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Novel Therapeutic Approaches for Ischemic Heart and Brain Injury: Modulation of Toll-Like Receptor-Mediated Signaling Pathways and PI3K/Akt Signaling

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by

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ABSTRACT

Novel Therapeutic Approaches for Ischemic Heart and Brain Injury: Modulation of Toll-Like Receptor-Mediated Signaling Pathways and PI3K/Akt Signaling

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Innate immune and inflammatory responses contribute to myocardial and cerebral ischemia/reperfusion (I/R) injury. Toll-like receptors (TLRs) play a critical role in the induction of innate immune and inflammatory responses via activation of nuclear factor kappa B (NF-κB). We have shown that activation of NF-κB contributes to myocardial and cerebral I/R injury. Indeed, inhibition of TLR4-mediated NF-κB activation significantly decreased myocardial and cerebral I/R injury via activation of PI3K/Akt signaling. PI3K/Akt signaling is an important pathway in regulating cellular survival and inflammatory responses. Therefore, an important question is how to differentially modulate PI3K/Akt signaling and TLR/NF-κB-mediated signaling pathway during I/R injury?

We demonstrated that pretreatment of mice with Pam3CSK4, a specific TLR2 ligand, significantly decreased cerebral I/R injury and improved neuronal functional recovery. Importantly, therapeutic administration of Pam3CSK4 also markedly decreased cerebral I/R injury. The mechanisms involved suppression of NF-κB binding activity and activation of PI3K/Akt signaling. We also demonstrated that CpG-ODN, a specific TLR9 ligand, induced protection against cerebral I/R injury via activation of PI3K/Akt signaling. Our findings were consistent with our previous reports showing that administration of Pam3CSK4 or CpG-ODN

protected against myocardial I/R injury via a PI3K/Akt-dependent mechanism. In addition, we demonstrated for the first time that TLR3 located in endosomes played a deleterious role in myocardial I/R injury via activation of NF-κB.

To investigate how to activate PI3K/Akt signaling during I/R injury, we examined the role of microRNA (miRs) in regulating PI3K/Akt signaling during myocardial ischemic injury. We discovered that Pam3CSK4 or CpG-ODN treatment significantly increased the expression of miR-130a in the myocardium, while myocardial infarction markedly decreased the levels of miR-130a in the myocardium. The data indicated that miR-130a served a protective role in myocardial ischemic injury. Indeed, we demonstrated for the first time that increased expression of miR-130a significantly attenuated cardiac dysfunction and promoted angiogenesis after myocardial infarction. The mechanisms involved activation of PI3K/Akt signaling via targeting PTEN expression by microRNA-130a.

This dissertation discovers novel mechanisms of cerebral and myocardial ischemic injury and provides solid basis for developing new approaches for the treatment and management of stroke and heart attack patients.

DEDICATION

This manuscript is dedicated to my family members, my father, my mother, and my older sister. They have been my main source of support and encouragement, and I am forever grateful for their love, sacrifice, and patience through my graduate studies.

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CHAPTER 1

INTRODUCTION

Cardiovascular Disease

Cardiovascular disease (CVD) is a group of diseases that involve the heart and blood vessels, such as coronary heart disease and cerebrovascular disease. According to the report from World Health Organization (WHO), cardiovascular disease is the leading cause of death worldwide. In 2008 it is estimated that 17.3 million people died from CVD, accounting for 30% of all global death. The primary cause of death from CVD is from coronary artery disease and stroke.

Coronary artery disease is the leading cause of death worldwide and the number one killer in the United State. Heart attack is the main form of coronary artery disease. According to the American Heart Association (AHA), an estimated 915,000 heart attacks occur each year and approximately every 1 minute someone will die of heart attack in the United States (Go and others 2013). Currently available treatments for the heart attack patients include thrombolysis to dissolve the blood clot, angioplasty to open the blockage in the coronary artery, and bypass surgery to restore the blood supply. However, some patients are not amenable to these conventional treatments, thus a considerable amount of attention has been directed towards exploring alternative therapies.

Stroke is the third leading cause of death in the United State. An estimated 795,000 Americans suffer a stroke each year and approximately every 4 minutes someone will die of stroke in the United States (Go and others 2013). Stroke can be divided into 2 major types of

stroke, i.e. ischemic stroke and hemorrhagic stroke. Ischemic stroke accounts for 87% of all stroke cases (Go and others 2013). So far, tissue plasminogen activator (tPA) is the only treatment approved by the FDA for the ischemic stroke (Lansberg and others 2009). Therefore, it is urgent to explore new and novel therapeutic approaches for the treatment and management of ischemic stroke.

Overview of Ischemia/Reperfusion Injury

Ischemia/reperfusion (I/R) injury is a major contributor to adverse clinical outcomes of heart attack or ischemic stroke. Ischemia/reperfusion injury evokes a cascade of pathophysiological processes and results in severe tissue damage. When the blood supplying the tissues is blocked, ischemic injury occurs and leads to a reduction of oxygen and glucose levels (Taoufik and Probert 2008). Reperfusion injury is caused by the restoration of blood flow to the ischemic tissue. Although reperfusion restores blood flow to the ischemic tissue and results in infarct size reduction, it can lead to the secondary tissue injury and influence the outcomes of the patients (Yellon and Hausenloy 2007; Frank and others 2012; Hausenloy and Yellon 2013). Ischemia/reperfusion injury is a complex process that involves not only the significant alterations of intracellular compartments but also activation of inflammatory responses (Figure 1.1). As a consequence, ischemia and reperfusion leads to the activation of cell death programs, including apoptosis, autophagy, and necrosis. Due to the absence of oxygen and glucose to the tissue, energy-dependent processes are compromised and initiate a cascade of events that culminate in cell death. The function of membrane ion channels depends on energy in the form of ATP. ATP levels in the cells are not adequate after ischemia. Thus, the membrane ion channels are not capable of maintaining ionic gradients across the membrane, leading to an increase in

cytoplasmic calcium and sodium concentration (Levitsky 2006). As a result, calcium overload causes the opening of mitochondrial permeability transition pore (mPTP) and the collapse of mitochondrial membrane potential, causing membrane depolarization (Bano and Nicotera 2007; Garcia-Dorado and others 2012). Meanwhile, the accumulation of intracellular sodium induces an increase in the permeability of the plasma membrane. During reperfusion, with the return of oxygen, there is an increase in reactive oxygen species (ROS) produced (Sanderson and others 2013). Additionally, the large amount of intracellular calcium further triggers the production of ROS (Kaminski and others 2002). The excessive production of ROS induces DNA cleavage (Cooke and others 2003), complement activation (Yasojima and others 1998; Gorsuch and others 2012), and the opening of mPTP (Halestrap and others 2004). The opening of mPTP in turn results in the release of cytochrome C and other apoptotic molecules that initiate cell apoptosis (Halestrap and others 2004). Moreover, the excessive amounts of ROS conversely induce the release of calcium from endoplasmic reticulum (ER) to the cytoplasm (Camello-Almaraz and others 2006). The resultant accumulation of intracellular calcium and excessive amount of ROS propagate to neighboring cells through cell junction. Consequently, the signals for cellular injury and death are passed from cell to cell. The increased intracellular calcium and the excessive production of ROS together participate in the activation of inflammatory cascades, such as tumor necrosis factor- α (TNF-α), interleukin-1β (IL-1β), and NF-κB. NF-κB is an important transcription factor that mediates the expression of proinflammatory cytokine and chemokines (Deng and others 2009). The proinflammatory cytokines/cheomkines are thought to modulate the expression of cell adhesion molecules, such as ICAM-1 and VCAM-1 (Szmitko and others 2003) that are upregulated and mediate the infiltration of inflammatory cells in response to I/R injury (Smith and others 1991; Entman and others 1992). As a result, the

recruited inflammatory cells, such as neutrophils, migrate into the site of injury and further release proinflammatory cytokines and chemokines (Jordan and others 1999; Kaminski and others 2002; Stevens and others 2002; Jin and others 2010). Eventually, it promotes both local and remote tissue damage via the release of numerous inflammatory mediators.

As Figure 1.1 shows, NF-κB-mediated inflammatory responses play a central role in mediating cell death in the pathogenesis of I/R injury. Toll-like receptors are well known to play a critical role in mediating innate immune and inflammatory responses via activation of NF-κB. A large body of evidence shows that NF-κB is activated following I/R injury. Inhibition of NF-κB activity ameliorates the infarct size and neurodeficits after cerebral ischemia (Desai and others 2010; Jiang and others 2010). NF-κB is also activated following myocardial ischemia (Li and others 1999). Evidence has provided that prolonged activation of NF-κB produces detrimental consequences after heart ischemia and promotes heart failure by triggering the expression of proinflammatory cytokines (Gordon and others 2011). Prabhu and colleagues reported that chronic activation of the p65 subunit of NF-κB compromises cardiac remodeling, promotes apoptosis, and enhances endoplasmic reticulum stress after myocardial infarction (Hamid and others 2011). Blockade of NF-κB attenuates cardiac dysfunction and improves survival after myocardial infarction (Morishita and others 1997; Kawano and others 2006). Inhibition of NF-κB translocation by IκB phosphorylation blockade induces a significant reduction in infarct size after myocardial I/R (Onai and others 2004). Importantly, our laboratory found that inhibition of TLR4-mediated NF-κB activation significantly decreases myocardial and cerebral I/R injury via activation of PI3K/Akt signaling (Hua, Ma, and others 2007; Hua, Ha, and others 2007), thus PI3K/Akt signaling serves a protective role in myocardial and cerebral I/R

injury. Therefore, the hallmarks of TLRs may provide insights on ischemia/reperfusion injury and lead to a new target for ischemic disease intervention.

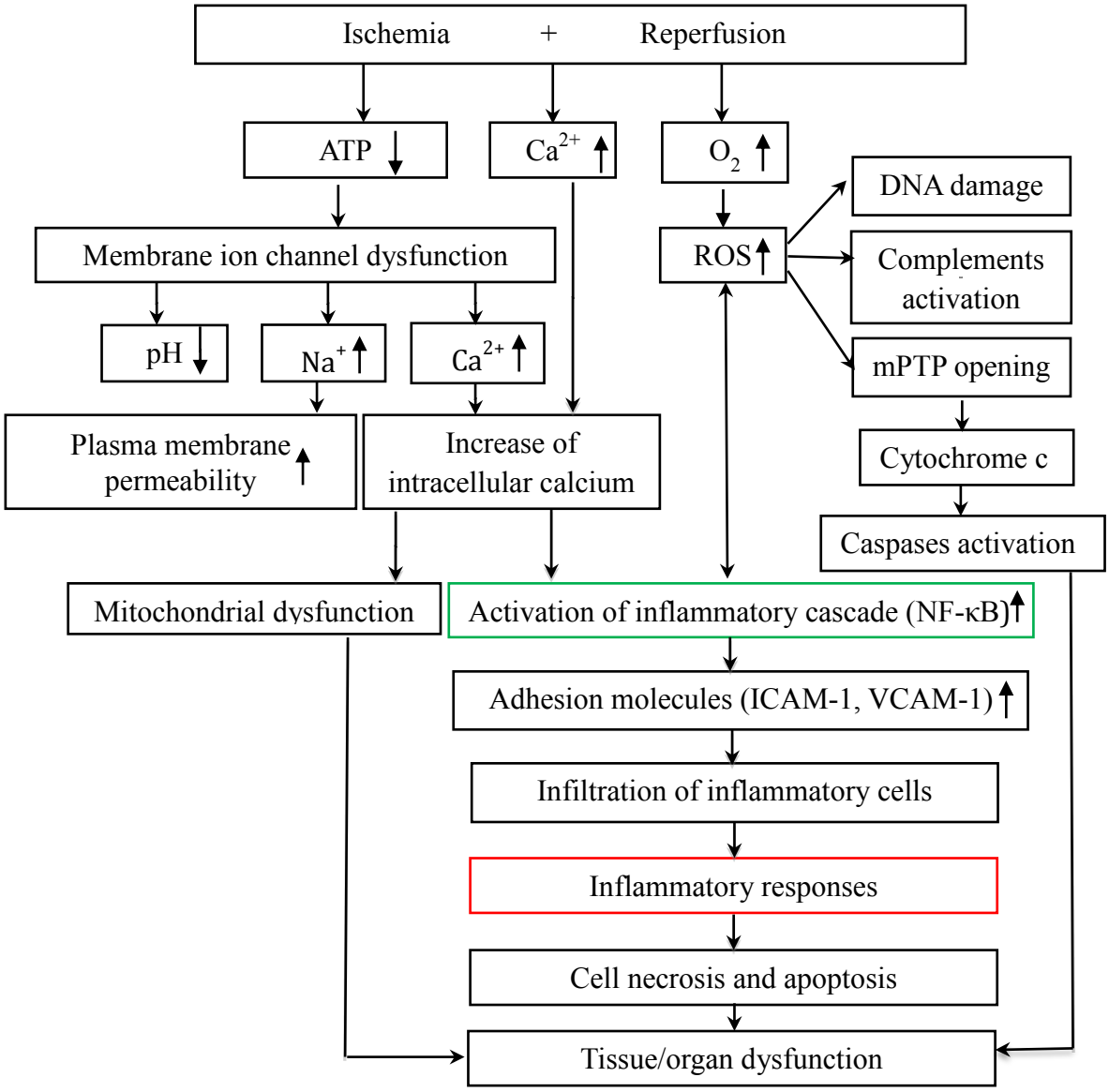

Figure 1.1 Schematic diagram of the processes in ischemia/reperfusion injury. In the setting of I/R injury, the cells are first exposed to hypoxia, leading to membrane ion channel dysfunction. The accumulation of calcium and sodium in the cytoplasm results in an increase of plasma membrane permeability and mitochondrial dysfunction. Then the cells suffer damage from reoxygenati that cause the excessive production of reactive oxygen species. Oxidative stress induces the DNA damage, complement activation, and the opening of mitochondrial

permeability transition pores. Subsequently, proinflammatory cytokines and chemokines are produced and the inflammatory cells infiltrate into the tissues. Furthermore, the opening of mPTP triggers caspases cascade in the cells. Taken together, these events eventually lead to cell necrosis and apoptosis.

In the cellular pathophysiological processes of I/R injury, several apoptotic molecules and apoptotic signaling pathways have been shown to play important roles in mediating cell death (Krijnen and others 2002; Broughton and others 2009). Ischemia/reperfusion injury generally induces activation of 2 apoptotic signaling cascades, including mitochondrial-dependent apoptosis and cell surface death receptor-mediated apoptosis. Among the apoptotic molecules, Bcl-2 is an important antiapoptotic protein, whereas Bax, Bak, and Bid are proapoptotic proteins in mitochondrial-dependent apoptotic signaling cascades. Emerging evidence indicate that the entry of proapoptotic proteins into the outer mitochondrial membrane induces the formation of a large conductance channel that mediates the release of cytochrome c from the mitochondria, resulting in activating caspases cascades (Vander Heiden and Thompson 1999; Newmeyer and Ferguson-Miller 2003). Kluck et al. have reported that Bcl-2 inhibited cytochrome c release from mitochondrial, thereby blocking the activation of caspases (Kluck and others 1997). Bcl-2 overexpression induces protection against neuron loss after cerebral I/R injury and inhibited cytochrome c translocation and casapse-3 activity (Zhao and others 2003). Overexpression of Bcl-2 also induces cardioprotection against myocardial I/R injury and attenuates apoptosis (Misao and others 1996; Chen and others 2001). Bax deficient hearts show protection against myocardia I/R injury and decrease caspases-3 activity (Hochhauser and others 2003). Following I/R injury, caspases activities are found to increase. Caspase-9 is found to be released from mitochondria during neuron apoptosis (Krajewski and others 1999). Namura and

colleagues found that cerebral ischemia induced the cleavage of caspase-3 (Namura and others 1998). Inhibition of caspases is reported to reduce infarct size after I/R injury. Yaoita et al. found that inhibition of caspases reduced myocardial I/R injury and attenuated cardiomyocyte apoptosis (Yaoita and others 1998). Caspase-3 deficiency is also resistant to cerebral ischemic injury *in vivo* and neuronal hypoxic injury *in vitro* (Le and others 2002). In addition, ischemia-induced apoptotic signaling is thought to play an important role in I/R injury. In cell surface death receptor-mediated signaling cascades, Fas/Fas-L pathway has shown to directly mediate cell death after myocardial ischemia (Jeremias and others 2000). The Fas/Fas-L pathway is also involved in cerebral ischemia-induced apoptosis in neurons (Martin-Villalba and others 1999). Thus, evidence suggests that inhibition of apoptosis ameliorates I/R-induced injury.

Cerebral Ischemia

Cerebral ischemia occurs when blood flow to the brain is limited. Blockage occurs locally resulting from thrombus or embolus caused by atherosclerosis, or globally by blood clots from the heart. During the progression of cerebral I/R injury, the absence of normal blood flow mainly results in death of neurons and glial cells (Hou and MacManus 2002) as well as glial cell over-activation. Due to decreased ATP levels, the activities of membrane ion channels are impaired, leading to cell membrane depolarization. The accumulation of intracellular calcium results in the release of ROS. Oxidative stress induces the opening of mPTP that causes the cytochrome C release from mitochondria, leading to activation of caspases that mediate cell apoptosis. Moreover, calcium overload causes the release of the neurotransmitter glutamate. Excessive amounts of extracellular glutamate depolarize adjacent cells and increase the concentration of intracellular calcium within these cells to toxic levels. As a result, the release of

glutamate and the influx of calcium further enhance the production of ROS. These events result in the production of proinflammatory cytokines and the activation of inflammatory responses.

In the central nervous system, microglial cells serve as the resident brain macrophages. Microglial cells are over activated in response to ischemic insults and secrete proinflammatory cytokines to trigger inflammation (Kreutzberg 1996; Stoll and others 1998; van and Hanisch 2004; del Zoppo and others 2007; Hanisch and Kettenmann 2007). As a consequence, inflammatory responses contribute to the disruption of blood brain barrier (BBB), leading to brain edema after cerebral I/R injury (Danton and Dietrich 2003). In cerebral ischemic stroke, neurons are extremely vulnerable to any reduction in blood supply and die within minutes of ischemia in the core (Oechmichen and Meissner 2006), whereas many neurons in the penumbra may undergo delayed neuronal death ranging from several hours to days (Zheng and others 2003). Of note, overproduction of proinflammatory cytokines from activated microglial cells is thought to result in the propagation of neuronal damage in the penumbra (Kaushal and Schlichter 2008). Moreover, endogenous processes such as oxidative stress and apoptosis contribute to neuronal cell death in the penumbra (Phan and others 2002; Allen and Bayraktutan 2009; Kaushal and Schlichter 2008). Thus, inhibition of inflammatory responses, apoptosis, and oxidative damage has the potential to salvage viable tissue and protect neurological function. It is still unclear how inflammatory responses contribute to brain damage after cerebral ischemia, whereas TLRs has been implicated in this process. TLRs detect “danger signals” and initiate inflammatory responses. Therefore, TLRs may play a pivotal role in the induction of inflammatory responses in ischemic stroke.

In general, neuron-to-microglia signals cause activation of microglial cells. Alternately, microglia-to-neuron signals affect neuronal response to injury in the brain disease (Bruce-Keller

1999; Bessis and others 2007; Biber and others 2007; Block and others 2007; Biber and others 2008). Microglia overactivation contributes to neuronal damage and may play a key role in orchestrating a cascade of pathological processes after cerebral damage (Bruce-Keller 1999; Bessis and others 2007). Thus, inhibition of microglia overactivation may protect neurons against further damage.

Myocardial Ischemia

Often myocardial ischemia is associated with atherosclerotic plaque rupture in the coronary artery. When a weakened plaque ruptures, it causes a clot to form. If the clot is big enough, it will block the blood flow to the coronary artery, resulting in heart attack. Myocardial ischemia leads to a reduction of oxygen and glucose to the myocardium and subsequently causes the necrosis and apoptosis of cardiomyocytes and nonmyocytes, including fibroblasts and endothelial cells (Saraste and others 1997; Anversa and others 1998). Reperfusion occurs after administration of recanalization therapy and restores coronary artery flow to the ischemic heart. Reperfusion after myocardial infarction has the potential to rescue the viable cells in the ischemic risk area, thereby limiting infarct development. However, reperfusion is associated with dramatic and further damage, such as myocardial stunning (Kloner and Jennings 2001a; Kloner and Jennings 2001b), arrhythmias (Hearse and Tosaki 1987), and severe intramyocardial hemorrhage (Asanuma and others 1997). After myocardial ischemia, an increase of intracellular calcium (Garcia-Dorado and others 2012), the excessive generation of ROS (Zweier and others 1987; Miyamae and others 1996), and the opening of mPTP occur. Consequently, these cellular events result in cell apoptosis. Mitochondrial-dependent apoptotic signaling and cell surface death receptors-mediated apoptotic signaling are activated in the ischemic zone. After

myocardial ischemia, inflammatory cells are activated and migrate to the site of injury (Hansen 1995; Jordan and others 1999). Subsequently, the expression of cell adhesion molecules on endothelial cells, such as ICAM-1 and VCAM-1, are upregulated by activated inflammatory cells. Consequently, these adhesion molecules facilitate the recruitment of inflammatory cells into the ischemic myocardium. The infiltration of inflammatory cells, including neutrophils and macrophages, further promote myocardium damage, leading to an excessive production of proinflammatory mediators and a massive activation of complements (Hansen 1995; Jordan and others 1999; Chakraborti and others 2000; Ren and others 2003). Thus, inhibition of cell apoptosis, reduction of inflammatory cell infiltration, and attenuation of the production of proinflammatory cytokines have the potential to reduce myocardial injury after myocardial ischemia.

