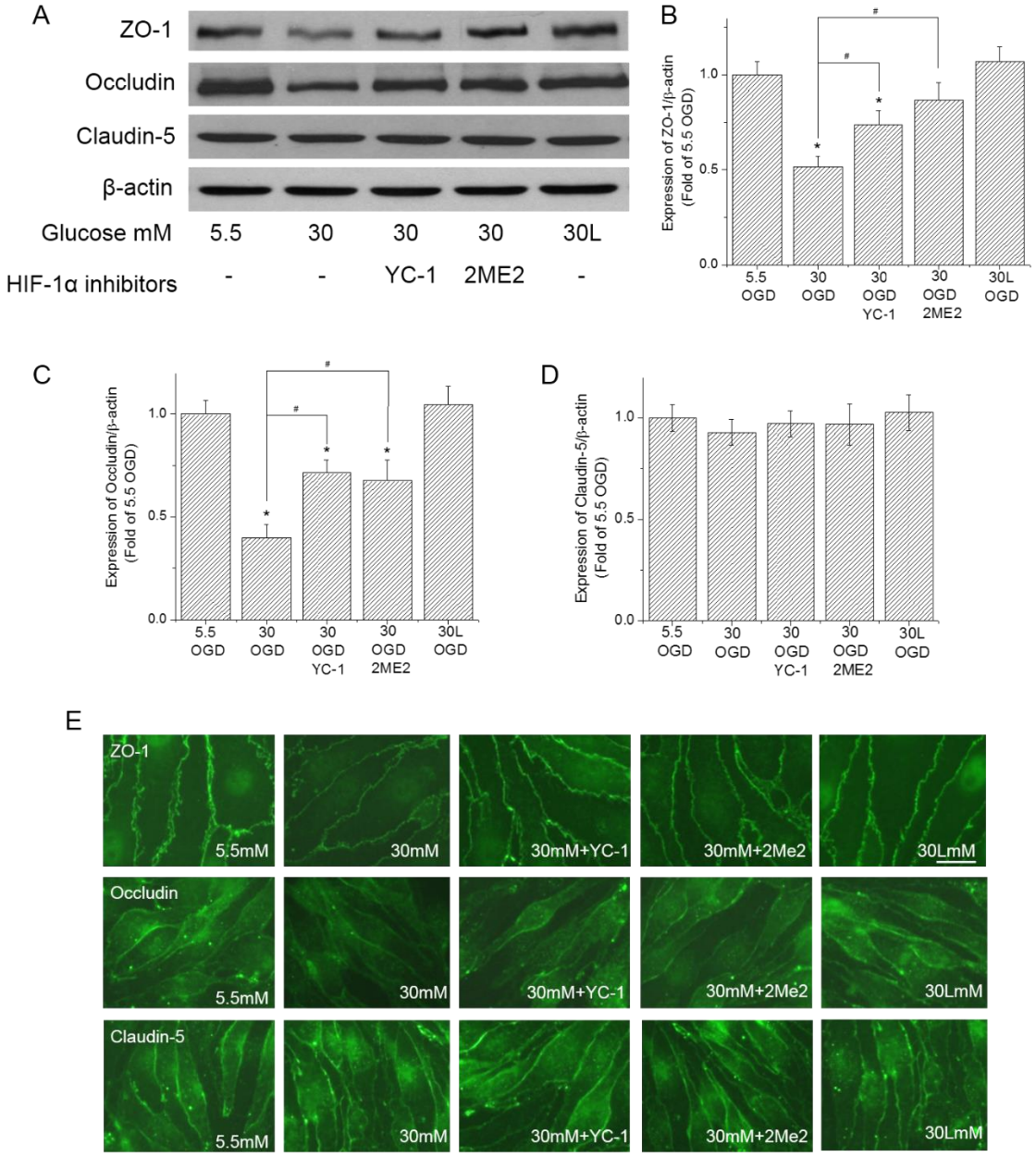

Fig. 3-8 Effect of HIF-1α inhibition on the expression and arrangement of ZO-1, occludin and claudin-5 in hCMEC/D3 cells. HIF-1α inhibitors (10 μM YC-1, or 10 μM 2ME2) were added to the culture medium at the onset of reoxygenation in high glucose (30 mM) treated groups.Cells were incubated in high glucose medium with or without HIF-1α inhibitors for 24 h. Cells treated with 30 mM L-glucose in the presence of 5.5 mM D-glucose serve as an osmotic control. **(A)** Representative Western blots of ZO-1,

occludin and claudin-5 after OGD/reoxygenation. β-actin serves as a protein loading control. **(B)** Quantification of ZO-1 protein level from 3 independent experiments. **(C)** Quantification of occludin protein level from 3 independent experiments. **(D)** Quantification of claudin-5 protein level from 3 independent experiments. **(E)** Representative images of ZO-1, occludin and claudin-5 immunostaining. Scale bar 20 μm. Values in B, C and D were normalized to β-actin and 5.5 mM OGD. Data are means ± SD, n = 3. $^{*}p < 0.05$ vs. 5.5 mM OGD. $^{\#}p < 0.05$ vs. 30 mM OGD.

VEGF contributes to HIF-1's effect on permeability

VEGF is transcriptionally induced by HIF-1 and has been reported to promote BBB permeability by altering TJs under ischemic conditions. We explored its role in high glucose-induced permeability increase after OGD. Coinciding with changes of HIF-1α expression, VEGF protein level was up-regulated in cells exposed to both 15 and 30 mM glucose**(Fig. 3-3)**. We also detected the VEGF expressionafter HIF-1α inhibition. BothYC-1 and 2ME2 and significantly reduced the protein levels of VEGF**(Fig. 3-7)**, indicating that high glucose-induced VEGF expression in hCMEC/D3 cells depends on HIF-1 activation. To verify the role of VEGF in high glucose-induced paracellular permeability increase, a VEGF antibody was used to block its effect, as reported previously[25]. As shown in **Fig. 3-9**, inhibiting VEGF attenuated paracellular permeability increase in 30 mM glucose treated cells after 24 h reoxygenation. Accordingly, these results support that a plausible pathway for the observed HIF-1-induced permeability in the cells exposed to high glucose may be through upregulating VEGF expression.

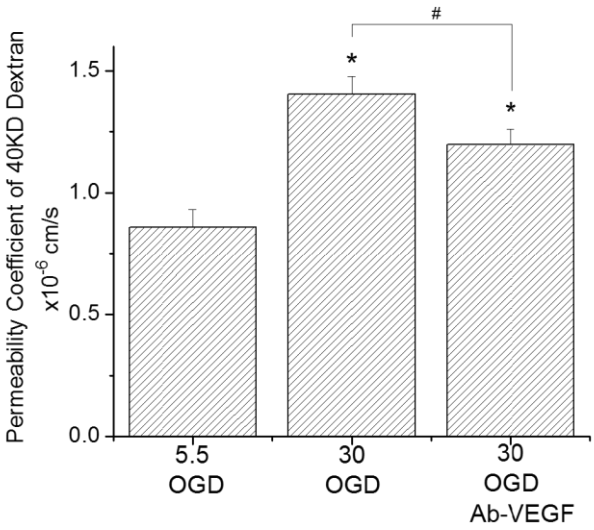

Fig. 3-9 Effect of VEGF on the paracellular permeability of hCMEC/D3 cells. VEGF antibody (Ab-VEGF) at 100 ng/ml (final concentration) was added to the cell culture medium at the onset of reoxygenation in high glucose (30 mM) treated group. Cells were incubated in high glucose medium with or without Ab-VEGF for 24 h. Data are means ± SD, n = 3. $^{*}p < 0.05$ vs. 5.5 mM OGD. $^{\#}p < 0.05$ vs. 30 mM OGD.

3.4 Discussion

There were several major findings of the present study. First, we demonstrated that high glucose could further induce HIF-1α expression in brain endothelial cells subjected to OGD/reoxygenation (detailed discussion in Chapter 4). Second, we investigated the effect of high glucose exposure on *in vitro* BBB permeability. We found that high glucose aggravated BBB opening and endothelial TJs disruption after OGD. Lastly, the effect of high glucose on BBB permeability was reversed by inhibition of HIF-1αactivity with YC-1 or 2ME2 during reoxygenation, suggesting that activation of HIF-1α in endothelial cells is an underlying mechanism by which permeability was increased. Thus, it appears that HIF-1α inhibition may be an important therapeutic target for prevention of BBB disruption which is relatively common during hyperglycemic stroke.

Many epidemiology studies have shown that elevated levels of blood glucose are frequently associated with a larger infarct size, poor clinical outcome and a higher risk of mortality in acute ischemic stroke[54-58]. Hyperglycemia was found to exaggerate edema formation, BBB injury and hemorrhagic transformation in both type I and type II diabetic animal models after cerebral ischemia [2-7,59,60]. Tissue plasminogen activator (tPA) is the only established treatment for acute ischemic stroke[61,62]. However, tPA treatment in stroke patients with admission hyperglycemia induced an incremental risk of death and spontaneous intracerebral hemorrhage as well as unfavorable 90-day outcomes[63,64]. Experimental studies have found that tPA treatment of stroke in diabetic and hyperglycemic animals significantly increases BBB permeability and brain hemorrhage in the ischemic brain[65-67]. Therefore, effective therapy of stroke in the normal glucose population may not necessarily transfer to the diabetic population, prompting the need to specifically developing therapeutic targets for cerebrovascular protection to prevent BBB dysfunction in the diabetic population.

One major finding of the present study is that HIF-1 contributes to high glucose-induced permeability of brain endothelial cells after OGD/reoxygenation. The results showed that (1) high glucose exposure in pretreatment and reoxygenation increased HIF-1α accumulation in hCMEC/D3 cells; (2) there was a positive correlation between HIF-1α expression and high glucose-induced endothelial

permeability; and (3) suppressing HIF-1α activity at the onset of reoxygenation attenuated the permeability increase caused by high glucose. To further confirm the role of HIF-1 and understand the mechanism of high glucose-induced permeability increase, VEGF expression and MMP-2/9 activities were examined in the brain endothelial cells subjected to OGD/reoxygenation. As shown in Fig. 3-3, VEGF expression was elevated concentration-dependently in high glucose-treated cells. This agrees with previous findings in retinal microvascular endothelial cells[68-70]. Also, thehigh glucose enhanced the MMP-2/9 gelatinolytic activity in hCMEC/D3 culture medium. More importantly, HIF-1 inhibition by YC-1 and 2ME2 suppressed the VEGF expression, which is in consistent with previous studies in endothelial cells [21] and in ischemic animal models[21,71,72]. Nonetheless, we did not conclude that HIF-1 is the sole inducer of the VEGF expression. Other factors may also be responsible for the VEGF expression, such as peroxisome proliferator-activated receptor γ cofactor-1α[73,74]. It is worth noting that YC-1 and 2ME2 completely abolished HIF-1α accumulation induced by high glucose, whereas only partially restored BBB function. These results suggested that besides HIF-1 activation, other pathways might also be involved in high-glucose-exacerbated BBB disruption after OGD/reoxygenation (discussed in Chapter 4).

It needs to be pointed out that although it is well accepted that YC-1 and 2ME2 are effective HIF-1 inhibitors, neither of them is a specific HIF-1 suppressor. For example, apart from inhibiting HIF-1α and its down-stream gene VEGF, YC-1 regulates the intracellular concentration of cGMP though enhancing the activity of soluble guanylate cyclase[75]. Nevertheless, inhibition of soluble guanylate cyclase did not change the effect of YC-1 on blood brain permeability[21]. In addition, 2ME2 has also been reported to have properties other than down-regulating HIF-1α, such as anti-proliferative, anti-angiogenenic and anti-neovacularization, and alteration of inflammatory response[40,76-78]. However, recent studies have reported that 2ME2 has a direct effect on HIF-1α inhibition and not as a result of a “side effect” of mitotic arrest. 2ME2 was specific for HIF-1α subunit, and had no effect on HIF-1β or other transcription factors. *In vitro* studies demonstrated that 2ME2 reduced the levels of nuclear and total HIF-1α in a dose-dependent manner[40]. Therefore, the protective effect of YC-1 and 2ME2 on *in vitro* BBB

model was largely dependent on HIF-1 pathway. Future study using HIF-1α specific siRNA to inhibit HIF-1 activity in hCMEC/D3 will further confirm the role of HIF-1 in high glucose-exacerbated BBB disruption after OGD/reoxygenation.

TJs in the BBB consist of both transmembrane proteins such as claudins, occludin and junctional adhesion molecules and cytoplasmic accessory proteins such as ZO-1 and -2. The TJs are responsible for the severe restriction of the paracellular diffusion pathway between the endothelial cells [79]. Generally, movement of TJ proteins away from the cellular borders or decreased expression at TJ cleft indicates a loss of junction integrity and increased paracellular permeability[13]. Occludin, claudin-5 and ZO-1 are the major structural proteins that make up the BBB and are considered sensitive indicators of normal and disturbed functional state of BBB [53]. In this study, we examined the expression and arrangement patterns of these 3 proteins. High glucose decreased the protein expression of ZO-1 and occludin in a concentration-dependent manner, whereas it had no effect on the claudin-5 protein level. Occludin is critically involved in regulating the TJs [80]. Altered expression of occludin causes disrupted BBB function in various pathologies including diabetes and stroke[22,81,82]. ZO-1 molecules connect transmembrane protein with the actin cytoskeleton [83]. Loss or dissociation of ZO-1 from the junctional complexes is associated with increased BBB permeability[17,84,85]. The reduced expression and discontinuous appearances of occludin and ZO-1 on hCMEC/D3 cell periphery may result in the high glucose-induced barrier hyperpermeability after OGD. In line with our results, previous studies have reported decreased protein expression of both occludin and ZO-1 in cerebral microvessels along with increased vascular permeability in STZ-induced type I diabetic rats[22]. The degree of downregulation of ZO-1 and occludin seems to be able to induce higher paracellular permeability than that observed. This may be due to stable expression and distribution of claudin-5, since the claudins form the primary seal of the TJ at BBB. The stably expressed claudin-5 may maintain part of the restriction of the TJs and prevent greater paracellular permeability. Inhibition of HIF-1α activity significantly elevated the protein levels of occludin and ZO-1 and effectively restored the staining of both TJ proteins to the cell borders, suggesting that HIF-1α activation is a predominate pathway which mediates high glucose-exacerbated TJs disruption.

VEGF has been reported as a potent inducer of endothelial permeability[20,49,86-89]. It is well studied that VEGF enhances vascular permeability by altering expression and organization of TJ proteins, such as ZO-1 and occludin [17,23,90]. Normally, VEGF's effects on vascular permeability are transduced by binding to its specific tyrosine receptors, VEGF receptor 1 (VEGFR-1, Flt-1) and VEGF receptor-2 (VEGFR-2, Flk-1)[91]. The activation of VEGFR-2 through dimerization and phosphorylation leads the activation of several intracellular signaling pathways including phospholipase Cγ, Src and phosphatidylinositol 3-kinase, which may be involved in alteration of vascular permeability[92-94]. VEGF antibodies combine with VEGF protein and block the interaction between VEGF and its receptor. Neutralization of VEGF with its antibodies has been proven to attenuate BBB disruption in both *in vitro* endothelial cell monolayer [17] and *in vivo* ischemic animal models[20,50,95]. Our results showed that a VEGF antibody efficiently reduced the increased permeability in cells exposed to high glucose. This proves the involvement of VEGF in high glucose-induced paracellular permeability after OGD/reoxygenation. It is noteworthy that the decrease of hCMEC/D3 permeability induced by VEGF antibody was less significant than that by HIF-1α inhibitors, suggesting other factors down-stream to HIF-1 may also contribute to BBB dysfunction (e.g., MMPs). The exact mechanisms of VEGF-induced TJ protein loss and delocalization are not completely understood. Two factors might be involved, decrease in mRNA expression and protein phosphorylation. VEGF is able to reduce the mRNA level of ZO-1 [21] and occludin[90]. Furthermore, VEGF may regulate endothelial permeability through phosphorylation of the TJ proteins such as ZO-1 and occludin[96], which are phosphoproteins. Changes in phosphorylation state affect their interaction, alter transmembrane protein localization and induce their redistribution (see review 97 for more details).

Upregulation of MMP-2 and -9 have been implicated in the BBB disruption in both diabetes and ischemic stroke pathologies. Increased plasma MMP-2 activity was found increased in STZ-induced diabetic mice, which leads to altered TJs and increased BBB permeability[22]. In *in vitro* studies, high glucose has been shown to increase MMP-2/9 activity in endothelial cell culture, which compromised BBB integrity by a mechanism involving proteolytic degradation of occludin[81,98]. Our study showed that high glucose induced significant higher level of MMP-2/9 secretion from hCMEC/D3 after

OGD/reoxygenation, which may exacerbate BBB dysfunction. These observations are consistent with other studies using animal models, in which greater MMP-2 and/or -9 activities were found in the ischemic brain of diabetic animals following stroke[3,7,60,99-101]. For example, previous study has observed significant increase of MMP-9 expression and gelatinolytic activity in the ischemic brain of the diabetic *db/db* mice compared to their non-diabetic littermates following a stroke. This increase activity was associated with greater degradation of occludin and basement membrane protein collagen IV, resulting in increased BBB permeability[7]. Inhibition of MMPs activity using broad-spectrum MMP inhibitor minocycline attenuated BBB breakdown and improved functional outcomes in diabetic animals after stroke[3].

There are some limitations of the current study. First, a single marker of paracellular transport, i.e., FITC-dextran (40 kDa) has been employed throughout the study. The use of FITC-dextran of higher molecular weights (e.g. 70 kDa) might have shed some light on the size of intracellular openings. Furthermore, the results presented in the report were based on an *in vitro* model of BBB consisting of human cerebral microvascular endothelial cells. In fact, the formation and function of BBB requires support of adjacent glial cells as well as neurons, pericytes, and extracellular matrix [102]. Cell-cell interactions and signaling occur in a coordinated manner between these multiple cell types play an important role in the physiological functioning of BBB [103]. This study using a cell culture model of human BBB established with only endothelial cells have its limitations. However, by reducing the number of cell types that are examined, it is possible to delineate specific cellular effects and to explore signal transduction mechanisms, while animal models allow these simplified observations to be tested in a more complex scenario. Thus, the study on *in vitro* BBB model provides proof of principle evidence for animal experiments and forms a necessary complement to studies carried out in animal models of stroke. Our experimental results from hCMEC/D3 cells were further confirmed in the mouse diabetic stroke model in the Chapter 4.

In summary, our study has demonstrated that high glucose enhances HIF-1α in brain microvascular endothelial cells after OGD/reoxygenation. The activation of HIF-1 pathway is involved in

high glucose-exacerbated brain endothelial leakage, which is associated with altering the expression and arrangement of TJ-associated proteins, such as occludin and ZO-1. These results suggest that HIF-1α inhibition may become a useful therapeutic approach for preventing the cerebrovascular disruption following ischemic stroke in patients with hyperglycemia.

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Chapter 4: HIF-1 is Involved in Hyperglycemia-Exacerbated Blood-Brain Barrier Disruption in Ischemic Stroke

Abstract

Diabetes is a major stroke risk factor and is associated with poor functional recovery after stroke. Accumulating evidence indicates that the worsened outcomes may be due to hyperglycemia-induced cerebral vascular complications, especially disruption of the blood-brain barrier (BBB).Increased BBB permeability contributes to the development of neurological damage in stroke. In the present study, we hypothesized that the activation of hypoxia inducible factor-1 (HIF-1) pathway was involved in hyperglycemia-aggravated BBB disruption in an ischemic stroke animal model.

Both the non-diabetic control and the streptozotozin (STZ)-induced type I diabetic mice were subjected to 90 min transient middle cerebral artery occlusion (MCAO) followed by reperfusion. Our results demonstrated that hyperglycemia induced higher expression of HIF-1α and its down-stream factor vascular endothelial growth factor (VEGF) in ischemic brain microvessels after MCAO/reperfusion. Diabetic mice showed exacerbated BBB damage and tight junction disruption, increased infarct volume as well as worsened neurological deficits. Suppressing HIF-1 activity by specific knock-out endothelial HIF-1α ameliorated BBB leakage and brain infarction in diabetic animals. Glycemic control by insulin treatment abolished HIF-1α up-regulation in diabetic animals and reduced BBB permeability and brain infarction. These findings strongly indicate that HIF-1 plays an important role in hyperglycemia-induced exacerbation of BBB disruption in ischemic stroke. HIF-1 inhibition warrants further investigation as a therapeutic target for the treatment of stroke in presence of diabetes.

4.1 Introduction

Ischemic stroke is a leading cause of death and long-term disability in the United States and worldwide and diabetes is the most rapidly increasing risk factor for stroke[1]. Approximately 30-40% of ischemic stroke patients present with admission hyperglycemia, either due to diabetes or due to a generalized stress reaction[2,3]. The relative risk of cerebrovascular disease or stroke is 2 to 6-fold higher in diabetes[1]. In addition to the increased stroke incidence, diabetes and hyperglycemia are associated with worsened stroke outcomes, leading to increased mortality and poor functional recovery[4-6]. Since diabetic patients are at a higher risk of stroke and have poorer prognosis compared to the non-diabetic population, a better understanding on how diabetes affects ischemic stroke outcome is pivotal for developing better prevention and treatment strategies before and after an ischemic insult.