If myocardial ischemia persists without reperfusion, it leads to death of cardiomyocytes and nonmyocytes in the ischemic zone. These changes induce an irreversible injury and lead to a deleterious cascade of intracellular processes that eventually cause cardiac remodeling, including dilation, hypertrophy, and the formation of collagen scar (Levitsky 2006). After myocardial ischemia, angiogenesis is a natural recovery process and plays a pivotal role in cardiac repair. Of note, the formation of new blood vessels may decrease the progression of cardiac remodeling. Stimulating angiogenesis may increase the levels of oxygen and glucose to sustain cellular metabolism. Promoting angiogenesis contributes to decreased cardiac fibrosis and lower the risk of cardiac rupture after myocardial infarction (Kutryk and Stewart 2003; van der Laan and others 2009). Accumulating evidence has demonstrated that PI3K/Akt signaling plays an important role in the progression of angiogenesis (Shiojima and Walsh 2002; Hamada and others 2005; Jiang and Liu 2009; Karar and Maity 2011). Moreover, PI3K/Akt signaling has been implicated in the

regulation of cardiac growth and coronary angiogenesis (Shiojima and Walsh 2006). Thus, the exploration of new intervention to promote angiogenesis and the regulation of PI3K/Akt signaling may be important for heart attack patients.

In addition, cardiomyocytes and nonmyocytes in the ischemic region die of apoptosis and necrosis during myocardial infarction. Increased apoptosis is a critical process in the pathogenesis of cardiac remodeling that occurs after myocardial ischemia (Takemura and Fujiwara 2004; Chandrashekhar 2005; Abbate and others 2006). Bax deficiency has shown to reduce infarct size and improve myocardial function following permanent coronary artery occlusion (Hochhauser and others 2007). Inhibition of caspase-3 activity is also found to limit cardiac dysfunction and remodeling after permanent coronary artery ligation (Balsam and others 2005). Hence, attenuation of apoptosis might attenuate cardiac remodeling after myocardial infarction.

Toll-Like Receptors

The innate immune system is the first line of defense against the foreign substances. The innate immune system is able to recognize the invading pathogens through a large group of pattern recognition receptors (PRRs) and eliminate the foreign pathogens (Modlin and others 1999). Amongst the PRRs, Toll-like receptors have a critical role in the induction of innate immune and inflammatory responses (Kopp and Medzhitov 1999). TLRs are transmembrane receptors characterized by a ligand recognition domain containing leucine-rich repeat (LRR) motifs and a cytoplasmic signaling domain containing a conserved region called the TIR domain (Botos and others 2011). TLRs have the ability to recognize pathogen associated molecular patterns (PAMPs) (Aderem and Ulevitch 2000). To date 12 TLRs have been identified in

mammals (11 in humans and 12 in mice) (Bowie and Haga 2005). As shown in Table 1.1, TLRs are generally divided into 2 subgroups based on their subcellular localization and respective PAMP ligands (Akira and others 2006). TLRs (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11) are expressed on the cell surface and they recognize cell wall products from bacteria or fungi, such as lipids, lipoproteins, and proteins (Kawai and Akira 2010). Other TLRs (TLR3, TLR7, TLR8, and TLR9) are located in the intracellular compartments, such as the endoplasmic reticulum (ER), endosomes, lysosomes and endolysosome. These TLRs are involved in the recognition of double-stranded RNA (dsRNA) or single-stranded RNA (ssRNA) (Kawai and Akira 2010). A diverse array of chemical PAMPs has been identified as TLRs agonists. For example, Pam3CSK4 (a synthetic triacylated lipopeptide) is recognized by TLR2; TLR4 recognizes lipopolysaccharide (LPS); TLR3 recognizes double-stranded RNA (dsRNA), such as polyinosine-polycytidylic acid (poly I: C) and TLR9 recognizes unmethylated CpG dinucleotides, such as CpG DNA. TLRs also recognize "danger" signals from damage tissue called damage associated molecular patterns (DAMPs), such as HMGB1, Hsp70, and Hsp 60 (Ionita and others 2010). Specially, recent evidence has highlighted microRNAs as small single-stranded noncoding RNAs bind to TLRs, such as TLR7 and TLR8 (Fabbri 2012; Fabbri and others 2012). Engagement of TLRs by PAMPs, including the host endogenous molecules and exogenous molecules, induces NF-κB activation and cytokines/chemokines production, leading to inflammatory responses (Mogensen 2009). TLRs are expressed on various immune cells, such as macrophages (Kadowaki and others 2001), neutrophils (Parker and others 2005), dendritic cells (Kadowaki and others 2001), and B cells (Dasari and others 2005), as well as on nonimmune cells, such as cardiomyocytes (Boyd and others 2006; Frantz and others 2009), endothelial cells

(Faure and others 2000), neurons (Prehaud and others 2005; Wadachi and Hargreaves 2006), and microglia cells (Aravalli and others 2007).

Table 1.1 The cellular localization of Toll-like receptors (TLRs) and their specific ligands

TLRS	Specific Ligands	Pathogens	Agonists	Localization
TLR1/TLR2	Triacyl lipoproteins, PGN	Bacteria, Fungi	Pam3CSK4	Cell surface
TLR3	Double-stranded RNA	Virus	Poly I:C	Intracellular
TLR4	Lipopolysaccharide	$Gram^-$ Bacteria	LPS	Cell surface
TLR5	Flagellin	Bacteria	-	Cell surface
TLR2/TLR6	Diacyl lipoproteins	Bacteria	FSL-1	Cell surface
TLR7	Single-stranded RNA	Virus	Imiquimod	Intracellular
TLR9	Unmethylated CpG DNA	Bacteria	CpG-ODN	Intracellular
TLR11	Uropathogenic bacteria	Bacteria	-	Cell surface

TLRs (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11) are expressed on the cell surface and recognize cell wall products, such as lipids, lipoproteins, and proteins. Other TLRs (TLR3, TLR7, TLR8, and TLR9) are located in intracellular compartments and recognize dsRNA or ssRNA.

Toll-Like Receptor-Mediated Signaling Pathways

Upon activation, TLRs induce the recruitment of adaptor proteins via the TIR domain, leading to activation of the transcription factors and production of proinflammatory cytokines and chemokines, thereby initiating inflammatory responses (Ha and others 2011). As shown in

Figure 1.2, there are 2 different pathways in TLR-mediated signaling that are dependent on the initiation of the different adaptor proteins, i.e. myeloid differentiation factor-88 (MyD88) or TIR domain-containing adaptor inducing IFN-β (TRIF). Each TLR family member, with the exception of TLR3, initiates intracellular signaling via the recruitment of MyD88, leading to the activation of IL-1 receptor associated kinase ($IRAK_{1, 2, 4}$) that interacts with TNF receptor-associated factor 6 (TRAF6), in turn, to activate IκB kinase (IKKα/β). IκB is phosphorylated and degraded by IKKα/β, thereby activating NF-κB to translocate into the nucleus and subsequently promoting the production of proinflammatory cytokines. MyD88 is also recruited to endosomal receptors TLR7 and TLR9, leading to activation of members of the IRAK family. The phosphorylated IRAKs bind to TRAF6. TRAF6 activate IκB kinase and NF-κB, resulting in proinflammatory cytokine gene expression. TLR3 is unique among the TLRs family due to its initiation of TRIF rather than MyD88. TRIF recruits TRAF3 and IKKi that activate IRF3, leading to IRF3 translocation to the nucleus with resultant Type I IFN production. Additionally, TRIF also recruits TRAF6 and RIP-1that activate the phosphorylation of IκB and the translocation of NF-κB to the nucleus. As a result, NF-κB as a critical transcription factor regulates innate immune and inflammatory responses (Tak and Firestein 2001; Li and Verma 2002). Activation of NF-κB has been noted in many cell types. NF-κB has been demonstrated to be activated in the neurons (Stephenson and others 2000), microglial cells (Kaushal and Schlichter 2008), astrocytes (Zhang and others 2007), endothelial cell (Howard and others 1998), and cardiomyocytes (Hamid and others 2011).

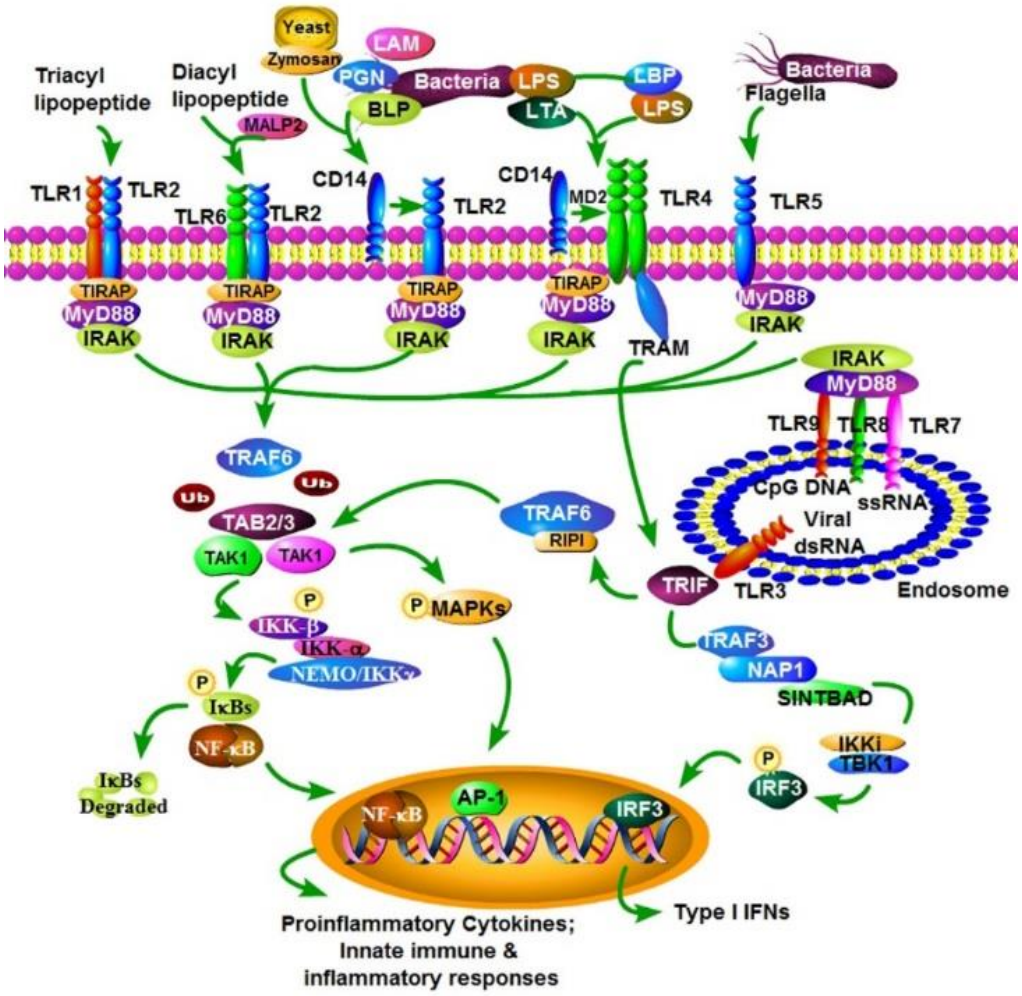

Figure 1.2 TLR-mediated signaling pathways. With the exception of TLR3, other TLR mediate cellular signaling through the MyD88-dependent pathway. MyD88 interacts with IRAKs that then associate with TRAF6 that activates a complex of TAK and TAB. The complex leads to phosphorylation of IκB kinase that phosphorylates IκB. IκB undergoes degradation by the ubiquitin-proteasome system. The released NF-κB translocates into the nucleus and activates the expression of proinflammatory cytokines genes. The TRIF-dependent pathway mediates the activation of both IRF3 and NF-κB signaling pathways. Stimulation of TLR3 and TLR4 activates the transcription of IRF3 and subsequent induction of IFN-β and IFN-inducible genes. TLR3 directly recruits TRIF. TRIF interacts with TBK1 and IKK-I that leads to IRF3 phosphorylation, nuclear translocation, and DNA binding. TRIF-dependent signaling also activates the NF-κB pathway. TRIF recruits TRAF6 and activates TAK1that in turn activates the NF-κB pathway. Adapted from Ha et al.2011 (*94*)

Toll-Like Receptors in Ischemia/Reperfusion Injury

Innate immune and inflammatory responses have been demonstrated to be involved in the pathogenesis and progression of cardiovascular disease, such as ischemic stroke and heart attack (Libby and others 2002; Rivest 2009). Ischemia-induced inflammatory responses are initiated via the recognition of injury-induced molecules from damaged cells by Toll-like receptors (TLRs). Toll-like receptors play a critical role in the innate immunity and inflammatory responses. Endogenous TLR2 and TLR4 ligands have been detected in I/R injury, such as HSP60, HSP70, and HMGB1 (Kinouchi and others 1993; Faraco and others 2007; Lehnardt and others 2008). Additionally, TLR3 can detect endogenous RNAs, such as microRNAs released by hypoxia and I/R (Chen and others 2014). Activation of TLRs results in massive inflammatory responses, leading to NF-κB activation and the production of proinflammatory cytokines/chemokines. Hence, modulation of TLRs seems to be capable of regulating the pathophysiological processes of cardiovascular disease.

Toll-Like Receptors in Cerebral Ischemia/Reperfusion Injury

Inflammatory responses play a pivotal role in cerebral ischemic injury (Jin and others 2010). However, the mechanisms that attempt to limit the inflammatory responses, to promote neurons survival, and to maintain cell homeostasis after cerebral ischemia remain unclear. Ischemia causes an early increase of TLR2 and TLR4 on neurons and a delayed increase on microglia, astrocytes, and endothelial cells (Carpentier and others 2005; Lehnardt and others 2007; Tang and others 2007; Ha and others 2011). Ablation of certain TLRs has been shown to decrease infarct size, improve neurological function, and attenuate inflammatory responses in the context of cerebral ischemia. TLR4 deficiency is resistant to focal and global cerebral ischemic

injury and attenuates neurological deficits (Cao and others 2007; Caso and others 2007; Hua, Ma, and others 2007; Caso and others 2008). TLR4 deficient mice increase the levels of phosphorylated Akt and GSK-3β as well as decreases NF- κB binding activity after cerebral ischemia (Hua, Ha, and others 2007). Ziegler et al. and Lehnardt et al. have reported that TLR2 deficient mice decreases infarct size compared with wild-type mice after cerebral ischemia (Lehnardt and others 2007; Ziegler and others 2007). However, blocking TLR2 with an anti-TLR2 antibody does not reduce infarct size (Ziegler and others 2011). We have shown that TLR2 deficiency does not protect against cerebral I/R injury (Hua and others 2009). Hyakkoku et al. reported that deficiency of TLR3 and TLR9 did not show a neuroprotective effect against cerebral I/R injury (Hyakkoku and others 2010).

Modulation of TLRs has also shown to attenuate brain damage after cerebral ischemia. Administration of TLRs ligands induce protection against ischemic injury through a preconditioning mechanism. Treatment with a small dose of TLR ligands prior to stroke is termed “preconditioning”. TLRs ligands administered systemically induce a state of tolerance to subsequent ischemic injury. Activation of TLRs by their ligands before cerebral ischemia reprograms TLR-mediated signaling pathway, resulting in suppression of the production of proinflammatory cytokines (Kariko and others 2004). TLR4-induced tolerance to cerebral ischemia is first demonstrated with low-dose systematic administration of LPS that protects rats from MCAO (Tasaki and others 1997). LPS preconditioning suppresses neutrophil infiltration into the brain and microglia activation in the ischemic brain (Medvedev and others 2002). Stimulation of TLR2 for 24 hours prior to cerebral ischemia increases the resistance to brain ischemia (Hua and others 2008). Pam3CKS4 preconditioning preserves blood brain barrier (BBB) functions after cerebral I/R injury by modulation of vascular tight junction proteins (Hua and

others 2008). Recently, it has been reported that TLR9 activated by its specific ligand 24 hours in advance of MCAO reduces ischemic damage (Stevens and others 2008). Notably, CpG preconditioning significantly increases serum TNF-α levels prior to MCAO. TNF-α knockout mouse fails to show protection in CpG preconditioning. TLR7 preconditioning is found to mediate neuroprotection against cerebral ischemic injury (Leung and others 2012). However, tolerance-induced protection is achieved by pretreatment with TLR agonists that are administered at least 24 hours in advance. By way of example, Pam3CSK4 administered 24 hours, CpG-ODN administered 24 hours, or gardiquimod administered 72 hours prior to MCAO have been shown to be effective. Therefore, it leads us to investigate whether there exists a similar neuroprotection when administration of Pam3CSK4 or CpG-ODN 1 hour prior to MCAO. Thus, the effect of TLR2 or TLR9 stimulation after cerebral I/R injury has been investigated in this dissertation.

Toll-Like Receptors in Myocardial Ischemia/Reperfusion Injury

The innate immune system plays an important role in the pathogenesis of myocardial ischemic injury. However, the complex mechanisms that are involved and the precise role of TLRs in modulating of myocardial ischemic injury remain unclear.

The role of TLR2 or TLR4 in myocardial ischemic injury is well documented. Blockade of TLR2 by anti-TLR2 antibody shows resistance to ischemia/reperfusion injury in the heart (Arslan and others 2010). TLR2 deficiency attenuates a decrease in left ventricular dysfunction and the production of inflammatory cytokines following myocardial I/R (Sakata and others 2007). TLR2 knockout mice also reduces infarct size and shows a decreased in neutrophil infiltration (Favre and others 2007). In addition, administration of the TLR4 antagonist (Eritoran)

intravenously shows the reduction of infarct size compared with vehicle-treated mice after myocardial infarction (Vanagt and others 2006). We and others have reported that TLR4 deficiency results in protection against myocardial I/R injury (Oyama and others 2004; Hua, Ha, and others 2007). We found that NF-κB binding activity was significantly decreased in TLR4 knockout mice in response to myocardial I/R injury compared with wild type mice (Hua, Ha, and others 2007). This indicates that TLR4/NF-κB-mediated signaling pathway contributes to myocardial I/R injury. We also observed that the levels of phosphorylated Akt in the myocardium of TLR4-deficient mice were remarkably increased compared with wild type mice. Moreover, modulation of TLR/NF-κB-mediated signaling pathway significantly reduces myocardial injury following I/R injury, improves cardiac functional recovery, and upregulates PI3K/Akt signaling (Li and others 2004). It is now well accepted that there is a cross talk between TLR/NF-κB-mediated signaling pathway and the PI3K/Akt signaling. Taken together, it is suggested that TLR2 and TLR4 deficiency lead to the significant reduction of cardiac dysfunction, the levels of myocardial inflammation, and TLR/NF-κB-mediated the production of cytokines after myocardial I/R injury. Thus, it is possible that the reduction of myocardial inflammatory responses may reduce cardiac injury in TLR deficient mice. Therefore, the role of other TLRs after myocardial ischemia remains to be investigated. The role of TLR3 following myocardial ischemic injury has been investigated in this dissertation.

PI3K/Akt Signaling

The phosphoinositide 3-kinases (PI3Ks) is a conserved family of signal transduction enzymes that plays an important role in regulating cellular survival, inflammatory response, and apoptosis (Fruman and Cantley 2002; Williams and others 2006). As shown in Figure 1.3, PI3K

catalyzes the conversion of phosphatidylinositol 4, 5 biphosphate PI (4, 5) P2 (PIP2) to PI (3, 4, 5) P3 (PIP3). Signaling proteins with pleckstrin homology domains bind to PIP3. These signaling proteins include phosphoinositide dependent kinase-1 (PDK1), PDK2 and Akt (protein kinase B, PKB). PDK activates Akt by phosphorylation of Ser473 and Thr308 (Oudit and Penninger 2009). Akt is an important physiologic mediator of the PI3K signaling pathway. However, phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a tumor suppressor that negatively regulates PI3K/Akt signaling. Phosphorylated Akt can phosphorylate several molecules, including phosphorylation of glycogen synthase kinase-3β (GSK-3β), phosphorylation of Bcl-2 associated death protein (Bad, Bax, and Bim), and inactivation of p53, thereby blocking the release of cytochrome C from mitochondrial to cytosol and subsequently inhibiting caspases activation (Datta and others 1997; Meek and Knippschild 2003; Hausenloy and Yellon 2004). PI3K/Akt signaling plays an important role in I/R injury. Our previous reports have shown that activation of PI3K/Akt signaling induces protection in TLR4 deficiency after myocardial and cerebral I/R injury. Activation of TLR2, TLR4, or TLR9 has a protective effect against myocardial I/R injury via a PI3K/Akt-dependent mechanism (Ha, Hua and others 2008; Ha and others 2010; Cao and others 2013). In line with our findings, others also found that Akt activation promoted cardiomyocyte survival *in vitro* and induced cardioprotection after myocardial I/R injury (Fujio and others 2000). Activation of PI3K and Akt induce protection against hypoxic injury in cardiomyocytes and inhibit cardiomyocyte apoptosis (Matsui and others 1999). PI3K/Akt survival signaling is also implicated in neuronal survival after stroke (Noshita and others 2001; Zhao and others 2006). Notably, PI3K/Akt signaling pathway has reported to negatively regulate TLR/NF-κB-mediated signaling pathway to attenuate inflammatory responses (Fukao and Koyasu 2003; Martin and others 2005; Chaurasia and others

2010). Indeed, our laboratory has confirmed that there is a cross talk between PI3K/Akt signaling and TLR/NF-κB-mediated pathway after myocardial I/R injury (Ha and others 2010; Cao and others 2013). Activation of PI3K/Akt signaling by PGN or CpG-ODN after myocardial I/R injury negatively regulates TLR/NF-κB-mediated signaling pathway and thus serves a protective role in I/R injury (Ha and others 2010; Cao and others 2013). Thus, activation of PI3K/Akt signaling may contribute to reduce organ damage after I/R injury.

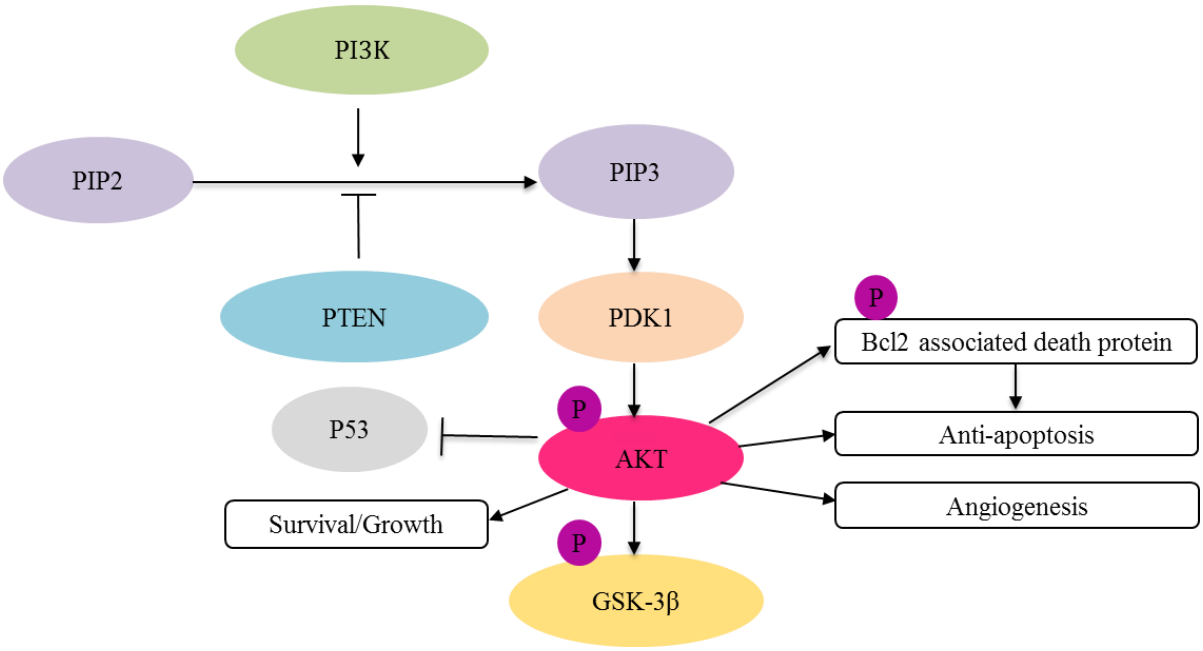

Figure 1.3 PI3K/Akt signaling. Upon activation, PI3K converts PIP2 to PIP3 that subsequently phosphorylates Akt through PDK1. Phosphorylated Akt in turn phosphorylates GSK-3β, phosphorylates Bcl2 associated death protein, and inhibits p53 expression. However, PTEN is a tumor suppressor that negatively regulates the pathway by converting PIP3 back to PIP2. Therefore, loss of PTEN leads to PI3K/Akt signaling activation that in turn results in survival, growth, antiapoptosis, and promoting angiogenesis.

MicroRNAs

As mentioned above, either TLRs deficiency or modulation of TLRs protects the heart or brain against ischemic injury through inhibition of inflammatory responses. Moreover, upregulation of PI3K/Akt signaling induces protection against ischemic injury. Inhibition of apoptotic signaling also induces protection following ischemic injury. Therefore, inflammatory responses, PI3K/Akt signaling, and apoptotic signaling must be very tightly regulated.

In recent years microRNAs (miRNAs) have emerged as a novel group of cellular regulators. MicroRNAs are small, noncoding single-stranded RNAs (~21-23 nucleotides) that can bind to the 3′-untranslated region (UTR) of their target messenger RNAs with imperfect complementarity. As a consequence, miRNAs can cleave and degrade their target mRNAs, resulting in suppressing their target mRNAs translation (Kim 2005). To some extent miRNAs serve as negative regulators and are capable of modulating the protein contents in the cells. Indeed, evidence has revealed that miRNAs can regulate various aspects of cellular activity, including cellular differentiation, development, proliferation, metabolism, survival, and apoptosis (Hwang and Mendell 2006). miRNAs have been implicated to influence the physiological and pathological processes in humans based on their dynamic nature. In the past decade miRNAs have been investigated as the potential candidate drug targets in a variety of diseases.

MicroRNAs in TLR/NF-κB-Mediated Signaling Pathway

There are numerous studies of miRNAs targeting TLR adaptor molecules to mediate immune responses (Virtue and others 2012). Several miRNAs are thought to regulate innate immune and inflammatory response through targeting the components in the TLR/NF-κB-

mediated signaling pathway. miR-155 has shown to regulate the expression of MyD88 (Tang and others 2010). miR-155 also modulates TLR/IL-1 inflammatory pathways via directly targeting TAB2 to downregulate inflammatory cytokines production in activated human monocyte-derived dendritic cells in response to microbial stimuli (Ceppi and others 2009). Taganov et al. reported that miR-146 negatively regulated TLR4-mediated signaling via directly suppression of IRAK1and TRAF6 expression to control inflammatory responses (Taganov and others 2006). IKK-α expression is regulated by miR-223, miR-15a, and miR-16 (Li and others 2010). IKK-β is targeted by miR-199a (Dai and others 2012). miR-155 and miR-9 are shown to target NF-κB mRNA (Ma and others 2011). miR-210 are reported to target NF-κB in response to LPS stimulation (Qi and others 2012). NF-κB is also regulated by miR-21 and miR-181b (Ma and others 2011). Of note, it is thought that NF-κB is capable of regulating the expression of miRNAs. miR-146 and miR-21 are found to be upregulated by NF-κB (Niu and others 2012). miR-125b is discovered to be downregulated by NF-κB (Tili and others 2007; Kim and others 2012). Additionally, miR-29b (Liu and others 2010), miR-10a (Huang and others 2010), miR-30b, and miR-130a (Zhou and others 2009; Zhou and others 2010) are dependent on the levels of NF-κB activity.

MicroRNAs in PI3K/Akt Signaling

Recent evidence suggests that microRNAs are important regulators in the PI3K/Akt signaling. Akt activity is positively or negatively regulated by several factors, such as a principal negative regulator phosphatase and tensin homolog (PTEN). PTEN, as a negative regulator of PI3K, blocks Akt phosphorylation and subsequent activation. PTEN is targeted by a number of microRNAs either directly or indirectly, resulting in the suppression of PTEN expression and

activation of PI3K/Akt signaling. PI3K/Akt signaling is involved in growth, proliferation, survival, and apoptosis (Cantley 2002; Engelman and others 2006). Therefore, miRNAs are capable of regulating PI3K/Akt signaling to further modulate cell activity through targeting PTEN. miR-21 is first identified to directly target PTEN to regulate PI3K/Akt signaling (Meng and others 2007). miR-181 (Henao-Mejia and others 2013), miR-486 (Small and others 2010), miR-214 (Yang and others 2008), and miR-29 (Tumaneng and others 2012) have also shown to regulate the PI3K/Akt signaling through PTEN suppression. For example, miR-21 has been reported to downregulate PTEN expression and thus promotes growth and invasion of NSCLC or HCC cells (Meng and others 2007; Zhang and others 2010).

MicroRNAs in Myocardial Ischemic Injury

The role of miRNAs in cardiovascular diseases has been highlighted recently (Small and Olson 2011; van 2011). Several miRNAs have been shown to regulate the pathophysiological processes in myocardial ischemic injury by regulating key signaling elements, thereby providing some evidence of their potential as therapeutic targets.

Myocardial ischemic injury affects the expression of miRNAs. Ren and colleagues have reported that the expression of miRNAs is altered in mouse heart after 30 minutes of ischemia followed by 24 hours of reperfusion (Ren and others 2009). They found that the levels of miR-146b, miR-21, miR-491, and miR-7 were significantly increased after myocardial I/R injury, whereas the levels of miR-320 were significantly decreased. Tang et al. found that the levels of miR-1, miR-126, and miR-208 were increased after myocardial I/R injury, whereas the levels of miR-21, miR-133, and miR-195 were decreased (Tang and others 2009). Dong and colleagues have shown that the expression of miRNAs is changed in heart at 6 hours after myocardial

infarction. miR-130a, miR-1, miR-24, miR-143, miR-30e, miR-16, miR-92a, and miR-126 levels are unregulated in noninfarcted myocardium compared with the infarcted myocardium (Dong and others 2009). However, miR-145, miR-133b, miR-181a, miR-146a, and miR-290 are downregulated in noninfarcted myocardium compared with the infarcted myocardium. Rooij et al. found that the levels of miR-21, miR-146b, miR-214, and miR-223 were upregulated by 2 or more fold in the border zone region of mouse heart both 3 days and 14 days after myocardial infarction when compared with sham control. In contrast, miR-29, miR-30, miR-130a, miR-145, and miR-499 levels were downregulated by 2 or more fold in border zone region of mouse heart both 3 days and 14 days after myocardial infarction when compared with sham control (van and others 2008).

Among the altered miRNA in the ischemic heart, miR-21 regulates matrix metalloprotease-2 (MMP-2) expression in cardiac fibroblasts via a PTEN pathway after myocardial I/R injury (Roy and others 2009). miR-214 has a cardioprotective effect against myocardial I/R injury by controlling Ca^{2+} overload and cell death (Aurora and others 2012). miR-216 improves angiogenesis and cardiac function via stimulation of Akt/ERK after permanent ligation of coronary artery (Chen and Zhou 2011). Overexpression of miR-29 in cardiac fibroblasts reduces collagen expression after coronary artery occlusion (van and others 2008). Moreover, miR-146b, miR-145, miR-16, and miR-223 have been shown to regulate TLR/NF-κB-mediated signaling pathways. miR-181a, miR-21, miR-29, and miR-214 have been reported to upregulate PI3K/Akt signaling through suppression of PTEN expression. For example, miR-29 directly targets PTEN in endothelial cells, leading to activation of Akt and promotion of angiogenesis (Wang and others 2013a).

It is noteworthy that increased expression of miRNAs induces cardiopretection in myocardial ischemic injury through either the regulation of TLR/NF-κB-mediated signaling pathway or PI3K/Akt signaling. Wang et al. found that increased miR-146a expression induced cardioprotection against myocardial I/R injury through suppression of IRAK and TRAF6 (Wang and others 2013b). Sayed et al. have reported that overexpression of miR-21 transgenic mice remarkably reduces cardiac I/R-induced infarct size compared with wild type mice. Moreover, miR-21 overexpression in transgenic mice significantly increases phospho-Akt levels and decreases PTEN expression after myocardial I/R injury (Sayed and others 2010). Of interest, the levels of miR-130a are downregulated in infarcted heart compared with noninfarcted heart by the investigators (van and others 2008; Dong and others 2009). It has been suggested that miR-130a may be required for the protection against myocardial ischemic injury. miR-130a has been reported to downregulate the antiangiogenic genes GAX and HoxA5 in endothelial cells, resulting in stimulation of angiogenesis (Chen and Gorski 2008). In contrast, downregulation of miR-130a expression contributes to dysfunction of endothelial progenitor cells (Jakob and others 2012; Meng and others 2013). These data indicates that miR-130a may potentially contribute to angiogenesis. miR-130a is predicated to target PTEN by TargetScan and miRBase. Thus, it is likely that miR-130a activates PI3K/Akt signaling through supersession of PTEN expression. In addition, inhibition of PTEN has shown to reduce cardiac injury after myocardial ischemic injury through upregulation of PI3K/Akt signaling (Keyes and others 2010). Moreover, PI3K/Akt signaling plays an important role in the progression of angiogenesis after myocardial ischemic injury (Shiojima and Walsh 2002; Hamada and others 2005; Jiang and Liu 2009; Karar and Maity 2011). Taken together, it is suggested that miR-130a possibly controls the progression of angiogenesis in ischemic heart after myocardial infarction via suppression of PTEN expression.

Therefore, the role of miR-130a after myocardial infarction has been investigated in this dissertation.

Specific Aims

We have demonstrated that TLR/NF-κB-mediated signaling pathway plays a deleterious role in myocardial and cerebral ischemia/reperfusion while activation of PI3K/Akt signaling protects heart and brain from I/R injury. Interestingly, there is a cross talk between TLR/NF-κB-mediated signaling pathway and PI3K/Akt signaling. PI3K/Akt signaling serves as a negative regulator for TLR/NF-κB-mediated signaling pathway.

The overall goal of the present study is to investigate how to differentially modulate PI3K/Akt signaling and TLR/NF-κB-mediated signaling pathway during I/R injury to develop novel therapeutic approaches for the patients with myocardial or brain ischemic injury. We investigate the hypotheses as follows: 1) modulation of TLRs by their specific ligands at small dose induces activation of PI3K/Akt signaling after cerebral ischemic injury, 2) modulation of TLRs by their specific ligands inhibits NF-κB activation via a PI3K/Akt-dependent mechanism after cerebral ischemic injury, 3) TLR/NF-κB-mediated signaling pathway contributes to myocardial ischemic injury, 4) upregulation of PI3K/Akt signaling via microRNA-130a induces protection against myocardial ischemic injury.

To critically investigate our hypotheses, we perform the following experiments in murine model of myocardial or cerebral ischemia. 1) We determine whether modulation of TLR2 by its specific ligand (Pam3CSK4) induces neuroprotection in MCAO stroke model. We examine whether modulation of TLR2 by Pam3CSK4 induces activation of PI3K/Akt signaling after cerebral I/R injury. We also examine whether there is a cross talk between TLR2 and PI3K/Akt signaling. 2) We evaluate whether modulation of other TLRs, such as TLR9 by its ligand CpG-

ODN, also induces protection against cerebral I/R injury through activation of PI3K/Akt signaling. 3) Previous studies have shown that TLR2 or TLR4 deficiency induces protection against myocardial I/R injury (Ha and others 2010; Ha and others 2011). However, the role of TLR3 located on endosomes has not been investigated. Hence, we determine whether TLR3 contributes to myocardial ischemic injury via TLR/NF-κB-mediated signaling pathway. 4) Activation of PI3K/Akt signaling has shown to protect organ against I/R injury. We examine whether microRNAs, such as microRNA-130a, regulates PI3K/Akt signaling during myocardial ischemic injury. PTEN is a negative regulator of PI3K/Akt signaling. Hence, we examine whether miR-130a targets PTEN expression. We also examine whether miR-130a promotes angiogenesis and inhibits cell apoptosis during myocardial ischemic injury.

The research described in this dissertation may provide a better understanding of the effects of modulation of TLRs, such as TLR2, TLR3, TLR9, and the role of activation of PI3K/Akt signaling via microRNA in organ ischemic injury. This knowledge may provide solid basis for developing novel therapeutic approaches for stroke and heart attack patients.

CHAPTER 2

TLR2 LIGAND INDUCES PROTECTION AGAINST CEREBRAL ISCHEMIA/REPERFUSION INJURY VIA ACTIVATION OF PI3K/AKT SIGNALING (*167*)

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Abbreviated Title: TLR2 Ligand Protects Brain from Ischemic Injury

Abstract

This study examined the effect of TLR2 activation by its specific ligand, Pam3CSK4, on cerebral ischemia/reperfusion (I/R) injury. Mice (n=8/group) were treated with Pam3CSK4 one hr before cerebral ischemia (60 min) followed by reperfusion (24 hrs). Pam3CSK4 was also given to the mice (n=8) 30 min after ischemia. Infarct size was determined by triphenyltetrazolium chloride staining. The morphology of neurons in brain sections was examined by Nissl staining. Pam3CSK4 administration significantly reduced infarct size by 55.9% ($p<0.01$) compared with untreated I/R mice. Therapeutic treatment with Pam3CSK4 also significantly reduced infarct size by 55.8%. Morphologic examination showed that there was less neuronal damage in the hippocampus of Pam3CSK4 treated mice compared with untreated cerebral I/R mice. Pam3CSK4 treatment increased the levels of Hsp27, Hsp70, Bcl2 and decreased Bax levels and NF-κB binding activity in the brain tissues. Administration of Pam3CSK4 significantly increased the levels of phospho-Akt/Akt and phospho-GSK-3β/GSK-3β compared with untreated I/R mice. More significantly, either TLR2 deficiency or PI3K inhibition with LY29004 abolished the protection by Pam3CSK4. These data demonstrate that activation of TLR2 by its ligand prevents focal cerebral ischemic damage through a TLR2/PI3K/Akt dependent mechanism. Of greater significance, these data indicate that therapy with a TLR2 specific agonist during cerebral ischemia is effective in reducing injury. (*J Immunol. 2011 Aug 1; 187(3):1458-66.*)

Introduction

Recent evidence suggests that innate immune and inflammatory responses play a critical role in cerebral ischemic stroke (1). We and other investigators have demonstrated that modulation of innate immune and inflammatory responses significantly attenuates cerebral I/R injury (2-6). However, the mechanisms by which innate immune and inflammatory responses contribute to cerebral ischemic stroke have not been completely elucidated.

Toll-like receptors (TLRs) have been demonstrated to play a critical role in the induction of innate and inflammatory responses (7-10). TLR-mediated signaling pathways predominately activate NF-κB which is a critical transcription factor regulating gene expression involved in innate and inflammatory responses (11). Indeed, recent studies have shown that modulation of the TLR-mediated NF-κB activation pathway significantly attenuated organ ischemic injury. We and others have recently demonstrated that TLR4 deficient mice showed decreased injury following cerebral I/R (2, 4, 6, 12). In addition to TLR4, TLR2 has been reported to play a role in focal cerebral ischemic injury (6, 13, 14). Focal ischemic injury was reduced in TLR2 deficient mice (13, 14). We reported recently that TLR2 deficiency did not protect the brain from focal cerebral I/R injury (6). We have also reported that activation of TLR2, with a specific ligand, induced protection against myocardial I/R injury and attenuated cardiac dysfunction in severe sepsis (15, 16). Recent studies have shown that TLR2 is involved in preconditioning-induced protection against cardiac dysfunction (17) and cerebral I/R injury (5). Collectively, these observations suggest that TLR2 may serve a protective role in focal cerebral ischemic injury (5, 6). However, whether a TLR2 ligand will have a therapeutic effect on cerebral I/R injury has not been investigated.

Activation of the phosphoinositide 3-kinase (PI3K) pathway has been shown to prevent neuronal apoptosis and protect the brain from cerebral I/R injury (18, 19). Recent evidence suggests that there is crosstalk between TLR signaling and the PI3K pathway (20, 21). Stimulation of TLR2 results in the recruitment of active Rac1 and PI3K to the TLR2 cytosolic domain, leading to activation of the PI3K pathway (20). Mal, an adaptor protein in the TLR-mediated signaling pathway, plays a role in TLR2 mediated PI3K activation (22). Therefore, it is possible that activation of TLR2 will activate the PI3K/Akt signaling pathway during focal cerebral I/R.

In the present study, we investigated the effect of activation of TLR2 by its specific ligand, Pam3CSK4, on focal cerebral ischemic injury. We observed that Pam3CSK4 administration significantly reduced infarct volume following cerebral I/R. Of greater clinical significance, administration of Pam3CSK4 30 min after induction of ischemia also significantly protected against cerebral I/R injury. However, the protective benefit of Pam3CSK4 was abolished either by TLR2 deficiency or by PI3K inhibition. The data suggest that activation of TLR2 prevents focal cerebral ischemic damage, in part, through a TLR2/PI3K dependent mechanism.

Materials and Methods

Animals

Age- and weight-matched male C57BL/6 mice and TLR2 knockout (TLR2 KO) mice (B6.129-TLR2tm1kir/J) were obtained from Jackson Laboratory (Indianapolis, IN). The TLR2 KO mice were backcross with C57BL/6 for 9 interbreeding generations. The mice were maintained in the Division of Laboratory Animal Resources at East Tennessee State University

(ETSU). The experiments described in this manuscript conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animal care and experimental protocols were approved by the ETSU Committee on Animal Care.