During ischemic stroke, cerebral ischemia and subsequent reperfusion result in harmful consequences, including the breakdown of the blood-brain barrier (BBB), which leads to severe neurologic deficits through aggravation of edema formation and brain hemorrhage[7-9]. The incidence and severity of BBB damage is markedly higher in stroke patients with diabetes or stress hyperglycemia than those without, implying a prominent role for high blood glucose in the development and exacerbation of BBB disruption[2,4]. However, the mechanisms involved remain largely unexplored and are of great importance to identify novel therapeutic targets for cerebrovascular protection.

Hypoxia-inducible factor 1 (HIF-1) is a key transcription factor in response to hypoxia/ischemia[10]. It is a heterodimer of two subunits, the regulatable HIF-1α and constitutively expressed and stable HIF-1β [11]. The activity of HIF-1 is primarily determined by the level of the α subunit[12,13]. HIF-1 is implicated in cerebral vascular disorders in various pathological conditions, e.g. ischemic stroke[14-17], sabarachnoid hemorrhage[18,19], and traumatic brain injury[20]. Inhibition of HIF-1 ameliorates hypoxia-induced BBB disruption and the subsequent brain damage in both adult[14,21]and neonatal rodent ischemic stroke models[15]. Previous studies have shown that HIF-1 disrupts BBB integrity by enhancing the expression of its target gene vascular endothelial growth factor (VEGF)[14]. VEGF is a potent vascular permeability factor that promotes BBB leakage and cerebral edema after ischemic

injury[22-25]. Furthermore, it has been reported that high glucose treatment upregulates HIF-1 activity in *in vitro* endothelial cell culture[26]. In addition, increased HIF-1α expression was found in the retina[27,28]and renal tubular epithelial cells [29]of diabetic animal models. However, regulation of cerebrovascular HIF-1α level in diabetic models and the subsequent effect on stroke pathology is not clear.

In light of the above, we hypothesized that HIF-1α activation was involved in hyperglycemia-induced BBB damage in ischemic stroke in the present study. Firstly, we investigated the effect of hyperglycemia on the expression of HIF-1α and its down-stream factor VEGF using a mouse model of 90 min middle cerebral artery occlusion (MCAO) with reperfusion. Secondly, we examined whether BBB dysfunction and brain damage could be prevented by inhibition of endothelial HIF-1α. Finally, we determined if normalization of blood glucose levels in diabetic animals could prevent enhanced BBB breakdown through modulation of HIF-1α pathway.

4.2 Materials and Methods

Animals

All procedures using animals were approved by the Institutional Animal Care and Use Committees of University of Kansas and conformed to the *National Institutes of Health*Guidelines for use of animals in research. Animals were maintained in a climate-controlled vivarium with a 12 h light-dark cycle with free access to food and water. Male wide type (WT) C57BL/6mice were from Charles River Laboratory (Wilmington, MA). Mice (B6.129-*hif-1α*^{tm3Rsjo}/J) carrying homozygous HIF-1α floxed alleles (HIF-1α$^{F/F}$) were generated by engineering loxP sites flanking exon 2 of the HIF-1α gene as described previously [30] and bought from the Jackson Laboratory (stock number: 007561, Bar Harbor, Maine, USA).*Tie2-Cre* transgenic mice (B6.Cg-Tg(*Tek-cre*)1Ywa/J) expressing *cre* recombinase under the control of the receptor tyrosine kinase *Tek* (Tie2)promoter were generated as described previously [31]and also bought form the Jackson Laboratory (stock number: 004128). All mice strains were maintained on a C57BL/6J background. All animals were acclimated to the environment for 7 days before the experiments. The mouse strain B6.Cg-Tg(*Tek-cre*)was crossed with homozygote HIF-1α$^{F/F}$ mice to generate Cre$^{+/-}$: HIF-1α$^{F/Wt}$, which were crossed with homozygote HIF-1α$^{F/F}$ mice to generate HIF-1α mutants Cre$^{+/-}$: HIF-1α$^{F/F}$, designated as endothelial specific HIF-1α knock-out HIF-1α$^{\Delta/\Delta}$ as described previously[32]. Littermates with the Cre$^{-/-}$: HIF-1α$^{F/F}$ genotypes were used as controls for each group of experiments. For genotyping, genomic DNA was isolated from tail biopsies collected at 21 d of age using the DNeasy genomic DNA isolation kit (Qiagen, Valencia, CA, USA). HIF-1α^{F} and wild-type alleles were detected using the following primers: 5′-CGT GTG AGA AAA CTT CTG GAT G- 3′ and 5′-AAA AGT ATT GTG TTG GGG CAG T-3′. Transgenic mice expressing Cre recombinase were identified using primers: 5′-GCG GTC TGG CAG TAA AAA CTA TC-3′ and 5′-GTG AAA CAG CAT TGC TGT CAC TT-3′. The PCR reactions were performed with the Omni Clenttaq polymerase (DNA Polymerase Company, St. Louis, Missouri, USA). The products were run on a 3% agarose gel for HIF-1α or Cre.

Middle cerebral artery occlusion (MCAO)

Transient focal cerebral ischemia was induced by surgical occlusion of the middle cerebral artery. The procedure of MCAO followed by reperfusion was conducted using an intraluminal model as previously described[33]. For the anesthesia, 2.0% isoflurane in medical O_2 was used for induction, and 1.0% for maintenance. Duration of anesthetic exposure was kept the same for each animal.Following a midline neck incision, external carotid artery (ECA), internal carotid artery (ICA), and pterygopalatine artery of ICA were exposed. A silicone rubber-coated monofilament nylon suture (Doccol Corporation, Sharon, MA)with a diameter of 0.23 mm was inserted into the ICA via a slit on the ECA. The suture was advanced along the ICA to the extent of 9 to 10 mm from the bifurcation of mice. Reperfusion was produced by gently withdrawing the suture until the suture tip reached the bifurcation and the incision closed 90 min after the onset of ischemia. After surgery, the animals were allowed to recover from anesthesia while being given food and water ad libitum. Buprenorphine was administrated at 0.1mg/kg subcutaneously as post-operative analgesia.For all animals used in this study, successful MCAO was confirmed by laser Doppler flowmetry (LDF) (Moor Instruments, Wilmington, DE) as described in the literature [34]. During ischemia, LDF regional cerebral blood flow dropped to 17.2 ± 4.3% of the pre-ischemic level; and after reperfusion the blood flow was restored to 88.4 ± 4.6% of pre-ischemic level. During the experiment, the mice body temperature wasmaintained within the range of 37.0 ± 0.5^{o}C by the heating pad. Sham-operated animals underwent the same anesthesia and surgical procedures without the occlusion of MCA. Animals that did not show any neurological deficits or died within 24 h after MCAO were excluded.

Induction of diabetes and administration of insulin

Six-week-old mice were rendered diabetic with three daily doses of freshly prepared streptozotocin (STZ) (85, 70 and 55 mg/kg, dissolved in 0.2ml sodium citrate buffer, pH 4.5) given by intraperitoneal injection. Control mice received injections of vehicle. Three days after the last injection, mice with fasting blood glucose > 290mg/dl were deemed diabetic (One-Touch Ultra glucometer).

Because it has been reported that increasing BBB permeability in STZ-induced diabetic animals requires at least 4 weeks, the mice were subjectedto MCAO 4 weeks after the STZ injection[35].

For the long-term insulin treatment, insulin (0.7 U/mouse/d, subcutaneously) was administrated daily from third day after the last STZ injection until the mice were sacrificed [36]. For the mice treated with insulin only at the onset of reperfusion, continuous administration of intermediate-acting insulinwas performed during the 24 h reperfusion.Mice were treated with three times insulin injection at 0h, 8h, and 16h reperfusion at a total dose of 0.7 U. Another group of STZ mice were treated with a low dose of insulin which was not able to correct the mice blood glucose level.Mice were injected with insulin at the onset of reperfusion at2 U/kg (about 0.04-0.05 U/mouse) [37,38]. The mice blood glucose in each group before and after ischemia was summarized in **Table 4-1**.

Isolation of cerebral microvessels

Since the BBB is formed at the level of cerebral microvessels, the microvessels were isolated from mice brain to analyze protein expression of HIF-1α, VEGF, and tight junction (TJ) proteins. At the indicated time points of reperfusion, mice were anesthetized and euthanized bydecapitation.The brain microvessels were isolated from freshly removed brains as described previously [39]. Briefly, brains were removed from the skull and immediately immersed in ice-cold PBS. Choroid plexus, meninges, cerebellum, and brain stem were removed and ipsilateral and contralateral hemispheres were separated and homogenized in isolation buffer separately. Then 26% dextran was added to the homogenate and samples were centrifuged (5800g, 4ºC) for 10 min. The supernatants were discarded; pellets were resuspended and filtered through a 100 µm mesh filter. The filtered homogenates were re-pelleted by centrifuge (1500 g, 10 min) and either smeared on microscope slide for immunostaining and flurescence microscopy or resuspended in lysis buffer for Western blotting analysis.

Table 4-1. Mice body weight and blood glucose levels.

	Weight (g)		Blood Glucose (mg/dl)	
	Baseline	Final	Before MCAO	After MCAO (admission)
Control	22.1±1.8	27.8±2.3	116±15	127±28
Diabetic	21.9±1.6	22.4±2.1	473±32*	465±38*
Long-term insulin	21.6±1.9	26.9±2.5	129±32	167±35
Insulin at reperfusion	22.5±1.5	23.1±1.7	489±43*	158±39
Low dose insulin	22.3±1.5	22.9±1.2	467±28*	423±37*

Data are expressed as means ± SD.

*$p < 0.05$ vs. control animals.

Western blotting

Cerebral microvessels isolated from contralateral and ipsilateral hemispheres were homogenized in an ice-cold RIPA buffer with 1μg/ml of a protease inhibitor cocktail (Thermo scientific, Rockford, IL, USA), and sonicated for 10 s. The homogenates were centrifuged at 14,000 rpm for 15 min at 4ºC; the supernatants were collected. Standard Western blotting procedures were followed as described previously[40]. The primary antibodies were rabbit anti-HIF-1α (Millipore, Billerica, MA), ZO-1 (40-2200, Invitrogen, Carlsbad, CA), occludin (33-1500, Invitrogen), claudin-5 (34-1600, Invitrogen), VEGF (sc-507, Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (sc-1616, Santa Cruz). The secondary antibody was goat anti-rabbit IgG-HRP (sc-2030, Santa Cruz Biotechnology, Santa Cruz, CA). β-actin was used as an internal control.

Immunohistochemical staining

Freshly isolated microvessels were spread onto microscope slide and heat-fixed for 10 min at 95ºC, followed by treatment with 4% paraformaldehyde for 10 min. Themicrovessels were then washed with PBS and permeabilized in PBS containing 0.1% Triton-X100 for 10 min. The nonspecific binding sites were blocked with PBS containing 0.05 % triton-X100 and 0.25 % BSA for 1 h. Primary ZO-1 antibody were diluted 1:100 in blocking buffer and incubated with sections overnight at 4°C [40]. Secondary antibody was donkey anti-rabbit Alexa 488 (Molecular Probes, Carlsbad, CA). Images were routinely captured with a Leica DMI 4000B fluorescent microscope. All immunohistochemical staining data were obtained in a blinded manner.

Evaluation of neurological deficits, infarct size and brain edema volume

At 24 h reperfusion,mice were evaluated for neurological deficits in a blinded fashion based on amodified scoring standard of Rogers *et al.*[41] with: 0=no deficit; 1=failure to extend right forepaw fully; 2=decreased grip of the right forelimb while tail gently pulled; 3=spontaneous movement in all directions, contralateral circling only if pulled by the tail; 4=circling or walking to the right; 5=walks only when

stimulated; 6=unresponsive to stimulation with a depressed level of consciousness. In addition, mortality was calculated at 24 h after MCAO/reperfusion.

Mice wereanesthetized and euthanized bydecapitation immediately after scoring.The brains were removed and sectioned into 2 mm slices. The slices were incubated in a 2% solution of TTC in PBS (pH 7.4) at 37°C for 30 min and fixed in 10% formalin. TTC staining has been widely used to reflect accurately the extent of irreversible ischemic damage in cerebral tissues in rodents [42]. TTC-stained brain sections were photographed using a digital camera (Powershot 400 digital camera, Canon) and analyzed using Image J for determination of infarct area and brain edema. To compensate for the effect of brain edema, the corrected infarct volume was calculated as previously described. The brain edema percentage was determined as 100% (ipsilateral volume–contralateral volume)/contralateral volume.

Determination of BBB permeability

BBB permeability was assessed by measuring extravasation of Evans blue (EB) dye. EB (2% in saline, 6 ml/kg body weight) was injected through tail vein right after reperfusion according to a previous report [43]. At the end of 24 h reperfusion, mice were transcardially perfused with saline under anesthesia until colorless perfusion fluid was obtained from the right atrium. After decapitation, the brain was removed and sectioned into 2 mm slices.The whole brain and brain sections were photographed using a digital camera (Powershot 400 digital camera, Canon). Then, the tissue from contralateral and ipsilateral hemispheres was separately weighed and soaked in 1 ml of 50% trichloroacetic acid solution. After homogenization and centrifugation, the extracted EB was diluted with ethanol (1:3); and fluorescence intensity was measured at 620 nm and 680 nm for excitation and emission, respectively, using a fluorescence reader. The tissue content of EB dye was quantified from a linear standard curve derived from known amounts of the dye and was expressed as micrograms per gram of tissues.

Statistical analysis

The results were presented as mean with a standard deviation of mean. Comparisons of Western blotting, EB leakage, edema formation, and infarct volumes were carried out by ANOVA test, followed by Tukey's correction (R 3.0.1). Neurological scores were compared using Kruskal-Wallis analysis followed by Bonferroni correction. A $p<0.05$ was considered statistically significant.

4.3 Results

Hyperglycemia enhanced endothelial HIF-1α expression in the ischemic brain throughout the reperfusion period.

To study whether HIF-1α was induced by hyperglycemia in ischemic brain, the control and STZ-induced diabetic mice were subjected to 90 min MCAO. Brain microvessels from ipsilateral brain were isolated at various time points after ischemia and homogenized to determine the expression of HIF-1α. As shown in **Fig. 4-1**, HIF-1αexpression was induced at 0 h after ischemic exposure, peaked at 6 h, and remained elevated for at least 24 h. The protein level of HIF-1αwas significantly increased at all time points (0h, 6h, 12 h, and 24 h) following MCAO in diabetic mice, suggesting that hyperglycemia enhanced cerebral endothelial HIF-1αexpression throughout the post-ischemic period. It is noteworthy that diabetic mice showed higher HIF-1αbasal level, which is in accordance with our previous study in *in vitro* cell culture. Because the maximal induction of HIF-1αby hyperglycemia appeared at 24 h reperfusion (44%), we chose 24 h as the time point for further study.

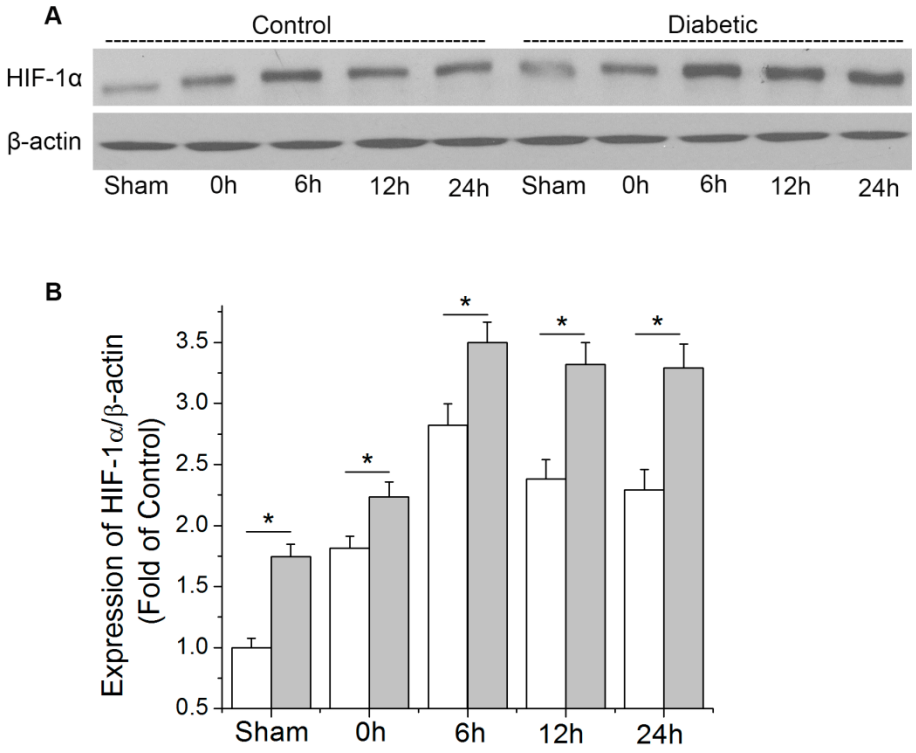

Fig.4-1 Effect of hyperglycemia on the expression of HIF-1α in ischemic brain microvessels. Control and diabetic mice were subjected to 90 min MCAO followed by reperfusion (0 h to 24 h). At 0 h, 6 h, 12 h, and 24 h post-ischemia, the protein levels of HIF-1α were analyzed by Western blotting in cerebral microvessels lysates from ipsilateral hemispheres of mice. **(A)**Representative Western blots of HIF-1α. **(B)** Quantification of the HIF-1α protein level. White bars, control ischemic brains; filled bars, diabetic ischemic brains. Values were normalized to β-actin and sham-operated control. Values are means ± SD, n = 5. $^{*}p < 0.05$ vs. control animals.

Diabetic mice demonstrated increased BBB permeability and worsened stroke outcomes after ischemia/reperfusion.

Cerebral ischemia leads to an impairment of BBB integrity and subsequent edema formation, which is greatly augmented by hyperglycemia. In this study, BBB permeability following 90 min ischemia and 24 h reperfusion was assessed using EB,a marker of albumin extravasation. EB leaked mainly into the ipsilateral hemisphere with only insignificant, low background level in the contralateral hemisphere of both control and diabetic mice, suggesting that 4-weeks diabetes does not elevate BBB permeability to macromolecular proteins such as albumin under basal condition. Diabetic mice showed remarkable increase of EB leakage in the ipsilateral hemisphere **(Fig 4-2 A and B)**, which indicated that hyperglycemia aggravated stroke-induced BBB breakdown. Consistent with the increased EB leakage, more pronounced brain edema was observed in the ipsilateral brain of diabetic mice **(Fig 4-2C)**. Because the BBB dysfunction after stroke may affect infarct expansion and the stroke outcome, we evaluated the infarct volume and functional recovery in control and diabetic mice. Diabetic mice demonstrated significantly increased lesion volume (82 mm^3, compared to 54 mm^3 in the control group) and more severe neurological impairments**(Fig 4-2 E and F)**.

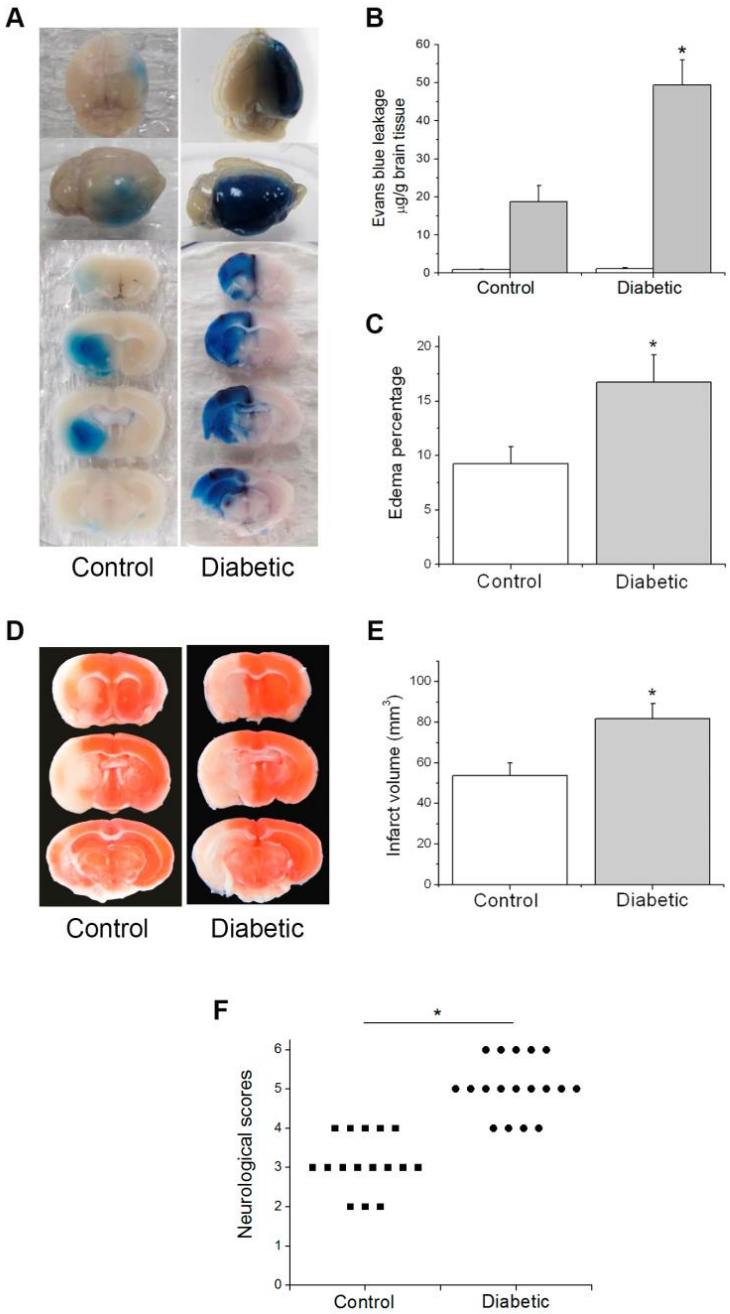

Fig.4-2 Effect of diabetesonischemia/reperfusion-induced EB extravasation, edema formation,brain infarction and neurological deficits.Control and diabetic mice were subjected to 90 min MCAO followed by 24 h reperfusion. **(A)** Representative images of EB extravasation in a whole brain and coronal sections. **(B)** Quantification of EB leakage in contralateral and ipsilateral hemispheres (n=5). White bars, contralateral hemisphere; filled bar, ipsilateral hemisphere. **(C)** Quantification of brain edema percentage (n=6 (control), 8 (diabetic)). **(D)**Representative TTC staining images of brain sections. **(E)** Quantification

of infarct volume estimated by TTC stained sections (n=6(control), 8(diabetic)). (**F**) Quantification ofneurological deficit scores rate(n= 16 (control), 18 (diabetic)). Values are means ± SD, $^{*}p < 0.05$ vs. control animals.