Focal Cerebral Ischemia/Reperfusion

Focal cerebral I/R was induced by occlusion of the middle cerebral artery (MCAO) on the left side as described in our previous studies (5, 6, 23). Briefly, mice (23-25 gram body weight) were anesthetized by 5.0% Isoflurane and anesthesia was maintained by inhalation of 1.5% to 2% Isoflurane driven by 100% oxygen flow. Mice were intubated and ventilated with room air using a rodent ventilator at a rate of 110 breaths per min with a total delivered volume of 0.5 ml. Body temperature was regulated at 37.0°C by surface water heating. Following the skin incision, the left common carotid artery (CCA), the external carotid artery (ECA) and the internal carotid artery (ICA) were carefully exposed. Microvascular aneurysm clips were applied to the left CCA and the ICA. A coated 6-0 filament (6023PK, Doccol Corp. CA, USA) was introduced into an arteriotomy hole, fed distally into the ICA. After the ICA clamp was removed, the filament was advanced 11 mm from the carotid bifurcation, and focal cerebral ischemia started. After ischemia for 60 minutes, the filament and the CCA clamp were gently removed (reperfusion starts). The collar suture at the base of the ECA stump was tightened. The skin was closed, anesthesia discontinued, and the animal allowed to recover in pre-warmed cages. Control mice underwent a neck dissection and coagulation of the external carotid artery, but no occlusion of the middle cerebral artery.

Measurement of Cerebral Blood Flow

Successful occlusion of the middle cerebral artery was verified and recorded by laser Doppler flowmetry (Model PeriFlux system 5000, Perimed, Stockholm, Sweden). Under anesthesia, a midline incision of the head was made and a probe holder was attached to the skull with crazy glue at 6 mm lateral and 1 mm posterior of bregma. A laser-Doppler probe was connected to the probe holder and regional cerebral blood flow (rCBF) was monitored and recorded. The data were continuously stored in a computer and analyzed using the Perimed data acquisition and analysis system. Regional CBF was expressed as a percentage of preischemic baseline values.

Experimental Design

To determine infarct volume, mice were subjected to focal cerebral ischemia for 60 min followed by reperfusion for 24 hrs. The brains were harvested and stained with triphenyltetrazolium chloride (TTC) (5, 6, 23).

To evaluate the role of a TLR2 ligand in focal cerebral I/R injury, Pam3CSK4 (Catalog number: tlrl-pms, InvivoGen, San Diego, CA) was dissolved in sterile endotoxin-free water and injected intraperitoneally (i.p., 2mg/Kg body weight, n=8) one hr prior to cerebral ischemia (60 min) followed by reperfusion for 24 hrs.

To examine the therapeutic effect of a TLR2 ligand on focal cerebral I/R injury, Pam3CSK4 (i.v., 2mg/Kg body weight, n=8) was administered to the mice 30 min after the beginning of cerebral ischemia. Focal cerebral ischemia was continued for an additional 30 min followed by reperfusion for 24 hrs.

To examine the role of TLR2 in Pam3CSK4-induced protection, TLR2 knockout (KO) mice (n=8/group) were treated with or without Pam3CSK4 (2mg/Kg body weight) 1 hr before the mice were subjected to focal cerebral ischemia (60 min) followed by reperfusion (24 hrs). The infarct size was determined by TTC staining (5, 6, 23).

To determine whether activation of the PI3K/Akt signaling pathway was involved in TLR2 ligand-induced protection, mice (n=8) were treated with the PI3K inhibitor Ly294002 (1mg/25 gram body weight) 15 min before administration of Pam3CSK4. The mice were subjected to focal cerebral ischemia (60 min) followed by reperfusion (24 hrs). The brains were harvested and infarct volume was determined (5, 6, 23).

Measurement of Infarct Volume

The infarct volume was determined as described previously (5, 6, 23). Twenty-four hrs after I/R, mice were sacrificed and perfused with ice cold phosphate buffered saline (PBS) via the ascending aorta. Brains were removed and sectioned coronally into 2-mm-thick slices. The slices were stained with 2% triphenyltetrazolium chloride (TTC) solution at 37°C for 15 min followed by fixation with 10% formalin neutral buffer solution (pH 7.4). The infarct areas were traced and quantified with an image-analysis system. Unstained areas (pale color) were defined as ischemic lesions. The areas of infarction and the areas of both hemispheres were calculated for each brain slice. An edema index was calculated by dividing the total volume of the left hemisphere by the total volume of the right hemisphere. The actual infarct volume adjusted for edema was calculated by dividing the infarct volume by the edema index (5, 6, 23). Infarct volumes are expressed as a percentage of the total brain volume ± S.E.M.

Evaluation of Neuronal Damage in the Hippocampal Formation (HF)

Neuronal damage in brain sections were determined by Nissl's method as described previously (5, 6, 23). Paraffin sections cut in the coronal plane at approximately 1.5 mm behind bregma with a thickness of 7 microns were deparaffinized and then stained with 0.1% cresyl violet for 2 min. The sections were evaluated using light microscopy.

Evaluation of Neurological Score

Neurological score was performed by a blinded investigator using a neurological evaluation instrument described in our previous studies (5). Briefly, the scoring system included five principle tasks: spontaneous activity over a 3-min period (0–3), symmetry of movement (0–3), open-field path linearity (0–3), beam walking on a 3 cm×1 cm beam (0–3), and response to vibrissae touch (1–3). The scoring system ranged from 0 to 15, in which 15 is a perfect score and 0 is death due to cerebral I/R injury. Sham controls received a score of 15.

Immunohistochemistry (IHC) Double Fluorescent Staining

Double fluorescent staining was performed to examine the response of microglia cells to Pam3CSK4 stimulation *in vivo* as described previously (4, 5, 15). Briefly, brain tissues were immersion-fixed in 4% buffered paraformaldehyde, embedded in paraffin, cut at 7 μm, and stained with an antibody directed against phospho-Akt (goat, Cell Signaling Technology, Inc, Beverly, MA) at 250C for 1 hr. After washing, the sections were incubated with Alexa 555 conjugated anti-goat IgG (GeneTex, San Antonio, TX) for 1 hr at 250C. The sections were washed again before incubation with anti-ionized calcium-binding adapter molecule 1 (IBA1, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 250C for 1 hr. After washing, the sections

were incubated with FITC conjugated anti-rabbit (GeneTex, San Antonio, TX) for 1 hr at 250C. The sections were covered with fluorescence mounting medium (Vector Labs). The images were viewed on an EVOS-fI digital inverted fluorescent microcopy (Advanced Microscopy Group, Bothell, WA).

In Vitro Experiments

BV2 microglial cells were provided by Dr. Keshvara at Ohio State University and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented 5% fetal bovine serum (FBS) under 5% CO2 at 37oC. When the cells reached 70-80% confluence, they were treated with Pam3CSK4 at a final concentration of 1 μg/ml for 0, 5, 15, 30, and 60 min with four replicates at each time point. The cells were harvested and cellular proteins were isolated for examination of Akt and GSK-3β phosphorylation by Western blot. TLR2 tyrosine phosphorylation and association with the p85 subunit of PI3K were examined by immunoprecipitation with specific anti-TLR2 antibody followed by immunoblots with anti-tyrosine and anti-p85 subunit of PI3K as described previously (15, 24).

Western Blots

Cellular proteins were prepared from brain tissues and Western blots were performed as described previously (4-6, 24). Briefly, the cellular proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ). The ECL membranes were incubated with the appropriate primary antibody [anti-phospho-Akt (Ser473), anti- phospho-GSK3β (Ser9), anti-phospho-IκB-α (Cell Signaling Technology, Inc, Beverly, MA), anti-GSK-3β, anti-Akt, anti-IκB-α, anti-Bcl2, anti-

Bax, anti-pTyr20, anti-p85, anti-Hsp27, and anti-Hsp70 (Santa Cruz Biotechnology, Inc.)] respectively, followed by incubation with peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.). The signals were detected with the ECL system (Amersham Pharmacia). To control for lane loading, the same membranes were probed with anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase, Biodesign, Saco, Maine) after being washed with stripping buffer. The signals were quantified by scanning densitometry using a Bio-Image Analysis System (Bio-Rad).

Caspase-3 Activity Assay

Caspase-3 activity in brain tissue was measured using a Caspase-Glo assay kit (Promega) according to the manufacturer's protocol as described previously (15).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins were isolated from ischemic cerebral hemispheres as described previously (4-6, 24). NF-κB binding activity was examined by EMSA in a 15 μl binding reaction mixture containing 15 μg of nuclear proteins and 35 fmols of [γ-32P] labeled double-stranded NF-κB consensus oligonucleotide.

Statistical Analysis

Data are expressed as mean ± SE. Comparisons of group mean levels between groups were made using one-way analysis of variance (ANOVA) and Tukey's procedure for multiple range tests was performed. $p < 0.05$ was considered significant.

Results

Pam3CSK4 Administration Decreased Focal Cerebral Infarct Volume Following I/R

To examine the role of a TLR2 ligand in focal cerebral I/R injury, we administered Pam3CSK4, a specific TLR2 ligand, to mice 1 hr before the mice were subjected to cerebral ischemia (60 min) followed by reperfusion (24 hrs). Figure 2.1A shows that Pam3CSK4 administration significantly reduced infarct volume by 55.9% (18.1 ± 2.72 vs. 7.98 ± 1.53) compared with the untreated I/R group. We also examined the therapeutic effect of TLR2 ligand on focal cerebral I/R injury. Administration of Pam3CSK4 30 min after the beginning of ischemia significantly reduced infarct volume by 55.8% (18.1 ± 2.72 vs. 7.99 ± 1.00) compared with untreated I/R mice. Figure 2.1B shows that cerebral blood flow was significantly reduced by 90% immediately following occlusion of middle cerebral artery and complete reperfusion after the occlusion was released. There was no significant difference in cerebral blood flow between the untreated cerebral I/R group and the Pam3CSK4-treated group.

TLR2 Deficiency Abolished Pam3CSK4-Induced Protection Against Cerebral I/R Injury

We examined the role of TLR2 in Pam3CSK4-induced protection against cerebral I/R injury. TLR2 knockout mice were treated with or without Pam3CSK4 one hr before the brains were subjected to ischemia (60 min) followed by reperfusion (24 hrs). Infarct volume was evaluated. As shown in Figure 2.1C, cerebral I/R induced cerebral infarction in TLR2 deficient mice which was comparable to WT mice. Pam3CSK4-induced protection against cerebral I/R injury was lost in TLR2 deficient mice. The data suggests that TLR2 is essential for mediating the beneficial effect of Pam3CSK4 on cerebral I/R injury.

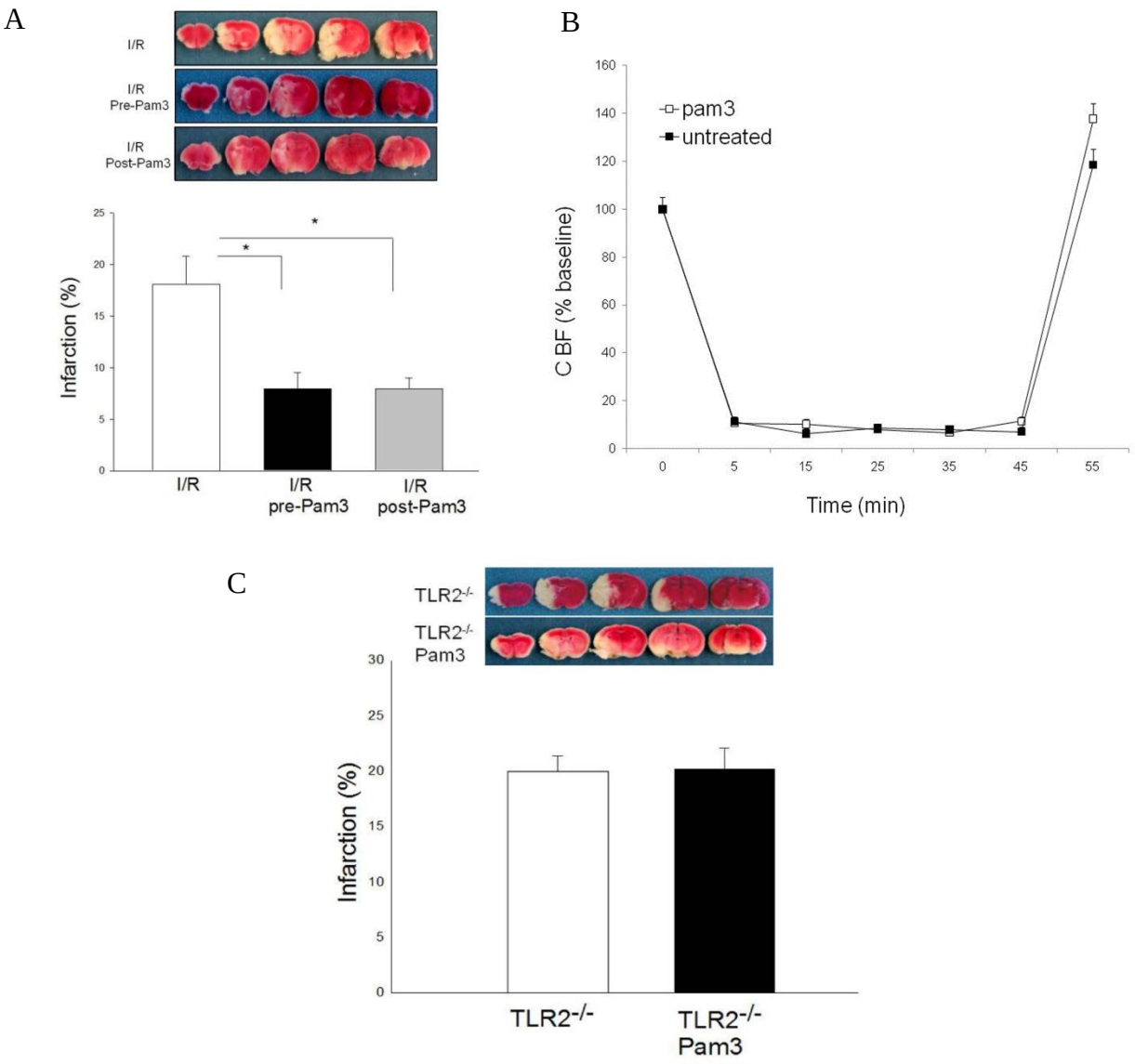


Figure 2.1 Pam3CSK4 administration reduces infarction following cerebral I/R. (A) Pam3CSK4 was administered to mice one hr before (pre-Pam3) or 30 min after ischemia (post-Pam3). Mice were subjected to cerebral ischemia (60 min) followed by reperfusion (24 hrs). (B) Cerebral blood flow measurement. (C) TLR2 deficiency abolishes the protection by Pam3CSK4 in cerebral I/R. TLR2 knockout mice were treated with or without Pam3CSK4 one hr prior to cerebral I/R. Infarct size was examined by TTC staining. * $p < 0.05$ compared with indicated group. N=8 each group.

Pam3CSK4 Attenuated Neuronal Damage in the Hippocampal Formation and Improved Neurological Deficits

We evaluated the effect of Pam3CSK4 on neuronal damage and neurological deficits following cerebral I/R. Nissl staining showed neuronal damage in the cornu ammonis 1 (CA1) field of the hippocampal formation (HF) characterized by shrunken cell bodies accompanied by shrunken and pyknotic nuclei in the I/R mice for 1 day and 3 days (Fig. 2.2A). Similar changes were variably expressed in the dentate gyrus (DG) and CA1. In the Pam3CSK4 treated mice, neuronal damage in the HC was significantly decreased and morphology was preserved.

Neurological score evaluation is an index for the degree of neurological deficits associated with stroke. Figure 2.2B shows that the neurological score was significantly decreased in I/R mice after 24 hrs and 72 hrs of reperfusion. In contrast, the neurological score of Pam3CSK4 treated I/R mice was significantly higher than that in the untreated I/R mice at all time periods, indicating that Pam3CSK4 administration attenuated the neurological deficits associated with stroke.

We also evaluated the effect of Pam3CSK4 treatment on I/R-induce brain apoptosis by measuring caspase-3 activity. Figure 2.2C shows that caspase-3 activity was significantly increased by 57.9% in I/R mice compared with sham control. In contrast, Pam3CSK4 treatment prevented I/R-increased caspase-3 activity in the brain tissues.

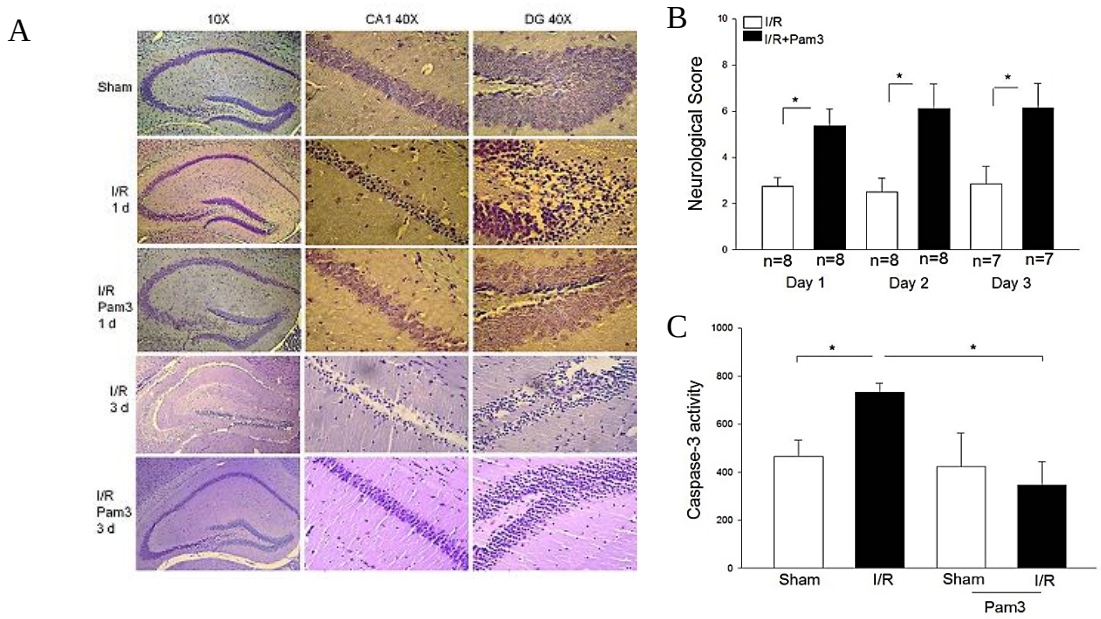


Figure 2.2 Pam3CSK4 treatment attenuates neuronal damage in the HF following cerebral I/R. (A) Mice (n=6/group) were treated with or without Pam3CSK4 one hr before cerebral I/R. Sham operated mice (n=4/each group) served as sham control. Brains were harvested 1, 2 and 3 days after reperfusion. Brain sections were stained with 0.1% cresyl violet. (B) Neurological function was evaluated using a scoring system ranging 0 to 15, with 15 being a perfect score and 0 being death due to cerebral I/R injury. (C) Pam3CSK4 decreases caspase-3 activity in the brain tissues following cerebral I/R. Mice were treated with or without Pam3CSK4 one hr prior to cerebral I/R (n=6/group). Sham surgical operation served as sham control (n=4/group). The brains were harvested and cellular proteins were isolated. Caspase-3 activity was measured using a Caspase-3 activity kit. * $P<0.05$ compared with indicated groups.

Pam3CSK4 Administration Attenuated I/R-Increased NF-κB Binding Activity in the Brain Tissues

NF-κB activation plays an important role in cerebral I/R injury (25). Figure 2.3 shows that I/R significantly increased the levels of NF-κB binding activity by 55.3% (A) and phospho-IκB-α by 62.4% (B) compared with sham control. However, Pam3CSK4 treatment significantly

prevented I/R-increased NF-κB binding activity and IκB-α phosphorylation in the brain tissues, respectively, compared with the cerebral I/R group.

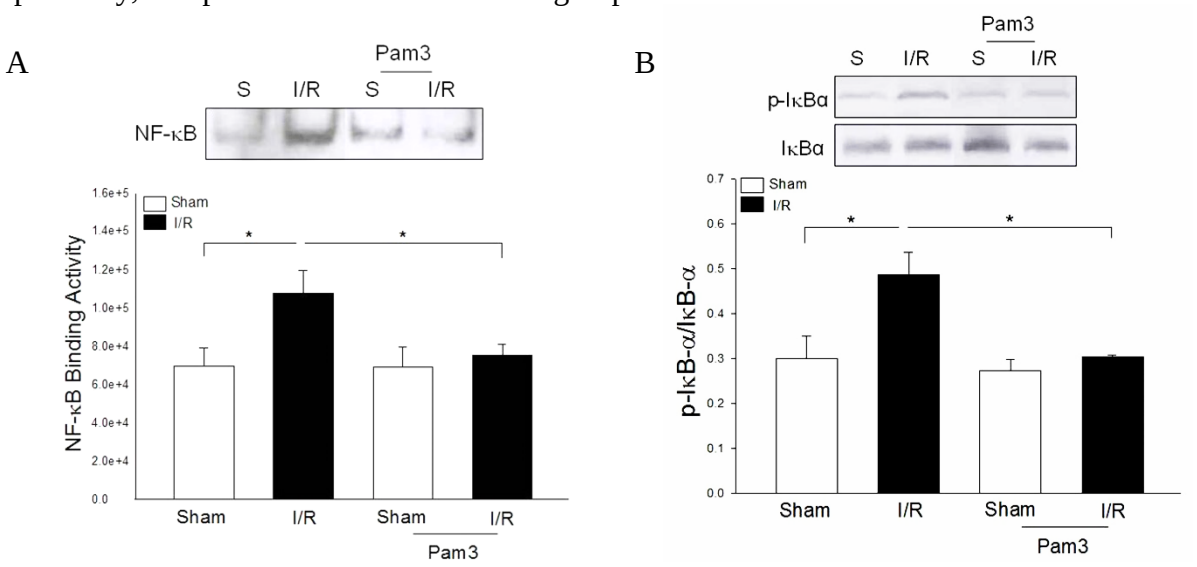


Figure 2.3 Pam3CSK4 administration decreases I/R-induced increases in NF-κB binding activity and phosphorylated IκBα levels in the brain tissues. Mice were treated with or without Pam3CSK4 one hr prior to cerebral ischemia (45 min) and reperfusion (6 hrs) (n=6/group). Sham surgical operation served as sham control (n=4/group). The brains were harvested and nuclear and cytoplasmic proteins were isolated. (A) NF-κB binding activity was determined by EMSA. (B) Phospho-IκBα levels were examined by Western blot with specific antibody. * $P<0.05$ compared with indicated groups.

Pam3CSK4 Increased Bcl-2 and Attenuated Bax Levels in Brain Tissues Following Cerebral I/R

Bcl-2 is important for cell survival and anti-apoptosis while Bax promotes apoptosis. We examined the effect of Pam3CSK4 treatment on the levels of Bcl-2 and Bax in the brain tissues following cerebral I/R. Figure 2.4A shows that Pam3CSK4 administration significantly increased the levels of Bcl-2 by 64.8% in the brain tissues following I/R compared with untreated I/R mice. Figure 2.4B shows that cerebral I/R significantly increased the levels of Bax in the brain tissues by 53.2% compared with sham control. In contrast, Pam3CSK4 treatment significantly prevented I/R-increased levels of Bax. The Bax levels in both Pam3CSK4 treated

sham control and I/R mice were significant lower than that of untreated sham and I/R mice (Fig. 2.4B).

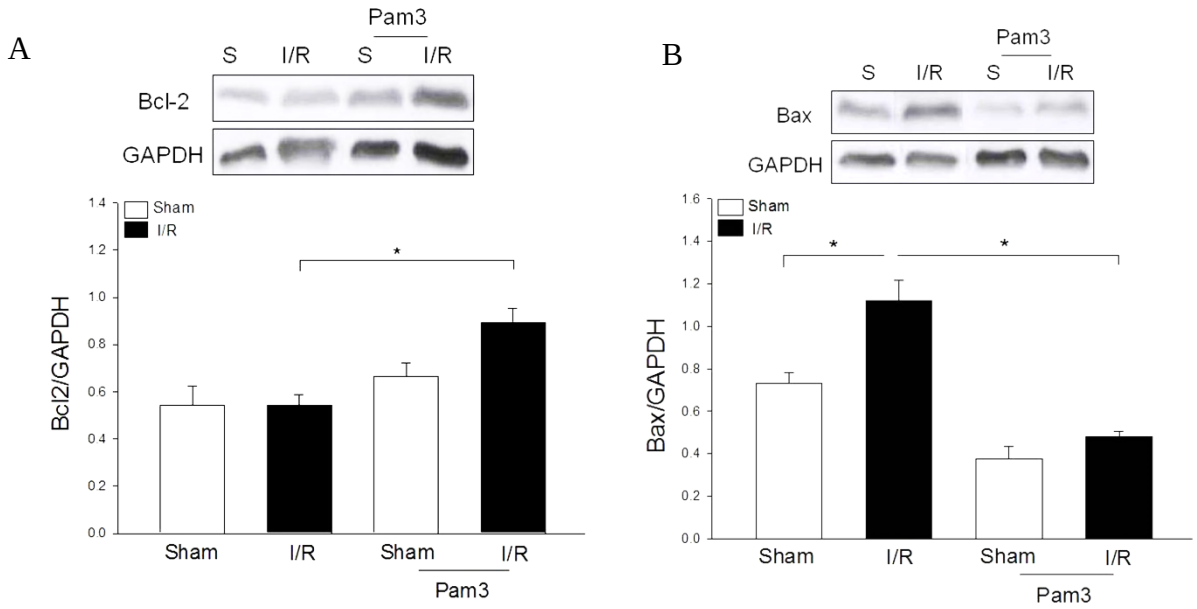


Figure 2.4 Pam3CSK4 administration increases Bcl-2 following cerebral I/R and decreases Bax levels in the brain tissues. Mice were treated with or without Pam3CSK4 one hr prior to cerebral ischemia (45 min) and reperfusion (6 hrs) (n=6/group). Sham surgical operation served as sham control (n=4/group). The brains were harvested and cellular proteins were isolated. Bcl-2 (A) and Bax (B) were examined by Western blot with specific antibodies. * $P<0.05$ compared with indicated groups.

Pam3CSK4 Increased the Levels of Hsp27 and Hsp70 in the Brain Tissues Following Cerebral I/R

Recent evidence has shown that Hsp27 (26, 27) and Hsp70 (28, 29) protect the brain from I/R injury. We examined the effect of Pam3CSK4 administration on the levels of Hsp27 and Hsp70 in the brain tissues following I/R. As shown in Figure 2.5A, I/R significantly increased the levels of Hsp27 in the brain tissues by 46.8% compared with sham control. Pam3CSK4 administration also significantly increased the levels of Hsp27 in the brain tissues in both sham control by 112% and I/R mice by 43.6% compared with that of untreated sham

control and untreated I/R groups. Figure 2.5B shows that cerebral I/R significantly decreased the levels of Hsp70 in the brain tissues by 34.9% compared with sham control. In the Pam3CSK4 treated group, the levels of Hsp70 both in sham control and in the I/R group were significantly higher than that of untreated sham control and untreated I/R groups.

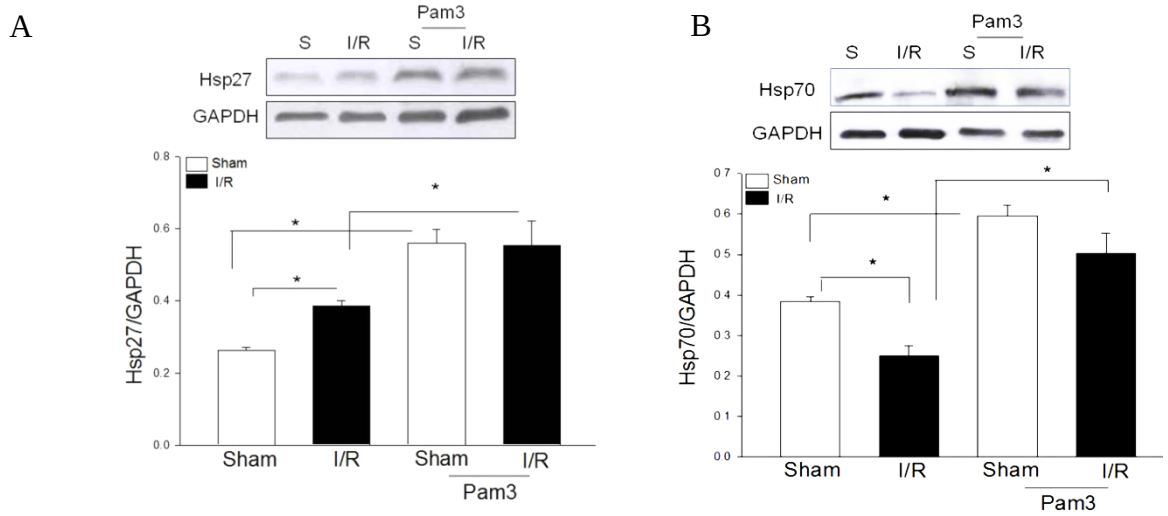


Figure 2.5 Pam3CSK4 administration increases Hsp27 and Hsp70 levels in the brain tissues. Mice were treated with or without Pam3CSK4 one hr prior to cerebral I/R (6 hrs) (n=6/group). Sham surgical operation served as sham control (n=4/group). The brains were harvested and cellular proteins were isolated. The Hsp27 (A) and Hsp70 (B) were examined by Western blot with specific antibodies. * P<0.05 compared with indicated groups.

Pam3CSK4 Treatment Increased the Levels of Phospho-Akt and Phospho-GSK-3β in Brain Tissues Following Cerebral I/R

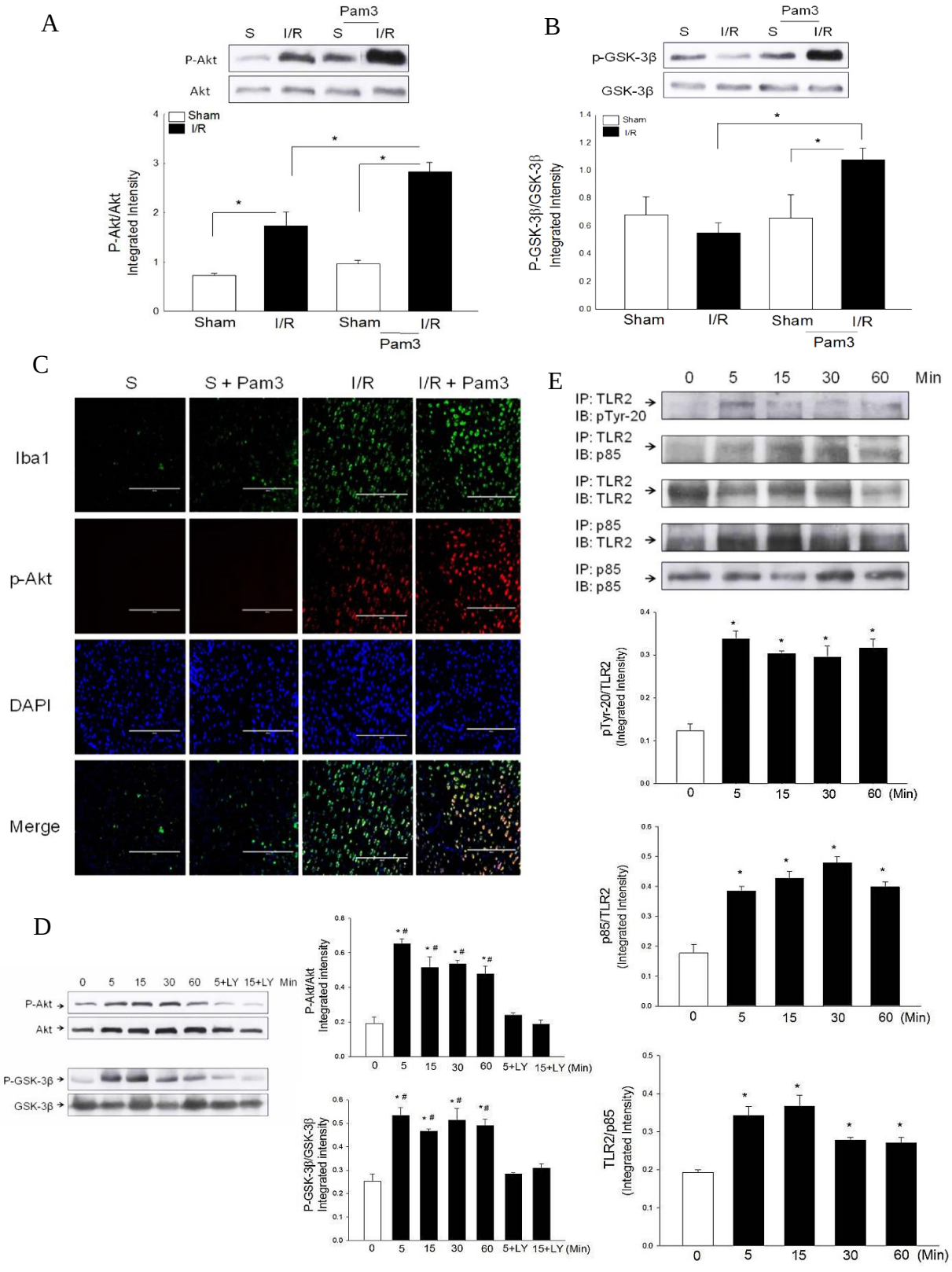
Activation of the PI3K/Akt signaling pathway protects against cerebral I/R injury (18). Recent evidence demonstrated that stimulation of TLR2 activates the PI3K/Akt signaling pathway (20, 21). We examined the effect of Pam3CSK4 on activation of the PI3K/Akt signaling pathway. Figure 2.6A shows that cerebral I/R increased the levels of phospho-Akt in the brain tissues by 140.0% compared with sham control. However, Pam3CSK4 treatment significantly increased the levels of phospho-Akt by 63.5% (2.6A) and phospho-GSK-3β by 95.3% (Figure

2.6B), respectively, compared with the untreated I/R group. Microglial cells are active sensors in response to pathogen associated molecular pattern (PAMP) stimulation (30). We examined whether microglial cells in brain tissue will respond to Pam3CSK4-increased Akt phosphorylation following cerebral I/R *in vivo*. Figure 2.6C shows that cerebral I/R induced Akt phosphorylation in microglial cells compared with sham control. However, Pam3CSK4 treatment increased Akt phosphorylation in microglial cells in response to I/R, which was consistent with Western blot results (Figure 2.6A).

Pam3CSK4 Administration Resulted in Association between TLR2 and the P85 Subunit of PI3K in Cultured Microglial Cells

Pam3CSK4 treatment increased Akt phosphorylation in microglia in brain tissues, therefore, we investigated how Pam3CSK4 treatment activated the PI3K/Akt signaling pathway. First, we examined whether Pam3CSK4 treatment will result Akt and GSK-3β phosphorylation. Microglial cells were treated with Pam3CSK4 (1 μg/ml) for 0, 5, 15, 30 and 60 min, respectively. Figure 2.6D shows that Pam3CSK4 administration significantly increased the levels of phospho-Akt and phospho-GSK-3β in the cells. LY29004 administration prevented Pam3CSK4-increased Akt and GSK-3β phosphorylation.

Next, we also examined whether Pam3CSK4 treatment will induce TLR2 tyrosine phosphorylation followed by association with the p85 subunit of PI3K. Figure 2.6ED shows that Pam3CSK4 stimulation increased TLR2 tyrosine phosphorylation and enhanced TLR2 association with the p85 subunit of PI3K. The data suggests that Pam3CSK4 administration activated the PI3K/Akt signaling pathway through TLR2 tyrosine phosphorylation and association with the p85 subunit of PI3K.


A
Pam3
S
I/R
S
I/R
P-Akt
Akt
Sham
I/R
P-Akt/Akt
Integrated Intensity
Sham
I/R
Sham
I/R
Pam3
B
Pam3
S
I/R
S
I/R
p-GSK-3β
GSK-3β
Sham
I/R
P-GSK-3β/GSK-3β
Integrated Intensity
Sham
I/R
Sham
I/R
Pam3
C
S
S + Pam3
I/R
I/R + Pam3
Iba1
p-Akt
DAPI
Merge
D
0
5
15
30
60
5+LY
15+LY
Min
P-Akt
Akt
P-GSK-3β
GSK-3β
P-Akt/Akt
Integrated intensity
0
5
15
30
60
5+LY
15+LY (Min)
P-GSK-3β/GSK-3β
Integrated intensity
0
5
15
30
60
5+LY
15+LY (Min)
E
0
5
15
30
60
Min
IP: TLR2
IB: pTyr-20
IP: TLR2
IB: p85
IP: TLR2
IB: TLR2
IP: p85
IB: TLR2
IP: p85
IB: p85
pTyr-20/TLR2
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60 (Min)
TLR2/p85
(Integrated Intensity)
0
5
15
30
60 (Min)

Figure 2.6 Pam3CSK4 administration increases the levels of Akt and GSK-3β phosphorylation and association of TLR2 with PI3K in the brain tissues following cerebral I/R. Mice were treated with or without Pam3CSK4 one hr prior to cerebral ischemia (45 min) and reperfusion (6 hrs) (n=6/group). Sham surgical operation served as sham control (n=4/group). The brains were harvested and cellular proteins were isolated. The phospho-Akt (A) and phospho-GSK-3β (B) were examined by Western blot with specific antibodies. * $P<0.05$ compared with indicated groups. (C) Double fluorescent staining of Akt phosporylation in microglial cells in brain tissue. Activated microglial cells were stained by an anti-Iba1 antibody (green); Akt phosphorylation was measured by a specific anti-phospho-Akt antibody (red); Nuclei were stained with DAPI (blue). Merged yellow color indicates phospho-Akt (red) localization in microglial cells (green). Three samples in each group were examined. (D) Treatment of Bv2 microglial cells with Pam3CSK4 increases Akt phosphorylation and (E) association of the p85 subunit of PI3K with TLR2. Microglial cells were treated with Pam3CSK4 for 0, 5, 15, 30, and 60 min, respectively. Cellular proteins were isolated and examined for Akt and GSK-3β phosphorylation (D) and for immunoprecipitation (IP) with specific anti-TLR2 antibody followed by immunoblots (IB) using antibodies to p85 and pTyr20 (E). Each time point was repeated four times. * $P<0.05$ compared with indicated groups or control group. # $P<0.05$ compared with LY treated groups.

Pharmacologic Inhibition of PI3K Abrogates the Protection Against Cerebral I/R Injury in Pam3CSK4-Treated Mice

To examine whether activation of PI3K/Akt signaling will be responsible for protection of the brain from I/R injury in Pam3CSK4-treated mice, we administered the PI3K inhibitor, LY294002 to Pam3CSK4 treated mice. Figure 2.7 shows that PI3K inhibition with LY294002 abrogated Pam3CSK4-induced protection against cerebral I/R injury. The infarct volume was significantly greater in Pam3CSK4 + LY294002 compared with Pam3CSK4 treated mice. There was no significant difference between LY294002 treatment alone and the untreated I/R group.

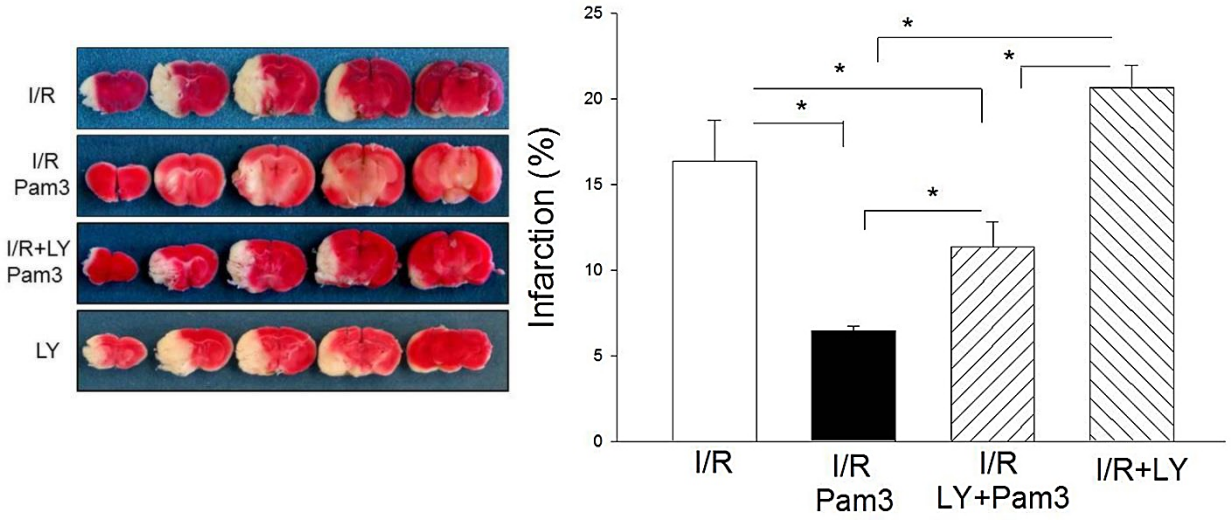

Figure 2.7 Pharmacologic inhibition of PI3K abrogates the protection by Pam3CSK4 against cerebral I/R injury. Mice were treated with or without LY294002 (1 mg/25 gram body weight) 15 min before administration of Pam3CSK4. The mice were subjected to cerebral ischemia (60 min) followed by reperfusion (24 hrs). LY294002 was also administered to mice 15 min prior to I/R. Infarct size was examined by TTC staining. *$p<0.05$ compared with indicated groups. N=8/group.

Discussion

A significant finding in the present study is that administration of the TLR2 ligand, Pam3CSK4, significantly reduced focal cerebral ischemic injury. Importantly, therapeutic administration of Pam3CSK4, 30 min after cerebral ischemia, also significantly protected against cerebral I/R. However, the beneficial effect of Pam3CSK4 was lost in TLR2 deficient mice, suggesting that TLR2 is required for its ligand-induced protection. In addition, PI3K inhibition abolished Pam3CSK4-induced protection. Thus, our data indicate that administration of a TLR2 agonist attenuates focal cerebral I/R injury via a TLR2 and PI3K/Akt dependent mechanism.