Hyperglycemia enhanced tight junction (TJ) protein degradation in ischemic brain microvessels

The TJs between endothelial cells of cerebral capillaries are critical in maintaining BBB integrity. Decreased TJ protein expression or variations in subcellular localization are associated with alterations in BBB permeability. TJs consist of the transmembrane proteins (e.g. claudins, occludin) and cytoplasmic accessory proteins (e.g. ZO-1) which connect the transmembrane proteins to the actin cytoskeleton. To determine whether exacerbated BBB disruption caused by hyperglycemia is associated with increased TJ proteins degradation following cerebral ischemia, we detected the protein level of ZO-1, occludin, and claudin-5 in the isolated brain microvessels. As shown in**Fig. 4-3**, consistent with the BBB permeability results, the level of all three proteins in the ischemic hemisphere of diabetic mice were significantly reduced comparing with that of control mice. Diabetic mice also showed lower level of occludin and ZO-1 in contralateral hemisphere. Furthermore, we studied the arrangement pattern of ZO-1 on brain microvessels by immunostaining. The immunostaining study revealed relatively continuous and linear staining of ZO-1 in the contralateral brain microvessels. Ischemia/reperfusion diminished staining intensity and disrupted the continuity of ZO-1. Moreover, the loss of ZO-1 was even more obvious in the diabetic ipsilateral brain microvessels. The results above demonstrated that diabetes further disrupted BBB by decreasing TJ proteins in ischemic brain.

HyperglycemiaupregulatedVEGF expression in the ischemic brain

VEGF is a potent vascular permeability enhancing factor and it is transcriptionally induced by HIF-1 in ischemic brain. It has been reported that VEGF induces brain vascular leakage in pathological conditions such as ischemia, possibly by decreasing ZO-1, occludin, and claudin-5 at the TJs. We found that VEGF expression in the contra- and ipsilateral brain of diabetic mice was significantly higher than that of control mice, respectively **(Fig. 4-4)**. The results demonstrated that diabetes enhanced expression of HIF-1 down-stream factor VEGF.

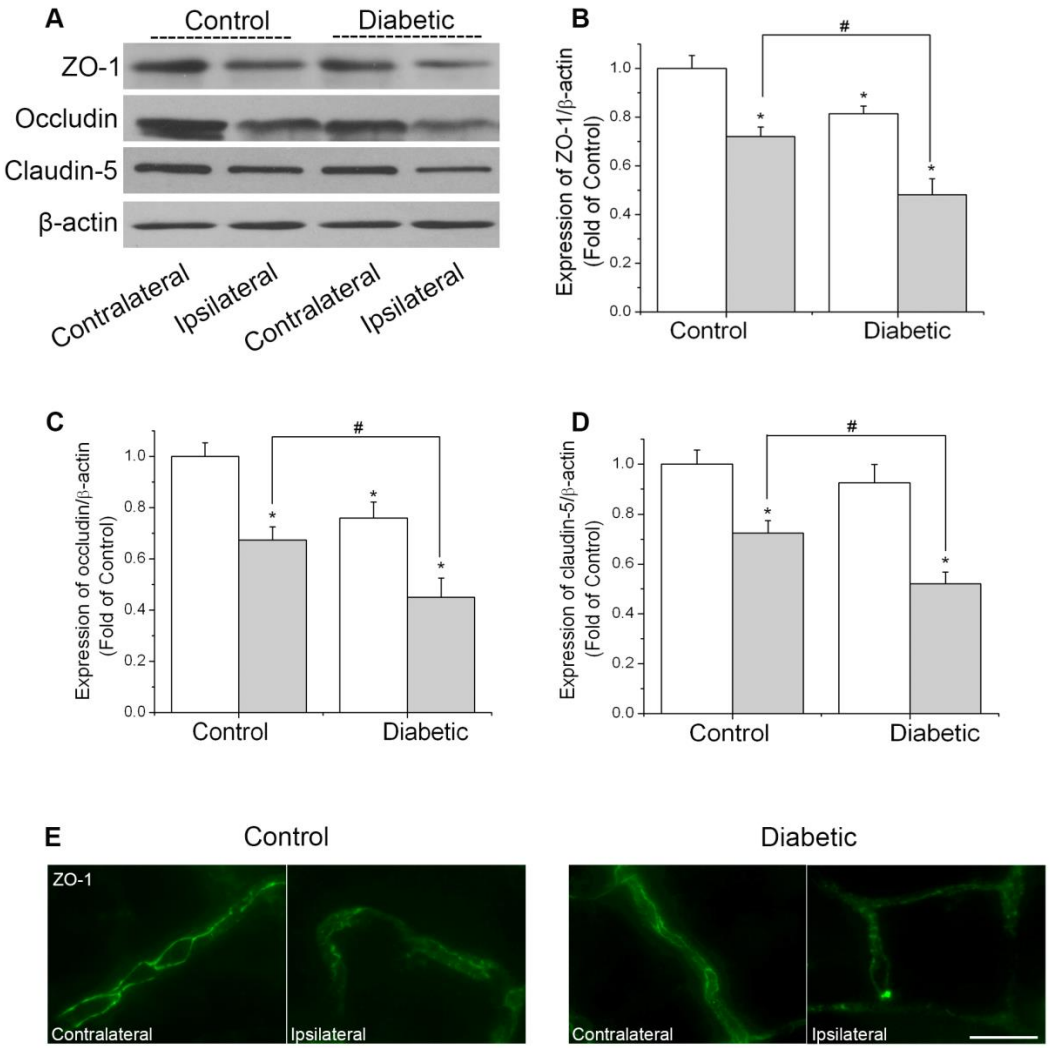

Fig.4-3 Effect of hyperglycemia on the expression of ZO-1, occludin, and claudin-5 in ischemic brain microvessels. The protein levels of ZO-1, occludin, and claudin-5 were analyzed in isolatedbrain microvesselsfrom contralateral and ipsilateral hemispheres of mice.**(A)** Representative Western blots of ZO-1, occludin, and claudin-5. **(B)** Quantification of the ZO-1 protein level. **(C)**Quantification of the occludin protein level.**(D)**Quantification of the claudin-5 protein level. White bars, contralateral hemispheres; filled bars, ipsilateral hemispheres. Values were normalized to β-actin and contralateral hemispheres of control animals. Values are means ± SD, n = 5. $^{*}p < 0.05$ vs. contralateral hemispheres from control animals.$^{\#}p < 0.05$ vs. ipsilateral hemispheres from control animals.**(E)** Immunostaining of ZO-1 on isolated brain microvessels. Scale bar, 20 μm.

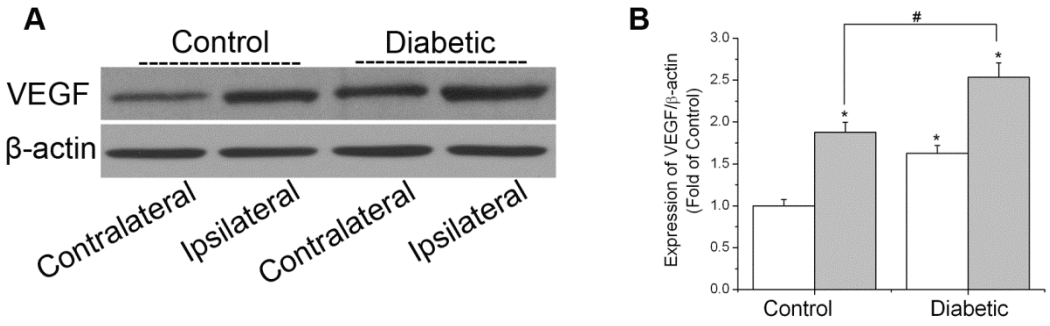

Fig. 4-4 Effect of hyperglycemia on the expression of VEGF in ischemic brain microvessels. The protein levels of VEGF were analyzed by Western blotting in cerebral microvessels lysates from contralateral and ipsilateral hemispheres of mice. **(A)**Representative Western blots of VEGF. **(B)**Quantification of the VEGF protein level. White bars, contralateral hemispheres; filled bars, ipsilateral hemispheres. Values were normalized to β-actin and contralateral hemispheres of control animals. Values are means ± SD, n = 5. $^{*}p < 0.05$ vs. contralateral hemispheres from control animals. $^{\#}p < 0.05$ vs. ipsilateral hemispheres from control animals.