TLR-mediated signaling pathways are involved in cerebral I/R injury. For example, deficiency of either TLR4 or TLR2 has been shown to protect the brain from cerebral I/R injury

(2, 6, 6, 12-14). Recently, Hyakkoku et al. reported that deficiency of TLR3 or TLR9 did not show a neuroprotective effect against cerebral I/R (31). Hua et al. reported that both MyD88 or TRIF knockout mice did not show a reduction of cerebral infarction and improvement of neurological deficits following cerebral I/R compared with wild type mice (32, 33). It is well known that all TLRs mediate signaling through MyD88 except TLR3 (8, 9). TLR3 and TLR4 mediate signaling through a TRIF-dependent pathway (8, 9). Collectively, current evidence suggests that TLR2 or TLR4 may recognize endogenous ligands (34) and mediate cerebral ischemic injury through, as yet, unidentified mechanisms.

Recent studies have shown that administration of TLR ligands induced protection against ischemic injury through a preconditioning mechanism (5, 17, 35). For example, pretreatment of mice with either a TLR9 ligand (36) or a TLR2 ligand (5) for 24 hrs significantly protected brain from cerebral ischemic injury. Preconditioning-induced protection requires at least 24 hrs of pretreatment. We have recently shown that administration of TLR2 ligands, either Pam3CSK4 or peptidoglycan, one hr prior to myocardial I/R significantly reduced myocardial infarct size and improved cardiac function (15). However, the myocardial protection by the TLR2 ligands was abolished in TLR2 deficient mice (15), suggesting that TLR2 is required for its ligand-induced protection. In the present study, we observed that administration of Pam3CSK4 one hr prior to cerebral I/R significantly reduced cerebral ischemic injury. More significantly, administration of Pam3CSK4 during the ischemic period also significantly reduced infarct volume following cerebral I/R injury. The data suggests that therapeutic administration of a TLR2 ligand will protect the brain from ischemic insult and may have significant clinical ramifications for the treatment of stroke.

The role of TLR2 deficiency in I/R injury of organs is inconsistent. For example, it has been shown that TLR2 contributes to ischemic injury in liver (37), kidney (38), and heart (39). In contrast, other studies have reported that TLR2 does not contribute to liver I/R Injury (40), protects against the small-bowel I/R injury (41) and could be essential for mediating mesenchymal stem cell-associated myocardial recovery following acute I/R injury (42). Reports on the role of TLR2 deficiency in cerebral I/R injury are also inconsistent. Ziegler et al., and Leemans et al., have reported that TLR2 deficient mice showed decreased infarct volume compared with WT I/R mice (13,14). Whereas blocking TLR2 by an anti-TLR2 antibody (T2.5) did not reduce infarct volume (43). We have previously shown that TLR2 deficiency did not protect against focal cerebral I/R injury (6). In the present study, we also observed that TLR2 deficiency abolished the protection against cerebral I/R injury by Pam3CSK4. It is unclear why there are conflicting observations. The observed differences may be caused by use of different animal models, size of the coated filament used for artery occlusion, animal body weights, different time periods for ischemia/reperfusion and differences in cerebral blood flow.

Activation of PI3K/Akt-dependent signaling protects the brain from I/R injury and prevents neuronal apoptosis (18, 19). Recent evidence has shown that the PI3K/Akt signaling pathway may be an endogenous negative feedback regulator of TLR/NF-κB-mediated pro-inflammatory responses (21, 44). We have previously reported that activation of the PI3K/Akt signaling pathway rapidly induces protection against myocardial I/R injury (15, 24). To investigate whether activation of the PI3K/Akt signaling pathway is involved in TLR2 ligand-induced protection, we examined the levels of phospho-Akt in the brain. We observed that Pam3CSK4 treatment significantly increased the levels of phospho-Akt in the brain tissues following cerebral I/R. Double fluorescent staining of brain tissues showed that Pam3CSK4

treatment increased Akt phosphorylation in microglial cells (Figure 6C), suggesting that microglia may play a role in the response to Pam3CSK4-induced protection against cerebral I/R injury. Indeed, microglial cells are active sensors and versatile effector cells in pathological and pathophysiological brain injury (30) and could be potential therapeutic targets for ischemic brain. Microglial cells are responsible for much of the TLR expression in the brain tissue. A recent study by Tang et al. showed that I/R induced high expression of TLR2 and TLR4 in neurons at early time periods, while 36 hrs after I/R, a high expression of TLR2 was observed in microglia(3). Interestingly, Tang et al. did not observe a response by neurons to the stimulation with TLR2 or TLR4 ligands (3). We observed in the present study that microglial cells responded to the TLR2 ligand, Pam3CSK4, stimulation by activating the PI3K/Akt signaling pathway both *in vivo* and *in vitro*. Published evidence suggests that there is cross-talk between the TLR2 and PI3K/Akt signaling pathways (20, 45). Stimulation of TLR2 resulted in activation of PI3K/Akt-dependent signaling (20, 22). A recent study reported that Mal, an adaptor protein in the TLR-mediated signaling pathway, connects TLR2 to PI3K activation (22). In the present study, we observed that treatment of cultured microglial cells with Pam3CSK4 rapidly induced TLR2 tyrosine phosphorylation and increased the association of the p85 subunit of PI3K with TLR2. When considered together, these data suggest that Pam3CSK4 administration increases phosphorylation of TLR2 with subsequent recruitment of the p85 subunit of PI3K, which results in activation of PI3K/Akt dependent signaling. We speculate that activation of PI3K/Akt signaling in Pam3CSK4 treated mice may be responsible for the protection against cerebral I/R injury. To test this hypothesis, we administered the PI3K inhibitor, LY294002, to Pam3CSK4 treated mice prior to focal cerebral I/R. We observed that pharmacologic inhibition of PI3K with LY294002 partially abrogated the protective effect of Pam3CSK4 on focal cerebral I/R injury.

Thus, we demonstrated that Pam3CSK4-induced protection is mediated, in part, through a TLR2 and PI3K/Akt-dependent mechanism.

The contribution of neuronal apoptosis to cerebral I/R injury has well been documented (46). We observed in the present study that Pam3CSK4 administration significantly attenuated caspase-3 activity in the I/R brain tissues, suggesting that TLR2 ligand administration will prevent apoptosis in the ischemic brain. In addition, activation of the PI3K/Akt signaling pathway has been demonstrated to have an anti-apoptotic effect (47). Therefore, activation of the PI3K/Akt signaling pathway could be an anti-apoptotic mechanism for the TLR2 ligand. To examine whether other anti-apoptotic effectors are involved in Pam3CSK4 attenuation of apoptosis following cerebral I/R, we examined the levels of Bcl-2 and Bax in the brain tissues. Bcl-2 is an important anti-apoptotic and anti-necrotic molecule while Bax is proapoptotic. We observed that Pam3CSK4 treatment significantly increased the levels of Bcl-2 and decreased Bax levels in the brain tissues of sham control and I/R mice. The data suggests that increased Bcl-2 and decreased Bax levels by Pam3CSK4 in the brain tissues could be another mechanism for the TLR2 ligand protection against cerebral ischemic injury.

Hsp27 has been reported to protect the brain from cerebral I/R injury (26, 27). We have previously reported that increased expression of Hsp27 significantly protects the myocardium from doxorubicin-induced cell death (48). In addition, Hsp70 has been shown to play a protective role in focal cerebral I/R (28, 29). In the present study, we observed that Pam3CSK4 treatment increased both Hsp27 and Hsp70 in the brain tissues of sham control and I/R mice. At present, we don't know how modulation of TLR2 by its ligand, Pam3CSK4, resulted in increased levels of Hsp27 and Hsp70 in brain tissues of both sham and I/R mice. Recent studies have suggested that there is a link between the PI3K/Akt signaling pathway and Hsp27 (35, 49)

and Hsp70 (50, 51). For example, we have previously reported that PI3K inhibition attenuated small dose of lipopolysaccharide-increased Hsp27 expression in the myocardium (35, 49). Since other studies have also reported that inhibition of PI3K resulted in decreased expression of Hsp27 and Hsp70 in brain tissues subjected to cerebral I/R, it is possible that Pam3CSK4 administration increased the levels of Hsp27 and Hsp70 in the I/R brain tissues by activation of the PI3K/Akt signaling pathway.

In summary, either prophylactic or therapeutic administration of a specific TLR2 ligand, Pam3CSK4, to mice significantly reduced cerebral I/R injury. TLR2 deficiency or PI3K inhibition abolished Pam3CSK4-induced protection against cerebral I/R injury, suggesting involvement of a TLR2 and PI3K/Akt-dependent mechanism. In addition, Pam3CSK4 increased the levels of Bcl2 and decreased the levels of Bax in I/R brain tissues after I/R, which indicates anti-apoptosis as an additional mechanism of the Pam3CSK4 protective effects against cerebral I/R injury. The data suggest that therapy using a TLR2 specific agonist could be effective in reducing stroke injury.

Acknowledgements

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CHAPTER 3

THE TLR9 LIGAND, CPG-ODN, INDUCES PROTECTION AGAINST CEREBRAL ISCHEMIA/REPERFUSION INJURY VIA ACTIVATION OF PI3K/AKT SIGNALING

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Running head: CpG-ODN Decreases Cerebral Ischemic Injury

Abstract

Background — Toll-like receptors (TLRs) have been shown to be involved in cerebral ischemia/reperfusion (I/R) injury. TLR9 is located in intracellular compartments and recognizes CpG-DNA. This study examined the effect of CpG-ODN on cerebral I/R injury.

Methods and Results —C57BL/6 mice were treated with CpG-ODN by i.p. injection one hr before the mice were subjected to cerebral ischemia (60 min) followed by reperfusion (24 hrs). Scrambled-ODN served as control-ODN. Untreated mice, subjected to cerebral I/R, served as I/R control. The effect of inhibitory CpG-ODN (iCpG-ODN) on cerebral I/R injury was also examined. In addition, we examined the therapeutic effect of CpG-ODN on cerebral I/R injury by administration of CpG-ODN 15 min after cerebral ischemia. CpG-ODN administration significantly decreased cerebral I/R-induced infarct volume by 69.7% (6.4 ± 1.80% vs 21.0 ± 2.85%, $p<0.05$), improved neurological scores, and increased survival rate, when compared with the untreated I/R group. Therapeutic administration of CpG-ODN also significantly reduced infarct volume by 44.7% (12.6 ± 2.03% vs 22.8 ± 2.54%, $p<0.05$) compared with untreated I/R mice. Neither control-ODN, nor iCpG-ODN altered I/R-induced cerebral injury or neurological deficits. Nissl staining showed that CpG-ODN treatment preserved neuronal morphology in the ischemic hippocampus. Immunoblot showed that CpG-ODN administration increased Bcl-2 levels by 41% and attenuated I/R-increased levels of Bax and caspase-3 activity in ischemic brain tissues. Importantly, CpG-ODN treatment induced Akt and GSK-3β phosphorylation in brain tissue and cultured microglial cells. PI3K inhibition with LY294002 abolished CpG-ODN-induced protection.

Conclusions— CpG-ODN significantly reduces cerebral I/R injury via a PI3K/Akt-dependent mechanism. Our data also indicate that CpG-ODN may be useful in the therapy of cerebral I/R injury.

Introduction

Stroke is the third leading cause of death and the leading cause of long-term disability in the United States. About 795,000 Americans suffer a new or recurrent stroke annually[1]. Approximately 610,000 of these are first attacks and 185,000 are recurrent attacks[1]. Cerebral ischemia/reperfusion (I/R) injury (ischemic stroke) accounts for about 83% of all stroke cases[1]. At present, there is no effective treatment for cerebral I/R injury. Numerous studies have demonstrated that the innate immune and inflammatory responses mediated by Toll-like receptors (TLRs) play an important role in cerebral ischemia/reperfusion (I/R) injury[2-4]. TLRs are pattern recognition receptors that play a critical role in the induction of innate immune and inflammatory responses[5]. Recent evidence suggests that TLRs may be the important targets for development of new treatment approaches for cerebral I/R injury[6-10]. We have demonstrated that TLR4 deficiency or TLR2 modulation significantly attenuates brain injury in response to cerebral I/R[10-12]. Published data have also shown that TLR agonists attenuate cerebral I/R) injury through a preconditioning mechanism[9, 13-15].

TLR9 is located intracellularly in endosomes and endoplasmic reticulum[16]. TLR9 recognizes unmethylated CpG-DNA from bacteria and endogenous DNA[16]. Synthetic CpG-oligodeoxynucleotide (ODN) has been reported to activate TLR9[16,17], improve cell survival, prevent cell apoptosis[18], attenuate cardiac dysfunction after I/R[19,20] and improve outcome in shock induced by polymicrobial sepsis[21-23] or trauma hemorrhage[24]. Scholtzova *et al*. reported

that administration of CpG-ODN effectively ameliorated Alzheimer's disease-related pathophysiology[25]. Stevens et al. reported pretreatment of animals with CpG-ODN for 24 hrs induced neuroprotection against ischemic injury through a preconditioning mechanism[14]. Since preconditioning requires a prolonged pretreatment time, it is important to investigate whether therapeutic administration of CpG-ODN will attenuate cerebral I/R injury.

Phosphoinositide 3-kinases (PI3Ks) and their downstream target serine/threonine kinase Akt are a conserved family of signal transduction enzymes which are involved in regulating cellular activation, inflammatory responses, and apoptosis[26]. Activation of PI3K/Akt-dependent signaling plays a role in protection against organ injury in response to I/R[20,27,28], septic shock[23] as well as trauma hemorrhage[24]. Recent evidence has also shown cross talk between PI3K/Akt signaling and TLR-mediated pathways[20, 23, 29]. We have previously reported that activation of PI3K/Akt signaling contributes to the protection against cerebral I/R injury by modulation of TLR2[30].

The goal of the present study was to investigate the effect of CpG-ODN on cerebral I/R injury. We observed that CpG-ODN administration significantly reduced infarct volume and improved neurological functional recovery after cerebral I/R injury. Therapeutic administration of CpG-ODN also markedly decreased infarct volume following cerebral I/R.

Materials and Methods

Animals

Male C57BL/6 mice were purchased from The Jackson Laboratory (Indianapolis, IN). The mice were maintained in the Division of Laboratory Animal Resources at East Tennessee State University (ETSU). The experiments outlined in this article conform to the Guide for the

Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication, 8th Edition, 2011). All aspects of the animal care and experimental protocols were approved by the ETSU Committee on Animal Care.

Focal Cerebral Ischemia/Reperfusion

Focal cerebral I/R was induced by occlusion of the middle cerebral artery (MCAO) on the left side as described in our previous studies[9,10,30,31]. Briefly, mice (23-25 gram body weight) were anesthetized by 5.0% Isoflurane and anesthesia was maintained by inhalation of 1.5% to 2% Isoflurane driven by 100% oxygen flow. Mice were intubated and ventilated using a rodent ventilator at a rate of 110 breaths per min with a total delivered volume of 0.5 ml. Body temperature was regulated at 37°C by surface water heating. Following the skin incision, the left common carotid artery (CCA), the external carotid artery (ECA) and the internal carotid artery (ICA) were carefully exposed. Microvascular aneurysm clips were applied to the left CCA and the ICA. A coated 6-0 filament (6023PK, Doccol Corp. CA, USA) was introduced into an arteriotomy hole, fed distally into the ICA. After the ICA clamp was removed, the filament was advanced 11 mm from the carotid bifurcation, and focal cerebral ischemia started. After ischemia for 60 minutes, the filament and the CCA clamp were gently removed (reperfusion starts). The collar suture at the base of the ECA stump was tightened. The skin was closed, anesthesia discontinued, and the animal allowed to recover in pre-warmed cages. Control mice underwent a neck dissection and coagulation of the external carotid artery, but no occlusion of the middle cerebral artery.

Measurement of Cerebral Blood Flow

Successful occlusion of the middle cerebral artery was verified and recorded by laser-Doppler flowmetry (Model PeriFlux System 5000; Perimed, Stockholm, Sweden) as described previously[30]. Briefly, under anesthesia, a midline incision of the head was made and a probe holder was attached to the skull with crazy glue at 6 mm lateral and 1mm posterior to the bregma. A laser-Doppler probe was connected to the probe holder, and regional cerebral blood flow (CBF) was monitored and recorded. The data was retrieved continuously stored in a computer and analyzed using the Perimed data acquisition and analysis system. Regional CBF was expressed as a percentage of preischemic baseline values.

Experimental Design

To examine the role of CpG-ODN on focal cerebral I/R injury, mice were treated with CpG-ODN (10 µg/25 gram body weight) or control-ODN (n=8, 10 µg/25 gram body weight) by intraperitoneal injection (i.p) one hr prior to cerebral ischemia (60 min) followed by reperfusion.

To evaluate the effect of TLR9 inhibition on cerebral I/R injury, we chose inhibitory CpG-ODN (iCpG-ODN) which binds C-terminal fragment of TLR9 preventing TLR9 activation[32]. iCpG-ODN (n=8, 100 µg/25 gram body weight) was administered to mice by i.p. injection one hr prior to focal cerebral I/R. Mice that did not receive any treatment served as untreated controls. The CpG-ODN (CpG-ODN 1826), Control-ODN (control-ODN 1826), and iCpG-ODN (iCpG-ODN 2088) were purchased from InvivoGen (San Diego, CA) and dissolved in sterile endotoxin free water[20, 23].

To examine the therapeutic effect of CpG-ODN on focal cerebral I/R injury, CpG-ODN was administered to mice 15 min after focal cerebral ischemia (n=8) by i.p injection. Focal cerebral ischemia was continued for an additional 45 min followed by reperfusion for 24 hrs.

To evaluate the effect of CpG-ODN on cerebral functional recovery and survival, CpG-ODN was administered to mice one hr prior to cerebral ischemia (60 min) followed by reperfusion for up to 21 days.

To determine the role of PI3K/Akt signaling in CpG-ODN induced protection against cerebral I/R injury, the PI3K inhibitor LY294002 (1 mg/25 gram body weight) was given to mice 15 min before CpG-ODN administration. The mice were subjected to focal cerebral ischemia (60 min) followed by reperfusion (24 h).

Measurement of Infarct Volume

The infarct volume was determined as described previously[9,10,30,31]. The infarct volume was measured by one blinded to experimental group. After completion of reperfusion, mice were sacrificed and perfused with ice cold phosphate buffered saline (PBS) via the ascending aorta. Brains were removed and sectioned coronally into 2-mm-thick slices. The slices were stained with 2% triphenyltetrazolium chloride (TTC) solution at 37°C for 15 min followed by fixation with 10% formalin neutral buffer solution (pH 7.4). The infarct areas were traced and quantified with an image-analysis system. Unstained areas (pale color) were defined as ischemic lesions. The areas of infarction and the areas of both hemispheres were calculated for each brain slice. An edema index was calculated by dividing the total volume of the left hemisphere by the total volume of the right hemisphere. The actual infarct volume adjusted for edema was calculated by

dividing the infarct volume by the edema index[9,10,30,31]. Infarct volumes are expressed as a percentage of the total brain volume ± S.E.M.

Evaluation of Neuronal Damage in the Hippocampal Formation (HF)

Neuronal damage in brain sections was examined by Nissl staining as described previously[9,10,30,31]. Paraffin sections cut in the coronal plane at approximately 1.5 mm behind bregma with a thickness of 7 microns were deparaffinized and then stained with 0.1% cresyl violet for 2 min. The sections were evaluated using light microscopy.

Evaluation of Neurological Score

Neurological score was performed by a blinded investigator using a neurological evaluation instrument described in our previous studies[9,30]. The neurological score was evaluated by one blinded to experimental group. Briefly, the scoring system included five principle tasks: spontaneous activity over a 3-min period (0–3), symmetry of movement (0–3), open-field path linearity (0–3), beam walking on a 3 cm×1 cm beam (0–3), and response to vibrissae touch (1–3). The scoring system ranged from 0 to 15, in which 15 is a perfect score and 0 is death due to cerebral I/R injury. Sham controls received a score of 15.

In Situ Apoptosis Assay

In situ neuronal cell apoptosis was examined by the TdT-mediated dUTP nick end-labeling (TUNEL) assay (Roche Applied Science, Indianapolis, IN) as described previously[30]. Fields of hippocampus were randomly evaluated for the percentage of apoptotic cells using the TUNEL assay. The images were viewed on an EVOS-fl digital inverted fluorescent microscopy

(Advanced Microscopy Group, Bothell, WA). Total cells were counted in each field, and apoptotic cells are presented as the percentage of total cells counted.

Immunohistochemistry Fluorescent Staining

Fluorescent staining was performed to examine caspase-3 activity and microglia activation after cerebral I/R as described previously[30]. Briefly, brains from each group were harvested and immersion-fixed in 4% buffered parafomaldehyde, embedded in paraffin, cut at 7 μm, and stained with an specific anti-cleaved caspase-3 antibody (Cell Signaling Technology, Inc.) or anti-Iba-1 antibody (Santa Cruz Biotechnology, Inc) as described previously[30]. After washing, the tissue sections were incubated with FITC-conjugated anti-rabbit (GeneTex) for 1 h at 25 °C and covered with fluorescence mounting medium (Life Technologies**,** Grand Island, NY). The images were viewed on an EVOS-fl digital inverted fluorescent microscopy (Advanced Microscopy Group, Bothell, WA). Fields of cortex were randomly examined using a defined rectangular field area for analysis of microglia activation. Total cells were counted in each field, and Iba-1-positive activated microglia cells are presented as the percentage of total cells counted.

In vitro Experiments

BV2 microglial cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and penicillin (Gibco) under 5% CO_2 at 37°C[30]. When BV2 cells reached 70-80% confluence, they were stimulated with CpG-ODN (100 nM) or Control-ODN (100 nM) respectively under normoxic conditions for 15, 30, and 60 min. The cells were harvested and

cellular proteins were isolated for examination of Akt and glycogen synthase kinase (GSK)-3β phosphorylation by Western Blot[30, 33, 34].

In separate experiments, BV2 cells were treated with CpG-ODN (100 nM) for 15, 30, and 60 min in the presence and absence of LY294002 (20 μM). BV2 cells were also treated with 20 μM LY294002 for 60 min. The cells were harvested for preparation of cellular proteins, which were used for examination of Akt phosphorylation by Western Blot. There were 6 replicates in each group.

Western Blot

Cellular proteins were prepared from brain tissues and Western blots were performed as described previously[8-10, 30, 31]. Briefly, the cellular proteins were separated by SDS-PAGE and transferred onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ). The ECL membranes were incubated with the appropriate antibodies respectively (anti-phospho-Akt [Ser473], anti-phospho-GSK-3β [Ser9], anti-Bax, anti-Cleaved Caspase-3 [Cell Signaling Technology], anti-Akt, anti-GSK-3β and anti-Bcl2 [Santa Cruz Biotechnology]) followed by incubation with peroxidase-conjugated secondary Abs (Cell Signlaing Technology). The signals were detected with the ECL system (Amersham Pharmacia). To control for lane loading, the same membrane were probed with anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase, Biodesign, Saco, Maine) after being washed with stripping buffer. The signals were quantified by scanning densitometry using a Syngene G: Box Image Analysis System.

Caspase-3/7 Activity Assay

Caspase-3/7 activity in brain tissue was measured using a Caspase-Glo assay kit (Promega) according to the manufacturer's protocol as described previously[34].

Statistical Analysis

Data is presented in figures as mean ± S.E.M for experimental groups. Group mean levels were compared with analysis of variance (one-way or multifactorial as dictated by the design structure) and the least significant difference procedure (since the F-test was statistically significant). The log-rank test was used to compare group survival trends (Kaplan-Meier plot in Figure 2B). Probability levels of 0.05 or smaller were used to indicate statistical significance.

Results

CpG-ODN Administration Decreased Focal Cerebral Infarct Volume Following I/R

To examine the role of CpG-ODN in focal cerebral I/R injury, we administered CpG-ODN, control-ODN or iCpG-ODN to mice 1 h before the mice were subjected to cerebral ischemia (1 h) and reperfusion (24 h). Figure 3.1A shows that CpG-ODN administration significantly reduced infarct volume by 69.7% compared with the untreated I/R group (6.4 ± 1.80% vs 21.0 ± 2.85%). Administration of either control-ODN or iCpG-ODN to mice did not alter I/R-induced cerebral infarct volume. We also evaluated the therapeutic effect of CpG-ODN on cerebral I/R injury. As shown in Figure 3.1B, therapeutic administration of CpG-ODN at 15 min after ischemia significantly reduced infarct volume by 44.7%, when compared with the untreated I/R group (12.6 ± 2.03% vs 22.8 ± 2.54%). Figure 3.1C shows that cerebral blood flow was significantly reduced by more than 80% immediately following occlusion of middle cerebral

artery and complete reperfusion was restored after the occlusion was released. There was no significant difference in cerebral blood flow between the CpG-ODN, control-ODN, iCpG-ODN and the untreated I/R groups.

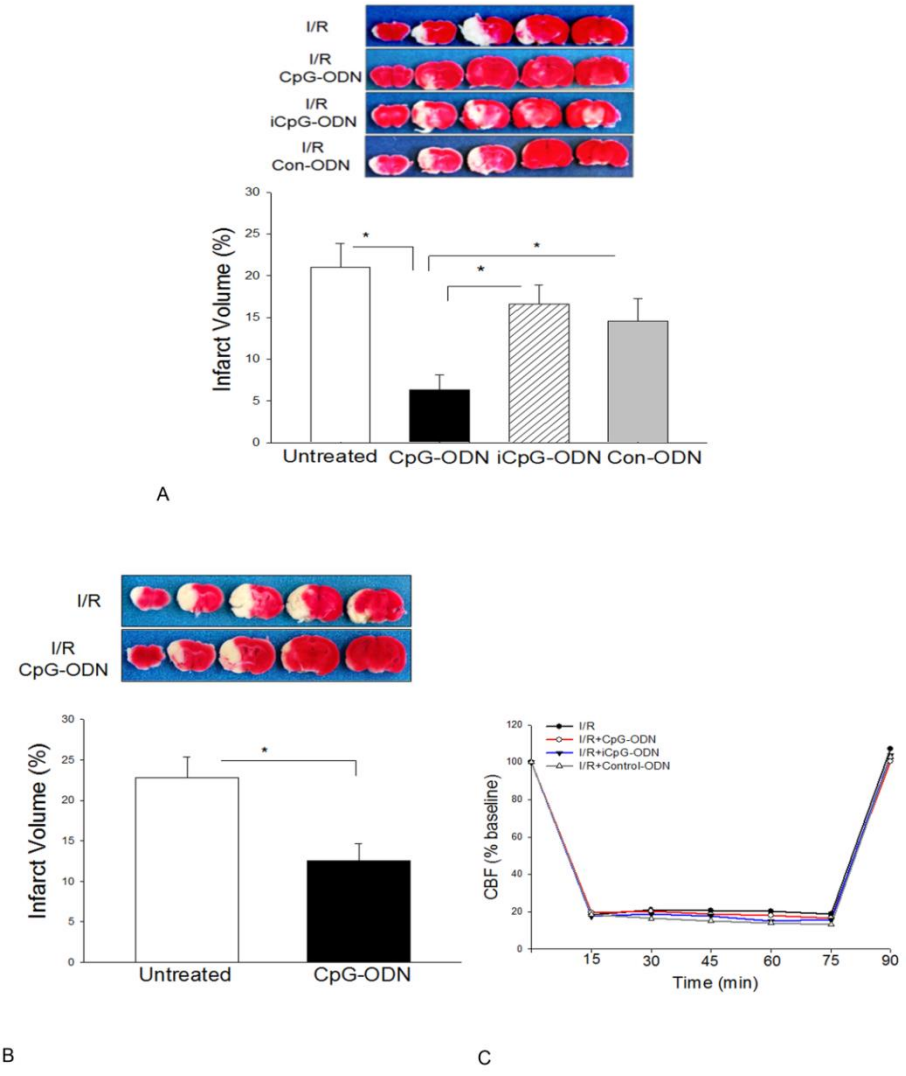


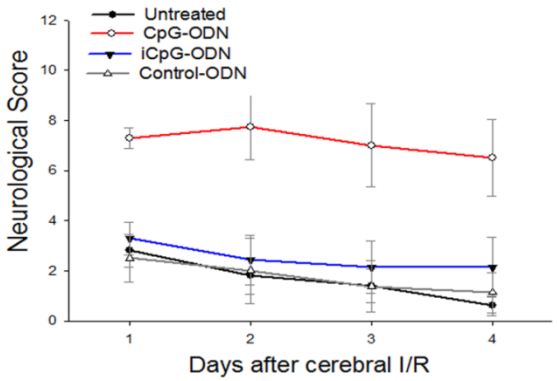
Figure 3.1 CpG-ODN administration reduces infarct volume following cerebral I/R. (A) CpG-ODN (10 µg/25 gram body weight), Control-ODN (10 µg/25 gam body weight) or iCpG-ODN (100 µg/25 g body weight) was administered to mice, respectively, by i.p injection one hr prior to cerebral ischemia (n=8/group). (B) CpG-ODN (10 µg/25 gam body weight) was given to mice 15 min after ischemia by intravascular injection. Mice were subjected to cerebral ischemia (60 min) followed by reperfusion (24 h). Representative image of infarct size from groups are shown on the top of bars (n=8 in untreated and n=6 in post treated group). (C) Cerebral blood flow

measurement before, during and after ischemia (n=6 in untreated, n=6 in CpG-ODN, n=7 in iCpG-ODN, and n=6 in control ODN group). * p <0.05 compared with indicated group.

CpG-ODN Administration Improved Neurological Deficits and Increased Survival Rate Following Focal Cerebral I/R

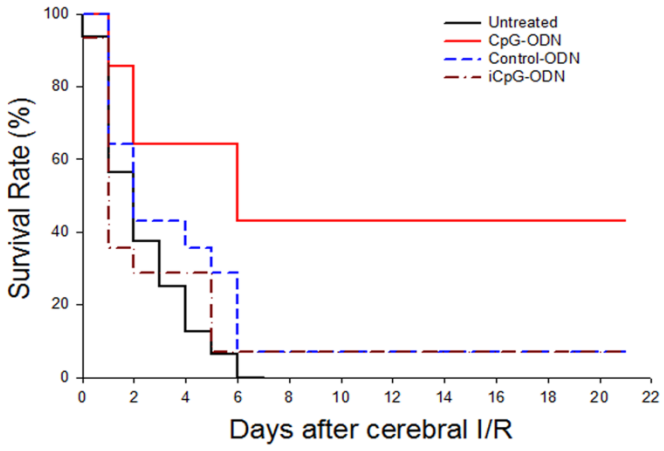
Neurologic score evaluation is an index for the degree of neurologic deficits associated with ischemic stroke[9, 30]. We evaluated the effect of CpG-ODN on neurological deficits following cerebral I/R. Figure 3.2A shows that the neurological score was significantly decreased in untreated I/R mice following cerebral I/R for up to 4 days. In contrast, the neurological scores in CpG-ODN treated I/R mice were significantly greater than in untreated I/R group at all time periods. Administration of either control-ODN or iCpG-ODN did not markedly affect cerebral I/R-induced neurological deficits.

We also evaluated the effect of CpG-ODN administration on survival rate following cerebral I/R. As shown in Figure 3.2B, in untreated mice, 50% of mice died at 48 h and 80% died at 96 h following cerebral I/R. In CpG-ODN treated mice, however, 80% of mice survived at 96 h and 50% survived at 21 days after cerebral I/R. There was no significant difference in the survival rate between control-ODN, iCpG-ODN and untreated I/R group.



A

Figure 3.2 (continued on next page)



B

Figure 3.2 CpG-ODN treatment improves neurological deficits and increases survival rate following cerebral I/R. Mice were treated with CpG-ODN, or Control-ODN, or iCpG-ODN, respectively, one hr prior to cerebral ischemia (60 min) followed by reperfusion for up to 4-21 days. Untreated I/R served as I/R control. Sham operation served as sham control. (A)*in vivo*Neurological function was evaluated using a scoring system ranging 0 to 15, with 15 being a perfect score and 0 being death due to cerebral I/R injury (n=7 in untreated, n=8 in CpG, n=7 in iCpG, n=7 in Control ODN group). (B) Survival rate was evaluated following cerebral I/R (n=16 in untreated, n=14 in CpG, n=14 in iCpG, and n=15 in control ODN group). * p <0.05 compared with indicated group.

CpG-ODN Administration Attenuated Neuronal Damage in the Hippocampal Formation

We evaluated the effect of CpG-ODN administration on neuronal morphology in the hippocampus after cerebral I/R. Nissl staining showed that neuronal damage in the cormu ammonis 1 (CA1) field of the hippocampal formation (HF) is characterized by shrunken cell bodies accompanied by shrunken and pyknotic nuclei in the untreated I/R mice (Fig. 3.3). Similar morphological changes were observed in the dentate gyrus (DG) field. In CpG-ODN treated I/R mice, neuronal damage in the HF was significantly decreased and morphology was

preserved. Administration of control-ODN or iCpG-ODN did not alter I/R-induced neuronal morphological changes in the hippocampus (Fig. 3.3).

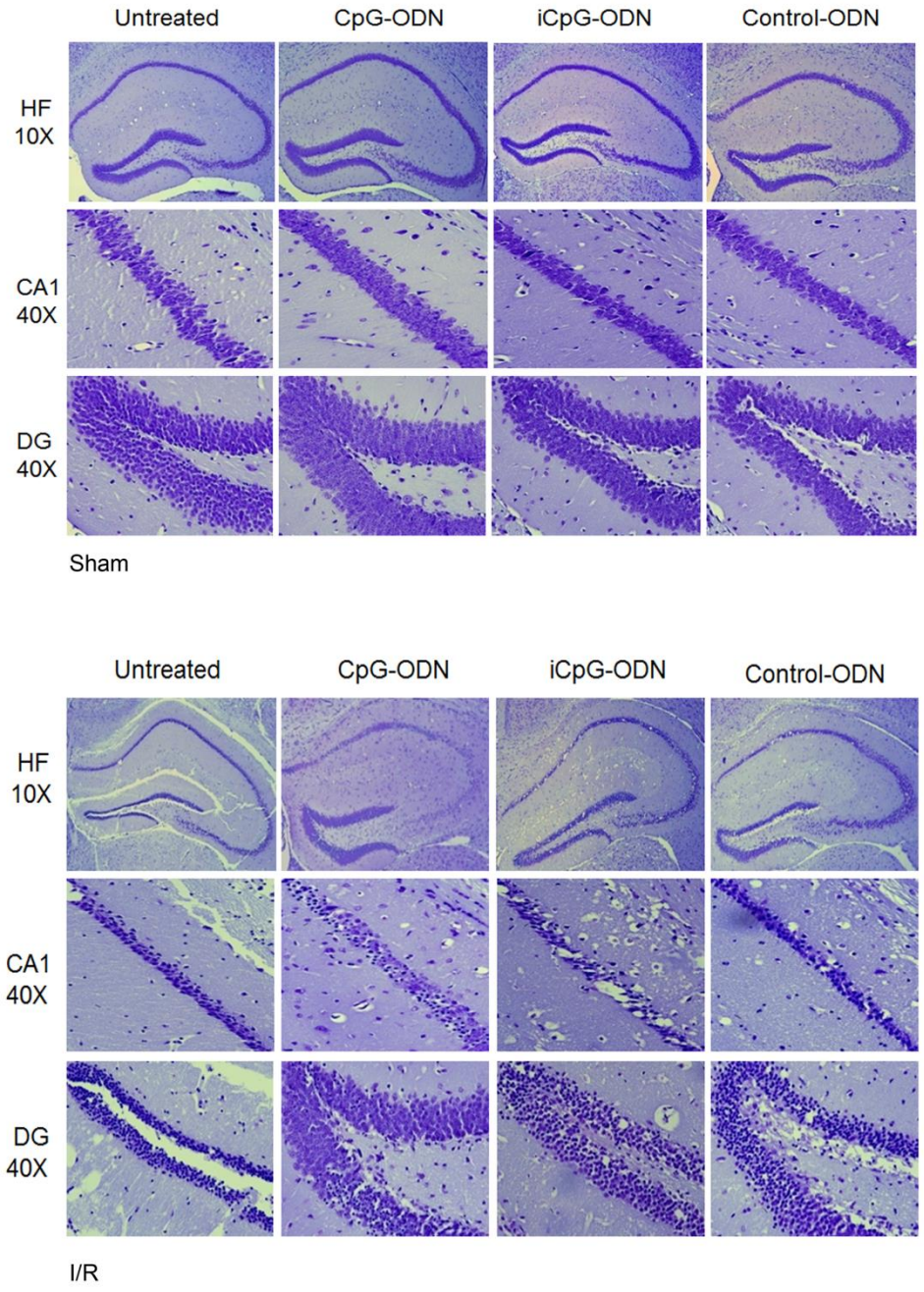


Figure 3.3 CpG-ODN treatment attenuates neuronal damage in the HF following cerebral I/R. Mice were treated with CpG-ODN, or Control-ODN, or iCpG-ODN, respectively, one hr prior to cerebral ischemia (60 min) followed by reperfusion for 24 h. Sham operation served as sham

control. Brains were harvested, sectioned, and stained with 0.1% cresyl violet (n=4 in each group).

CpG-ODN Administration Attenuated Apoptosis in the Brain Following Cerebral I/R Injury

Cerebral I/R induced apoptosis plays a role in brain tissue injury in response to I/R[35]. We examined whether administration of CpG-ODN will attenuate cerebral I/R-induced apoptosis in the brain tissues. TUNEL assay showed that cerebral I/R significantly induced apoptosis in the fields of CA1 and cortex (Figure 3.4A). In CpG-ODN treated I/R mice, however, the numbers of apoptotic nuclei were significantly reduced by 78.8% in CA1 region and by 91.7% in the cortex, respectively, when compared with untreated I/R mice. Cerebral I/R also significantly induced caspase-3 activity as evidenced by showing positive fluorescent staining with a specific anti-cleaved caspase-3 antibody (Figure 3.4B), when compared with sham control. Caspase-3/7 activity was also significantly increased in the brain tissues following cerebral I/R (Figure 3.4C). In contrast, CpG-ODN administration markedly reduced the numbers of positive fluorescent staining cells and attenuated caspase-3/7 activity, when compared with untreated I/R group. Treatment of mice with control-ODN or iCpG-ODN did not significantly alter cerebral I/R induced apoptosis and caspase-3/7 activity in the brain tissues.

CpG-ODN Administration Increased Bcl-2 and Attenuated Bax Levels in Brain Tissues Following Cerebral I/R

Bcl-2 is important for cell survival and anti-apoptosis while Bax promotes apoptosis[36]. We examined the effect of CpG-ODN administration on the levels of Bcl2 and Bax in the brain tissues following cerebral I/R. As shown in Figure 3.4D and Figure 3.4E, cerebral I/R increased the levels of Bax by 126.7% in the brain tissues compared with sham control. In contrast, CpG-

ODN administration prevented I/R-increased Bax levels in the brain tissues. CpG-ODN treatment also significantly increased Bcl2 levels by 41% in the brain tissues following cerebral I/R compared with untreated I/R mice (1.24 ± 0.10 vs 0.88 ± 0.14). Administration of either control-ODN or iCpG-ODN to mice did not affect the levels of Bax and Bcl2 in the brain tissues following I/R.

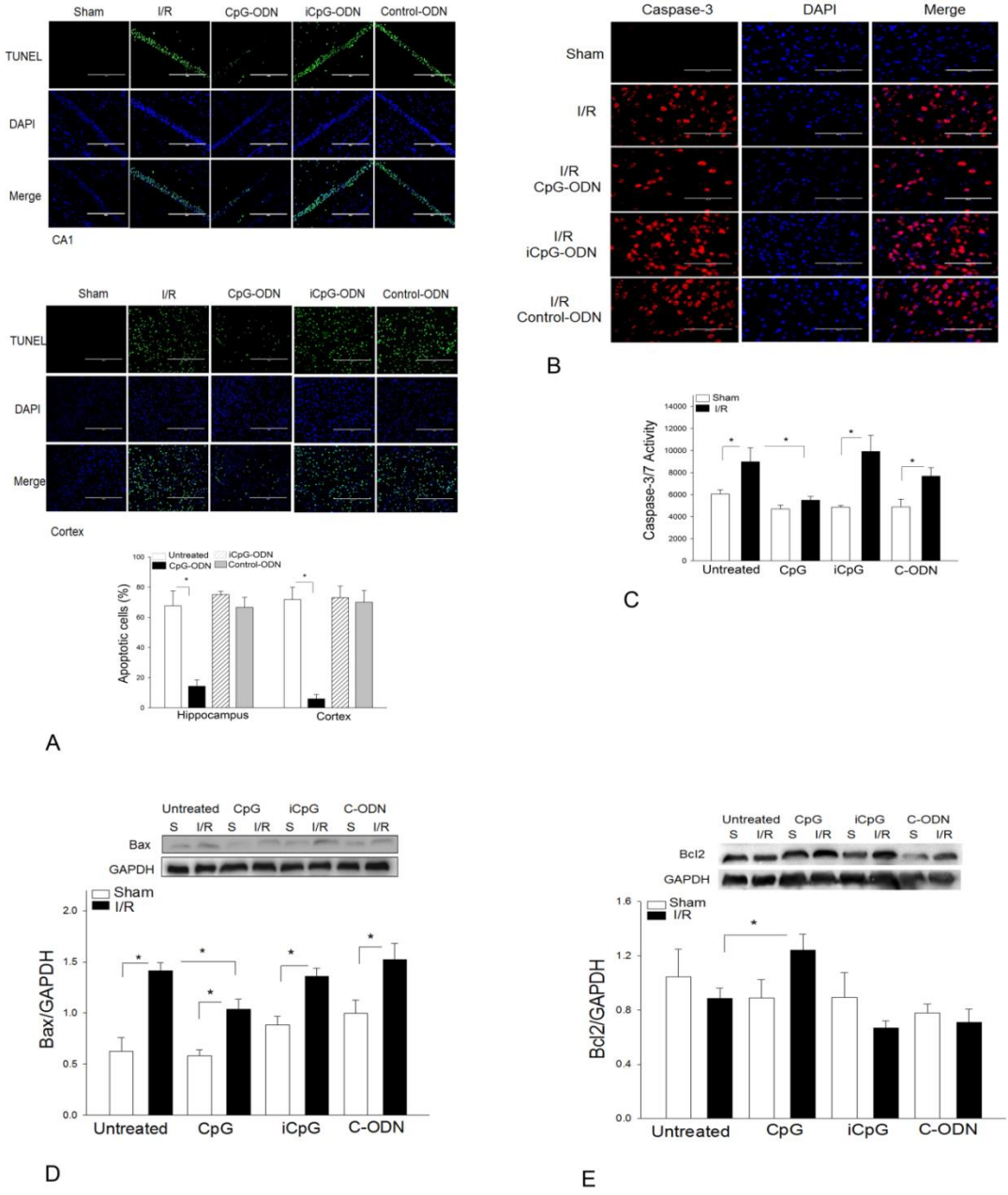


Figure 3.4 CpG-ODN administration attenuates I/R-induced apoptosis in the brain tissues. Mice were treated with CpG-ODN, or Control-ODN, or iCpG-ODN, respectively, one hr prior to cerebral ischemia (60 min) followed by reperfusion for 24 hrs (n=6/group). Sham surgical operation served as sham control (n=4). Brains were harvested and sectioned. Cellular proteins were prepared from the remaining brain tissues. (A) Apoptosis in brain tissue were examined by TUNEL assay (n=3/group) and (B) caspase-3 activity was stained by cleaved caspase-3 antibody (red). The nuclei were stained by DAPI (blue). (C) Caspase-3/7 activity was measured using Caspase-Glo 3/7 assay kit. (D, E) CpG-ODN increased Bcl2 (D) and decreased Bax (E) levels in the brain tissues following cerebral I/R. * $P<0.05$ compared with indicated groups.