Endothelial-specific HIF-1α knock-out reduced BBB permeability and brain infarction in diabetic mice

To investigate the specific role of HIF-1 in hyperglycemia-aggravated BBB disruption, we utilized endothelial-specific HIF-1α knock-out mice (HIF-1$\alpha^{\Delta/\Delta}$). The deletion was achieved by crossing mice bearing loxP-floxed HIF-1α alleles with transgenic mice expressing Cre recombinase under control of *Tek* promoter. Both wild-type and HIF-1α knock-out mice were rendered diabetes and subjected to 90 min MCAO and 24 h reperfusion. To evaluate the efficiency of HIF-1α ablation in endothelial cells of the HIF-1$\alpha^{\Delta/\Delta}$ mice, we detected HIF-1α expression in cerebral microvessel homogenates from contra- and ipsilateral hemisphere. As expected, post-ischemic accumulation of HIF-1α was significantly attenuated in the both control and diabetic HIF-1$\alpha^{\Delta/\Delta}$ mice **(Fig. 4-5A)**. We observed that the EB extravasation was significantly reduced in diabetic HIF-1$\alpha^{\Delta/\Delta}$ mice **(Fig. 4-5C)**, suggesting that inhibition of endothelialHIF-1α partially restored BBB integrity. Moreover, the brain infarct volume was reduced from 79 mm^3 in diabetic wild-type to 61 mm^3 in diabetic HIF-1$\alpha^{\Delta/\Delta}$ mice. It is of interest to point out that deletion of endothelial HIF-1α also attenuated BBB leakage and brain damage in non-diabetic mice, which is in line with previous reports that HIF-1 promotes BBB permeability in ischemic stroke [14]. In summary, we demonstrated that hyperglycemia-exacerbated BBB dysfunction was heavily dependent on its ability to activate HIF-1α since its detrimental effects were markedly alleviated in HIF-1$\alpha^{\Delta/\Delta}$ mice.

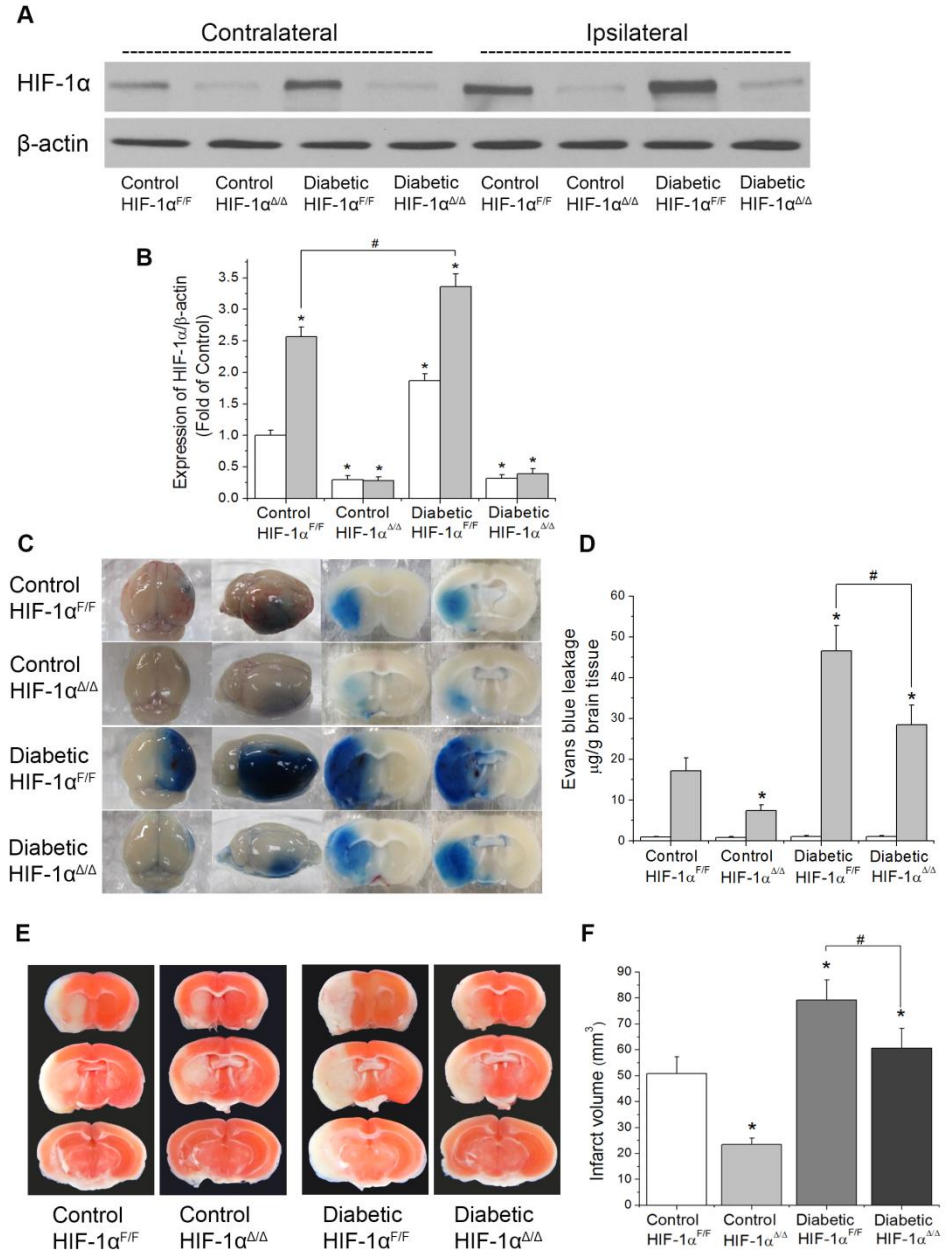

Fig. 4-5 Effect of endothelial-specific HIF-1α deficiency on the BBB permeability and brain infarction in control and diabetic mice. The protein levels of HIF-1α were analyzed by Western blotting in cerebral microvessel lysates from contralateral and ipsilateral hemispheres of mice.**(A)** Representative Western blots of HIF-1α. **(B)** Quantification of the HIF-1α protein level (n=5). Values were normalized to β-actin and contralateral hemispheres of control animals. $^{*}p< 0.05$ vs. contralateral hemispheres from control animals.$^{\#}p< 0.05$ vs. ipsilateral hemispheres from control animals.**(C)** Representative images of

EBextravasation in a whole brain and coronal sections. **(D)** Quantification of EB leakage in contralateral and ipsilateral hemispheres (n=5). White bars, contralateral hemisphere; filled bar, ipsilateral hemisphere. **(E)**Representative TTC staining images of brain sections. (**F**) Quantification of infarct volume estimated by TTC stained sections (n=4). Values are means ± SD, $^{*}p < 0.05$ vs. control animals.

Normalizing mice blood glucose by insulin abolished HIF-1α upregulation in diabetic mice

We normalized the diabetic mice blood glucose by long-term insulin administration to verify that the upregulation of HIF-1α and the exacerbated BBB disruption observed in diabetic mice were due to hyperglycemia.As expected, the long-term insulin-treated diabetic mice showed similar HIF-1α protein level in both contra-and ipsilateral hemisphere compared with the non-diabetic control. In addition, a group of diabetic mice only received insulin treatment at the time of reperfusion was included to further explore the effects of hyperglycemia on HIF-1α expression and BBB disruption at different stages of ischemia. We foundnormalizing the blood glucose during post-ischemic reperfusion did not significantly affect the HIF-1α expression in contralateral hemisphere compared with the diabetic mice. However, the HIF-1α accumulation in the ipsilateral hemisphere was decreased **(Fig. 4-6A)**, suggesting that hyperglycemia-induced HIF-1αupregulation in ischemic brain was mainly due to the high blood glucose at reperfusion rather than pre-ischemia. Both the long-term or reperfusion insulin administration attenuated EB extravasation and brain infarction in diabetic mice **(Fig. 4-6 D-G)**, which indicates that the adverse effects of hyperglycemia in stroke largely results from high blood glucose in reperfusion. It was unclear whether insulin protected ischemic brain by blood glucose correction or by its direct interaction with brain tissue. To differentiate the protective mechanism of insulin, a group of diabetic mice were treated with a low dose of insulin which was not able to correct the mice blood glucose levels. The low dose insulin did not affect HIF-1α expression, BBB permeability or brain injury comparing with the non-treated diabetic mice**(Fig. 4-7)**, indicating that the effects of insulin are predominantly via alteration in blood glucose rather than activation of insulin receptors signaling pathways.

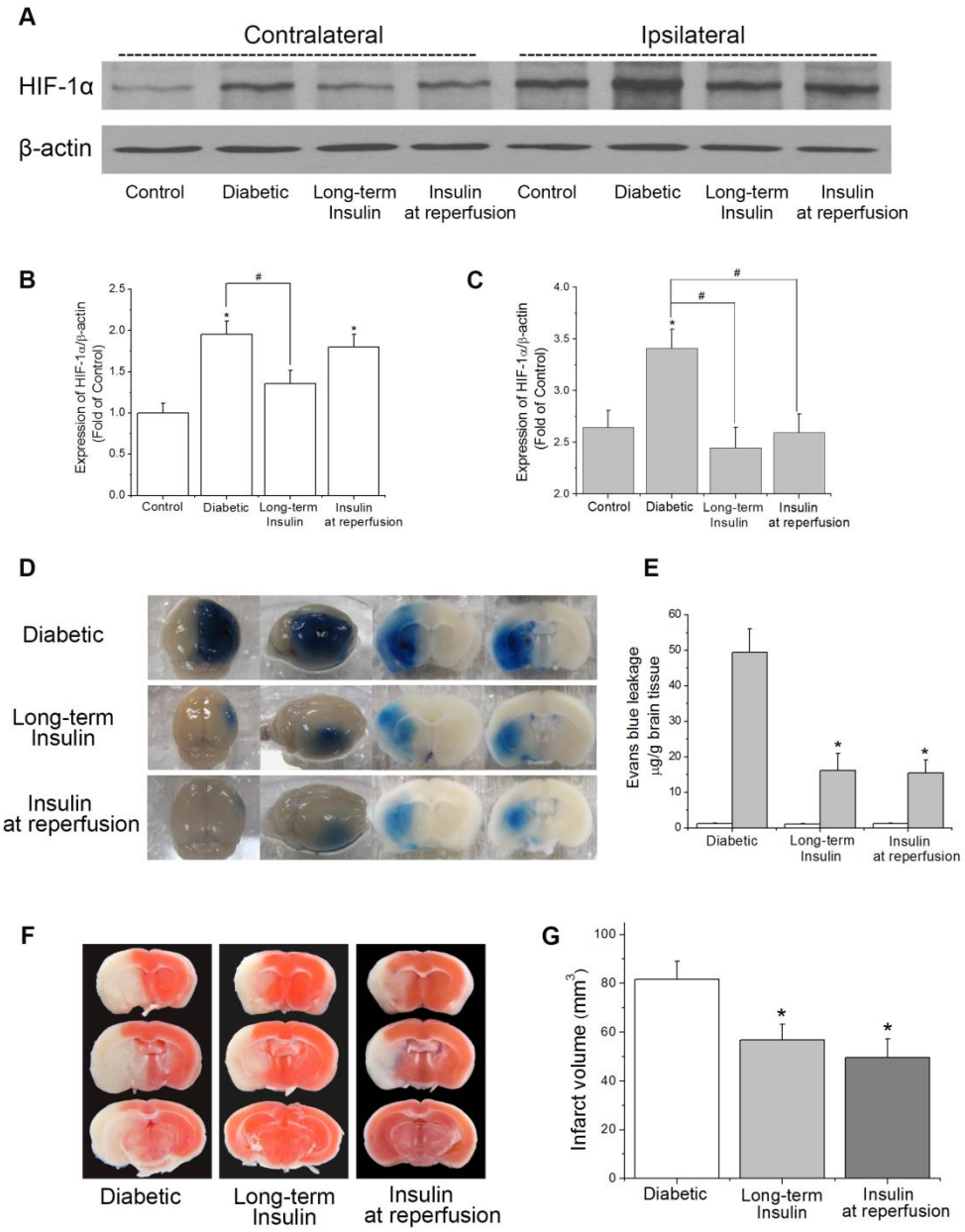

Fig. 4-6 Effect of normalizing blood glucose on HIF-1α expression, BBB permeability and brain infarction in diabetic mice. Long term insulin: mice were treated with insulin (0.7 U) daily from the 3rd day after the diabetes induction; Insulin at reperfusion:mice were administrated with continuous insulin (0.7 U) during the 24 h reperfusion.The protein level of HIF-1α was analyzed by Western blotting in cerebral microvessels lysates from contralateral and ipsilateral hemispheres of mice.**(A)** Representative

Western blots of HIF-1α. **(B, C)** Quantification of the HIF-1α protein level in contralateral hemispheres (white bars) and ipsilateral hemispheres (filled bars) (n=5). Values were normalized to β-actin and contralateral hemispheres of control animals. $^{*}p < 0.05$ vs. control animals. $^{\#}p < 0.05$ vs. diabetic animals. (**D**) Representative images of EB extravasation in a whole brain and coronal sections. (**E**) Quantification of EB leakage in contralateral and ipsilateral hemispheres (n=5). White bars, contralateral hemisphere; filled bar, ipsilateral hemisphere. (**F**) Representative TTC staining images of brain sections. (**G**) Quantification of infarct volume estimated by TTC stained sections (n=8 (diabetic), 6 (long-term insulin), 5 (insulin at reperfusion)). Values are means ± SD, $^{*}p < 0.05$ vs. diabetic animals.

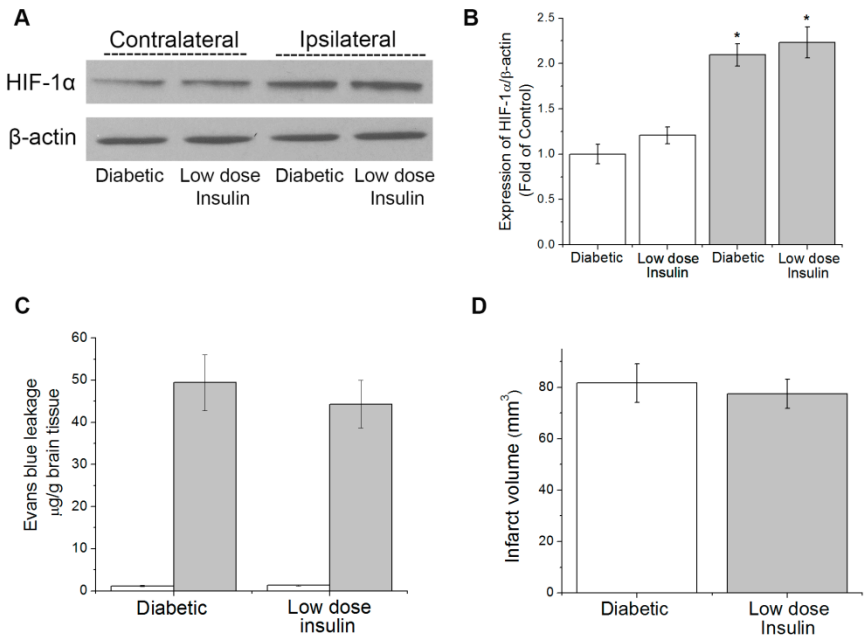

Fig. 4-7 Effect of low dose insulin on HIF-1α expression, BBB permeability and brain infarction in diabetic mice. Mice were treated with low dose insulin(2 U/kg) at the onset of reperfusion.The protein levels of HIF-1α were analyzed by Western blotting in cerebral microvessels lysates from contralateral and ipsilateral hemispheres of mice.**(A)** Representative Western blots of HIF-1α. **(B)** Quantification of the HIF-1α protein level in contralateral hemispheres (white bars) and ipsilateral hemispheres (filled bars) (n=5 (diabetic), 3 (low dose insulin)). Values were normalized to β-actin and contralateral hemispheres of diabetic animals. $^{*}p < 0.05$ vs.contralateral of diabetic animals.**(C)** Quantification of EB leakage in contralateral and ipsilateral hemispheres (n=5 (diabetic), 3 (low dose insulin)). White bars, contralateral hemisphere; filled bar, ipsilateral hemisphere. **(D)**Quantification of infarct volume estimated by TTC stained sections (n=8 (diabetic), 3 (low dose insulin)). Values are means ± SD, $^{*}p < 0.05$ vs. diabetic animals.

4.4 Discussion

This study provides several major findings on the effect of hyperglycemia on neurovascular and functional outcomes of stroke. First, we demonstrated that STZ-induced hyperglycemia enhanced the expression of HIF-1α and VEGF in ischemic brain microvessels, which was accompanied by increased BBB disruption, larger infarct volumes, more severe edema formation, and worsened neurological deficits. Second, the detrimental effects of hyperglycemia on cerebral vascular damage was partially reversed by specific inhibition of endothelial HIF-1α, suggesting that activation of HIF-1α is an underlying mechanism by which BBB permeability was increased. Lastly, both chronic and acute glycemic control abolished HIF-1α upregulation in the ischemic brain of diabetic mice and lessened neurovascular injury in diabetes.

Many lines of evidence have shown that hyperglycemia induces HIF-1α expression. In STZ-induced diabetic animal models, HIF-1α was found upregulated in hearts[44], peripheral nerves[45], pancreas [46], retina [27,28]and kidney glomeruli[47]. Studies based on samples from diabetic patients have reported increased HIF-1α levels in human preretinal membranes [48]and vitreous fluid [49]. Previous study from our lab has revealed that high glucose activates HIF-1α pathway in brain vascular endothelial cell culture [26]and in primary neurons exposed to hypoxia[50]. Our present report is first to demonstrate that HIF-1α is upregulated in the brain microvessels isolated from both contralateral and ipsilateral hemispheres in diabetic mice subjected to MCAO. The mechanism of high glucose-induced HIF-1α expression after ischemia was not clear. It is well established that HIF-1α subunit is stabilized in hypoxia and rapidly degraded under normoxic conditions. In normoxia, HIF-1α is continuously hydroxylated by prolyl hydroxylases (PHDs)[51]. The von Hippel-Lindau (pVHL) tumor suppressor protein binds to hydroxylated HIF-1α, thus targeting HIF-1α for ubiquitination and degradation by 26S proteasome[52,53]. The PHD enzymes require oxygen as cofactors[54,55]. Under hypoxic conditions, HIF-1α is accumulated because of decreased PHDs activities[10]. Besides hypoxia, PHDs activities can also be influenced by other factors. Some of these factors are involved in diabetes, e.g., advanced glycosylation end products [56]and Krebs cycle intermediates. In fact, it has been recently demonstrated that glucose metabolites fumarate and

succinate can inhibit PHDs with consequent stabilization of HIF-1α[57-61]. Diabetes is associated with an increased in the intracellular fumarate and succinate concentration[62]. Therefore, diabetes may stabilize HIF-1α by inhibiting PHDs activity. Moreover, high glucose has been shown to induce HIF-1α transcription via a carbohydrate response element binding protein (ChREBP)[47]. ChREBP binds to a specific target site in HIF-1α promoter and increases HIF-1α mRNA level, resulting in an enhanced expression of a broad range of HIF-1 target genes including VEGF. Future investigation is needed to elucidate the molecular mechanism responsible for the upregulation of HIF-1α in brain endothelial cells.

HIF-1's role in cerebral ischemia is arguable. On the one hand, HIF-1 regulates a broad range of genes that facilitate cellular adaption to low oxygen conditions[63]. Its targets include the genes that code for molecules participating in angiogenesis, erythropoiesis, energy metabolism, and cell proliferation[64,65]. Each one of these functions potentially contributes to neuronal survival under ischemic conditions. Indeed, HIF-1 has been reported to protect neurons against cerebral ischemic damage[66-68]. Furthermore, neuron-specific knockdown of HIF-1α was demonstrated to aggravate tissue damage and reduce survival rate of mice subjected to MCAO[69]. On the other hand, several groups have reported opposite effects of HIF-1 in cerebral ischemia, showing that HIF-1 is a likely mediator of BBB disruption. For example, Yeh *et al*. demonstrated that HIF-1α inhibitor YC-1was able to prevent ischemia/reperfusion-induced BBB hyperpermeability in both rat brain endothelial cell culture and in *in vivo* model[14]. Results by Chen *et al.* showed that early inhibition of HIF-1α by 2-methoxyestradiol (2ME2) provided neuroprotection after neonatal hypoxia-ischemia by preserving BBB integrity and attenuating brain edema. Moreover, HIF-1α upregulation by dimethyloxaylglycine (DMOG) increased the permeability of BBB and brain edema[15]. The discrepancy of these observations may be partly explained by distinctive effects of HIF-1 down-stream targets in different cell types. For example, the angiogenic factor VEGF is the best defined HIF-1 target protein in vascular biology[70]. It has been reported that VEGF mediates neuroprotection in cerebral ischemia by its angiogenesis and neurotropic effects[71-74]. However, VEGF is a strong inducer of vascular permeability, and it plays a critical role in causing BBB disruption and cerebral edema[22-25,75]. Experimental evidence has shown that VEGF down-regulated TJ proteins claudin-5[76], occludin[76]and ZO-

1[75,77] expression in brain microvasculature. In this study, we observed significant increase in both HIF-1α and VEGF expression in diabetic brain microvessels, which correlated with aggravated BBB leakage after stroke. This result supports the concept that enhanced HIF-1α activity in diabetic mice brain promotes BBB damage after ischemia, possibly through upregulating VEGF expression. To further evaluating the contribution of endothelial HIF-1α signaling in BBB disruption, we utilized an endothelial specific HIF-1α knock-out mouse model. We found that inhibition of HIF-1α attenuated BBB breakdown and brain infarction in both normoglycemic and hyperglycemic mice after MCAO/reperfusion, indicating that endothelial HIF-1α is an important mediator of BBB disruption in ischemic stroke.

Although genetic depletion of endothelial HIF-1α reduced BBB leakage and brain infarct volume in diabetic mice, the BBB permeability in diabetic HIF-1$\alpha^{\Delta/\Delta}$ mice was still significantly higher than the non-diabetic mice even though HIF-1α accumulation was largely diminished. These results suggested that there might be other pathways that are probably mediated independently of HIF-1α, and were involved in BBB disruption in diabetic stroke as well. For example, the activation of protein kinase C (PKC) is implicated in cerebral microvascular dysfunction in hyperglycemic stroke[78-80]. PKC activity is rapidly increased in endothelium in response to hyperglycemia due to de novo synthesis of doacyglycerol, the primary activator of PKC[81,82]. PKC activation can directly affect BBB permeability through its ability to phosphorylate ZO-1 and disrupt TJs [83,84]as well as promote excessive superoxide production through NADPH oxidase which leads to endothelial barrier dysfunction [78,79]. In a recent study, Cipolla *et al.* has demonstrated that the inhibition of PKC-β reversed the enhanced BBB permeability and prevented edema formation in STZ-induce diabetic rats subjected to MCAO[79]. Another important factor in vascular damage is the post-stroke inflammation. Hyperglycemia is known to be associated with increased expression of several pro-inflammatory transcription factors, such as NF-κB. These factors regulate the inflammatory responses by increasing the pro-inflammatory cytokines and promoting the adhesion of inflammatory cells to the vascular endothelium, which leads to BBB breakdown[5,6,85]. Moreover, our experiments only inhibited the HIF-1 signaling in endothelial cells of HIF-1$\alpha^{\Delta/\Delta}$ mice. In ischemic brain, VEGF is also expressed by reactive astrocytes and astrocytic end-feet lie in close proximity to microvascular

endothelium[76]. VEGF secreted from astrocytes may interact with VEGF receptors on the ischemic vessels and induce BBB leakage in a paracrine manner[86].

Numerous studies have reported increased brain injury in hyperglycemic animals after reperfusion. However, in the animals without reperfusion, hyperglycemia seemed to have no adverse effect and might even have been beneficial[87-89]. The question of when hyperglycemia has its most deleterious effects is of great clinical importance since it provides guidelines for optimal glycemic control in acute ischemic stroke. We tested the hypothesis that the adverse effect of hyperglycemia may become most evident during reperfusion. The current study showed that both chronic and acute insulin treatments suppressed HIF-1α expression in ischemic brain microvessels of diabetic mice, indicating that hyperglycemia-induced HIF-1α accumulation was mainly due to the high blood glucose during reperfusion. Moreover, acute glycemic control at post-ischemic reperfusion in previously untreated diabetic subjects inhibited hyperglycemia-induced BBB permeability and reduced brain injury. In conclusion, the results suggested that insulin treatment in reperfusion could abolish HIF-1α upregulation and ameliorate BBB disruption. Insulin is the most commonly used agent to regulate blood glucose and has been shown to reduce ischemic brain damage in animal models[90-93]. It is unclear whether its protective effects are due to blood glucose correction or due to its direct interaction with brain tissue. To differentiate the protective mechanism of insulin, we administrated STZ mice with a low dose of insulin (2U/kg) which was not able to correct the mice blood glucose levels. The dose was chosen based on previous studies which showed that insulin at 2U/kg was able to activate insulin receptor signaling pathway in the animal brain[94]. We found that the low dose insulin did not affect HIF-1α expression, BBB permeability or brain injury comparing with the non-treated diabetic mice, indicating that the effects of insulin are predominantly via alteration in blood glucose rather than activation of insulin receptors signaling pathways. Examining the levels of the phosphorylation of insulin receptors and the key down-stream kinases in the animal brain will surely help to validate the activation of insulin receptor signaling pathway and further differentiate the mechanism of insulin's effects. A previous study showed that most of insulin's protective effect was abolished by co-administration of a glucose infusion in a MCAO rat

model [93], which is in line with our observations that insulin benefits ischemic stroke by reducing the blood glucose. Although it is still debatable whether glucose-lowering treatment improves clinical outcome in patients with ischemic stroke, our study on mouse model suggested a favorable outcome of glycemic control given at the onset of reperfusion, which provides additional rationale for glucose control in hyperglycemic stroke patients.

There are several limitations that need to be recognized. First, this study used only male and young animals. Second, we used STZ-induced type I diabetic mice model with severe hyperglycemia (around 450-500 mg/dl). However, clinical evidence suggests that blood glucose levels in stroke patients present with hyperglycemia at admission range between 140-200 mg/dl[4,95,96]. A mild-moderate hyperglycemia animal model will more closely resemble the clinical situation. In addition, we only examined the BBB permeability and neurological outcomes at 24 h reperfusion. Additional studies are warranted under conditions of a longer post-ischemia period which will provide long-term functional recovery information in stroke animals. Third, the ischemia was induced by the intraluminal suture occlusion of MCA mouse models. Unlike clinical stroke, there is no true clot in this model and thus no platelet or thrombus breakdown products released into the post-ischemic tissue, which could modulate reperfusion-induced BBB damage. In the future studies, a humanized thromboembolic clot model can be used to better recapitulate the clinical scenario where the clot persists with slow spontaneous reperfusion.

In the present study, we found that hyperglycemia significantly increased HIF-1α and its downstream factor VEGF expression in the ischemic brain microvessels after MCAO. The enhanced HIF-1 expression might represent an important mechanism for aggravated ischemic damage, particularly BBB disruption, during hyperglycemic stroke. Our study suggested that targeting HIF-1α may provide a novel therapeutic option for BBB protection in ischemic stroke patients with admission hyperglycemia.

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CHAPTER 5 Conclusions and Outlooks

In summary, this dissertation systemically explored two aspects of HIF-1 in ischemic stroke. First, neuronal expression of HIF-1α and its target factors EPO and GLUT-3 was upregulated inthe ischemic brain of rodents pretreated withantioxidant NAC, which was correlated with reduction in brain infarct and neurological deficits. Suppressing HIF-1 activity by HIF-1α inhibitors or specific knock-out of neuronal HIF-1α abolished NAC's neuroprotective effects. NAC stabilized HIF-1α by enhancing its interaction with Hsp90. The study suggests that neuronal HIF-1 is an important mediator of NAC-induced neuroprotection in experimental ischemic stroke models. Indeed, many protective agents used to treat ischemic stroke in pre-clinical research have been reported to act via HIF-1 induction. HIF-1 activation can induce multiple target factors to minimize damage and promote repair. From a therapeutic stand-point, this is attractive, since clinical trials with agents targeting single aspect of cell death in ischemic cascade have had disappointing results. In general, the therapeutic potential of development and use of small molecule HIF-1α stabilizers (PHD inhibitors) to improve cell survival after injury is gaining popularity in many different fields. Such drugs could provide significant protection for a variety of different cells during injury and pathological situations such as stroke. However, a major caveat, clear from the study outlined above, is that not all consequences of HIF-1 activation are beneficial and some can even be deleterious.

A second focus of this dissertation explored the mechanism of hyperglycemia-aggravated BBB disruption in ischemic stroke. As shown in Chapter 4, hyperglycemia induced higher expression of HIF-1α and its down-stream VEGF in brain microvessels after MCAO/reperfusion, which was in correlation with exacerbated BBB leakage, TJ disruption and increased brain damage. Specific inhibition of endothelial HIF-1α ameliorated BBB breakdown and brain infarction in diabetic animals. The study suggests that HIF-1 plays an important role in hyperglycemia-induced exacerbation of BBB disruption in ischemic stroke. Since this study shows that HIF-1 may disturb BBB function, the applications of agents that induce HIF-1α must be treated with caution. Enhanced edema and influx of other blood-borne

molecules as a result of increased BBB permeability will increase intra-cranial pressure and the transport of potentially detrimental substances into brain parenchyma. Additionally, the doses applied and the chemical toxicity of the PHDs inhibitors could also significantly modify cellular responses and contribute to damage. Thus the adverse effects of disrupted BBB integrity must be carefully assessed when administrating HIF-1 stabilizing drugs. It must also be emphasized, however, the timing of the stabilization of HIF-1 and the regions being targeted will likely be instrumental in obtaining a positive outcome during and after treatment and must also be taken into consideration.

Overall considering its multifunctional roles, differential effects on different cell types and double-edged sword mode of action, it seems unlikely that that HIF-1α stabilization can be a magic solution to improving brain cell survival after injury. Future studies may focus on combination of thrombolytic treatment and HIF-1 stabilization. Additionally, since therapeutic activation of HIF-1 is likely to mimic the effects of hypoxia preconditioning, stabilization of HIF-1α in pretreatment may prove particularly useful in situations in which cerebral ischemia can be anticipated such as brain or heart surgery, cardiac arrest, and respiratory distress. Further studies to determine the full extent of HIF-1's beneficial and detrimental effects will undoubtedly provide better clarification of its therapeutic potential.

Although the study above indicates neuron-specific HIF-1α knock-out is detrimental while silencing HIF-1α in endothelial cells is protective via inhibiting BBB leakage, we do not exclude the possibility that HIF-1 can also play a negative role in neurons and a positive role in endothelial cells. In fact, HIF-1-mediated VEGF expression in endothelial cells is important for angiogenesis in the late recovery process. In addition, early upregulation of HIF-1 induces pro-apoptotic genes expression in neurons. Generally, in the view of risk of edema formation and deterioration of the neurological state, such risks are particularly relevant in the very early stroke phase. Since it is technically impossible to specific delivery HIF-1 stabilizer or inhibitor to a certain cell type, understanding the timing of HIF-1's effect is very important for developing novel therapeutics. Also, it is helpful to conduct an in-depth exploration and validation of the molecular targets of HIF-1, including temporal and cell-specific expression and function profile in relation to the pathophysiology of stroke. Development of therapeutic

strategies targeting HIF-1 must take both the positive and negative aspects of the stroke response into consideration, to ensure that the treatments are administrated under the conditions that are most appropriate and that will produce greatest benefit.

In the future, it will be important to explore the effect of inducing or inhibiting HIF-1α at different time point after ischemia. The current study is performed in a way in favor of a treatment effect by administrating NAC before experimental injury occurs. Moreover, by using transgenic mice, HIF-1α is also inhibited before the start of ischemia. This may be appropriate to prove HIF-1's differential role in different cell types, but the clinical situation is not sufficiently reflected. In order to test the whether HIF-1 stabilization may work in human clinical situation without inducing significant BBB leakage, the test HIF-1α stabilizer can be administrated at both early phase (less that 3 h after ischemia) and late phase (more than 12 h). In addition, longer follow-up period for assessment of the outcome will be used instead of 24 h. Apart from measuring histological infarct volume,the functional assessmentwill be evaluated by animal behavior studies. Furthermore, conflicting observations on how ROS modulate HIF-1α have been reported. Our study showed that antioxidant may stabilize HIF-1α in ischemic brain by enhancing its interaction with Hsp90. However, there is another school of thought that an increase in ROS stabilizes HIF-1α by inhibiting PHDs activity. Due to the controversial results, further study is needed to elucidate the exact mechanism of HIF-1α degradation/accumulation induced by ROS generated in ischemic brain.