CpG-ODN Administration Attenuated Cerebral I/R-Induced Microglial Cell Activation

Microglia are active sensor and effector cells in the pathophysiological brain injury[37]. We have previously reported that cerebral I/R induced microglia activation in brain tissues[30]. We examined whether CpG-ODN administration will attenuate cerebral I/R-induced microglial cell activation. Figure 3.5 shows that cerebral I/R induced microglial cell activation as evidenced by anti-Iba-1 positive staining cells in the cortex region of brain (green color). I/R also induced caspase-3 activity (red color) in microglial cells (yellow color in merge). In CpG-ODN treated mice, however, the numbers of anti-Iba-1 positive staining cells were significantly decreased compared with untreated I/R mice (52.2 ± 3.8% vs 74.9 ± 3%). CpG-ODN administration also markedly reduced caspase-3 activity in microglial cells. Administration of either control-ODN or iCpG-ODN did not markedly alter I/R-induced activation of microglial cells and caspase-3 activity in microglial cells.

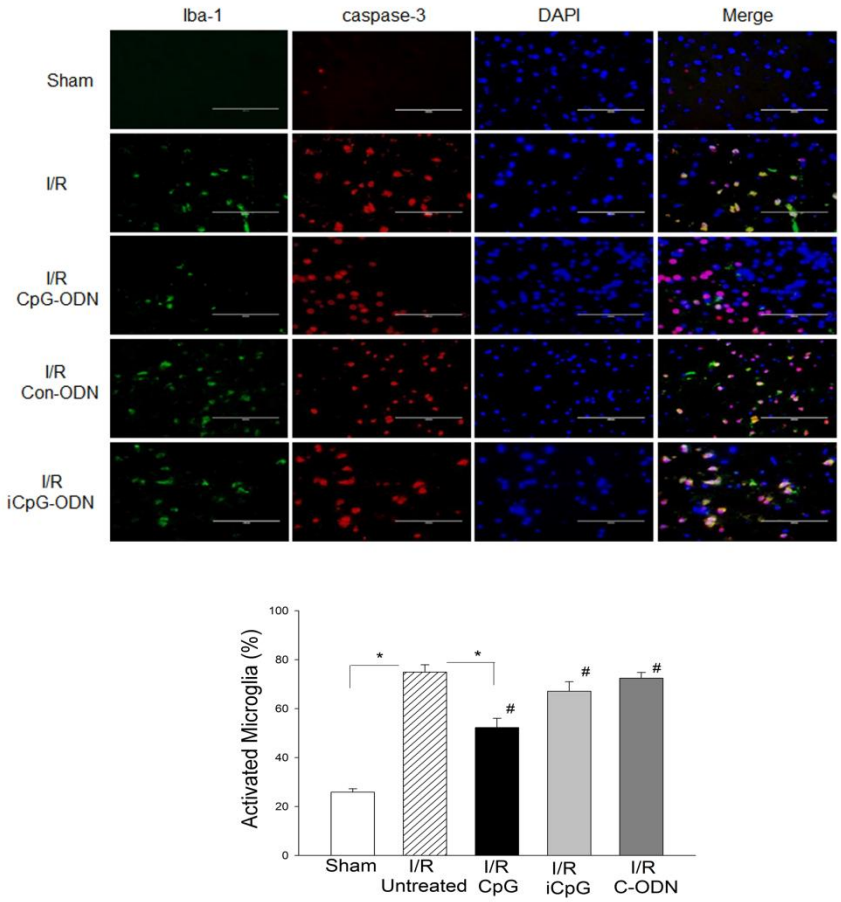


Figure 3.5 CpG-ODN administration attenuates I/R-induced caspase-3 activity in microglial cells in the brain tissues. Mice were treated with CpG-ODN, or Control-ODN, or iCpG-ODN, respectively, one hr prior to cerebral ischemia (60 min) followed by reperfusion for 24 hrs. Sham surgical operation served as sham control. Brains were harvested and sectioned. Activation of microglia was examined with specific antibody against Iba1 (green) and caspase-3 activity was measured by anti-cleaved-caspase-3 antibody (red). Nuclei were stained with DAPI (blue). Caspase-3 activity in activated microglial cells were yellow color (merge). (n=3/group). *P<0.05 compared with indicated groups. # P<0.05 compared with sham group.

CpG-ODN Treatment Increased the Levels of Phospho-Akt and Phospho-GSK-3β in the Brain Tissues

Activation of the PI3K/Akt signaling pathway induces protection against cerebral I/R[30,38]. We examine the effect of CpG-ODN on the activation of PI3K/Akt signaling in the brain tissues. Figure 3.6A shows that CpG-ODN treatment significantly increased the levels of phospho-Akt in the brain tissues of sham control mice. Cerebral I/R increased the levels of phospho-Akt compared with sham control. However, the levels of phospho-Akt in CpG-ODN treated mice were further increased following cerebral I/R, when compared with untreated I/R group.

GSK-3β is an important downstream target of Akt[26]. Figure 3.6B shows that CpG-ODN administration markedly increased the levels of phospho-GSK-3β in sham control mice. The levels of phosphor-GSK-3β in CpG-ODN treated mice were further increased following cerebral I/R compared with untreated I/R group. Treatment of mice with either control-ODN or iCpG-ODN did not significantly alter the levels of phospho-Akt and phosopho-GSK-3β in the brain tissues with and without cerebral I/R.

CpG-ODN Induced Akt and GSK Phosphorylation in Cultured Microglial Cells

We performed *in vitro* experiments using microglial cells (BV2) to investigate whether CpG-ODN can activate PI3K/Akt signaling. BV2 cells were treated with CpG-ODN for 0, 15, 30 and 60 min, respectively. Control-ODN served as control. Figure 3.6C, D shows that CpG-ODN treatment significantly induced Akt (Figure 3.6C) and GSK-3β (Figure 3.6D) phosphorylation at 30 and 60 min, compared with untreated group. PI3K inhibition by LY294002 completely prevented CpG-ODN-induced Akt phosphorylation (Figure 3.6E). Control-ODN did not markedly stimulate Akt and GSK-3β phosphorylation in microglial cells.

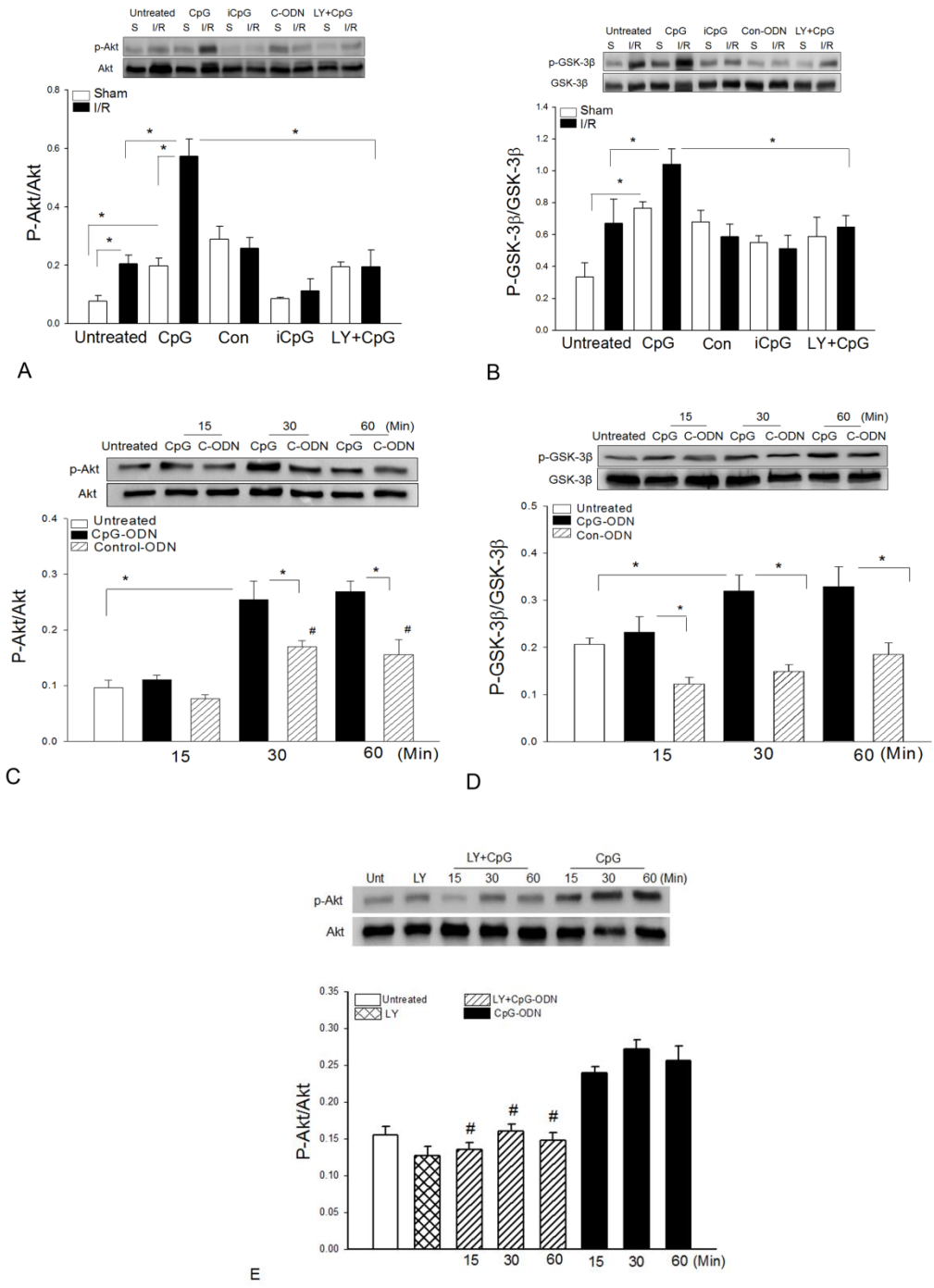


Figure 3.6 CpG-ODN administration increases the levels of Akt and GSK-3β phosphorylation in the brain tissues and in cultured microglial cells. Mice were treated with CpG-ODN (n=7), or Control-ODN (n=7), or iCpG-ODN (n=6), respectively, one hr prior to cerebral ischemia (60 min) followed by reperfusion (24 hrs). Untreated I/R mice served as I/R control (n=6). Sham surgical operation served as sham control (n=4). Brains were harvested and cellular proteins

were prepared. The phospho-Akt (A) and phospho-GSK-3β (B) were examined by Western blot with specific antibodies. (C, D) Microglial cells (BV2) were treated with CpG-ODN or Control–ODN for 15, 30, and 60 min. The cells were harvested and cellular proteins were prepared Western blot examination of (C) Akt and (D) GSK-3β phosphorylation. (E) Microglial cells (BV2) were treated with CpG-ODN for 15, 30, and 60 min in the presence and absence of LY294002. PI3K inhibition by LY294002 prevents CpG-ODN-induced Akt phosphorylation in cultured microglial cells (n=6 replicates/group). * $P<0.05$ compared with indicated groups. $^{\#}P<0.05$ compared with the respective CpG-ODN group.

Inhibition of PI3K/Akt Abolished CpG-ODN-Induced Protection Against Cerebral I/R Injury

To determine whether activation of PI3K/Akt signaling contributes to CpG-ODN induced protection against cerebral I/R injury, we treated mice with LY294002, a specific PI3K inhibitor[20, 23, 30] 15 min prior to CpG-ODN administration. As shown in Figure 3.7, CpG-ODN administration markedly reduced infarct volume compared with untreated I/R mice. However, the infarct volume in CpG-ODN treated mice was comparable with untreated I/R mice, when LY294002 was administered. Thus, PI3K/Akt inhibition by LY294002 completely abolished protection against cerebral I/R injury by CPG-ODN. There was no significant difference in the infarct volume between CpG-ODN-LY294002 I/R group and untreated I/R mice. Figure 3.6A & B show that LY294002 treatment prevented CpG-ODN-induced increases in the levels of phospho-Akt and phospho-GSK-3β in the brain tissues.

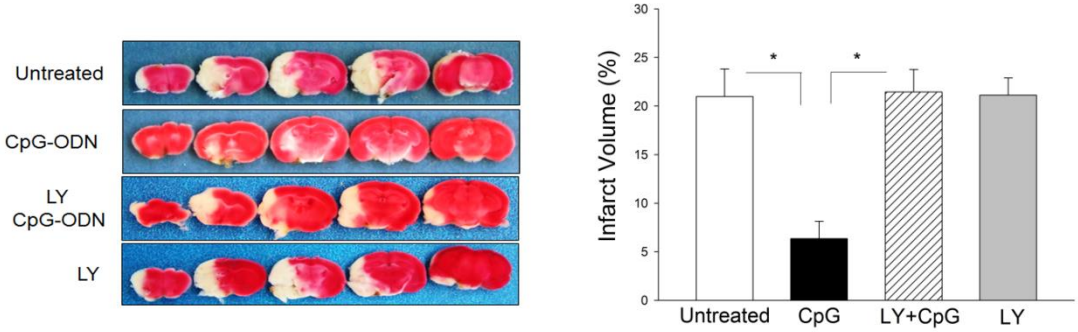


Figure 3.7 Pharmacologic inhibition of PI3K abrogates CpG-ODN-induced protection against cerebral I/R injury. Mice were treated with or without LY294002 (1 mg/25 gram body weight) 15 min before administration of CpG-ODN. The mice were subjected to cerebral ischemia (60 min) followed by reperfusion (24 h). LY294002 was also administered to mice 15 min prior to I/R. Infarct size was examined by TTC staining. n=8/group. * $p<0.05$ compared with indicated groups.

Discussion

The present study has shown that administration of CpG-ODN, a TLR9 ligand, to mice one hr prior to cerebral I/R significantly reduced infarct volume, attenuated neurological deflects and improved survival rate following cerebral I/R injury. More significantly, therapeutic administration of CpG-ODN, 15 min after ischemia, also markedly decreased cerebral I/R-induced infarct volume. In addition, CpG-ODN administration significantly increased the levels of phospho-Akt and phospho-GSK3β in the brain tissues. PI3K inhibition abolished CpG-ODN-induced protection against cerebral I/R injury. Thus our data suggest that CpG-ODN attenuates focal cerebral I/R injury via a PI3K/Akt dependent mechanism.

Innate immune and inflammatory responses are known to be involved in cerebral I/R injury[2-4]. TLRs play a critical role in the induction of innate immune responses[5]. TLRs activate signaling through MyD88 and/or TRIF[5]. Recent evidence has indicated that TLRs contribute to cerebral I/R injury[6,10,10-12,39]. For example, TLR4 deficiency protects the brain from cerebral I/R injury[6,10,10-12,39]. Hua et al. reported that MyD88 or TRIF knockout mice did not show a reduction of cerebral infarction or improvement of neurological deficits following cerebral I/R[40,41]. This observation indicated that the signaling mediated by MyD88 or TRIF may be required for protection. Indeed, we have previously reported that modulation of TLR2 by its ligand, Pam3CSK4 significantly reduced infarct volume and improved neurological score following

cerebral I/R injury[30]. Recently Stevens et al. reported that activation of TLR9 by its agonist, CpG-ODN, significantly induced protection against ischemic stroke[14]. Hyakkoku et al. reported that TLR3 or TLR9 deficiency did not show a neuroprotective effect against cerebral I/R[42], indicating that TLR3 or TLR9 may serve a protective role in cerebral I/R injury. Indeed, we demonstrated in the present study that administration of CpG-ODN decreased infarct volume and improved neurological score following cerebral I/R. We also observed that therapeutic administration of CpG-ODN decreased infarct volume. Collectively, the data suggest that modulation of TLR9 by its ligand CpG-ODN could be an approach for the treatment and management of ischemic stroke.

Cerebral I/R induces neuronal apoptosis which contributes to brain injury in response to I/R[35]. It is well known that Bax is a pro-apoptotic molecule whereas Bcl2 is an important anti-apoptotic molecule[36]. We observed that cerebral I/R significantly increased the levels of Bax and caspase-3/7 activity in the brain tissues, which are consistent with the data showing cerebral I/R, induced apoptotic cells in the brain tissues. Importantly, CpG-ODN administration significantly decreased I/R-induced apoptosis and attenuated I/R-increased levels of Bax and caspase-3/7 activity in the brain tissues. In addition, CpG-ODN markedly increased the levels of Bcl2 both in sham control and I/R mice. The data indicate that the anti-apoptotic effect of CpG-ODN[43] may be one of the mechanisms by which CpG-ODN attenuated cerebral I/R injury.

Microglia are active sensors and versatile effector cells in pathological and pathophysiological brain injury[37]. Interestingly, a recent study by Burguillos et al. reported that activation of caspase-8 is associated with microglial activation[44]. Activated microglia release substances that cause neuronal injury[37,45]. We have observed that cerebral I/R induces microglial activation in brain tissues. However, CpG-ODN treatment prevented I/R-induced activation of

microglial cells, suggesting that CpG-ODN can prevent microglial activation. Microglial cells are responsible for much of the TLR expression in brain tissue[45]. We have previously reported that TLR2 modulation increased PI3K/Akt signaling in microglial cells[30]. CpG-ODN treatment also increased the levels of Akt and GSK-3β phosphorylation, which may be a possible mechanism for preventing microglial activation by CpG-ODN.

We observed in the present study that CpG-ODN administration significantly induced Akt and GSK-3β phosphorylation both *in vivo* and *in vitro*, indicating that CpG-ODN can activate the PI3K/Akt signaling pathway[20,30]. We have previously reported that activation of PI3K/Akt signaling attenuates cerebral I/R-induced brain injury and neuronal apoptosis[30], protects the myocardium from I/R injury[20] and improves outcome of polymicrobial sepsis[23]. Activation of PI3K/Akt signaling has been reported to protect cells from apoptosis induced by I/R[27,33,46]. Activated PI3K/Akt can inhibit Bax conformational change, thus preventing Bax from translocating and integrating into mitochondrial membranes. PI3K/Akt activation also phosphorylates Bim, leading to dissociation of Bim from Bcl2. In addition, activation of PI3K/Akt may be a negative feedback regulator that prevents excessive innate immune and/or inflammatory responses[47,48].

We have previously reported that treatment of cardiomyoblasts with CPG-ODN induced TLR9 tyrosine phosphorylation followed by association with the p85 subunit of PI3K[20,23], resulting in activating PI3K/Akt signaling. We have observed in the present study that CpG-ODN-induced activation of PI3K/Akt signaling both *in vivo* and *in vitro* cultured microglial cells, indicating that activation of the PI3K/Akt signaling pathway may contribute to the CpG-ODN-induced protective effect against cerebral I/R injury. To evaluate our hypothesis, we treated mice with a PI3K specific inhibitor, LY294002, before CpG-ODN administration. We observed that

PI3K inhibition completely abolished CpG-ODN-induced protection against cerebral I/R injury. The data suggests that CpG-ODN induced protection against cerebral is mediated via the PI3K/Akt dependent mechanisms.

In summary, administration of CpG-ODN significantly decreased I/R-induced infarct volume and improved neurological score following cerebral I/R. Therapeutic administration of CpG-ODN also markedly reduced I/R-induced infarct volume. CpG-ODN-induced protection against cerebral I/R injury is mediated through activation of PI3K/Akt signaling. The data suggest that the CpG-ODN may be a new approach for the management and treatment of cerebral I/R injury.

Acknowledgements

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CHAPTER 4

TOLL-LIKE RECEPTOR 3 PLAYS A ROLE IN MYOCARDIAL INFARCTION AND ISCHEMIA/REPERFUSION INJURY (*168*)

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Abstract

Innate immune and inflammatory responses mediated by Toll like receptors (TLRs) have been implicated in myocardial ischemia/reperfusion (I/R) injury. This study examined the role of TLR3 in myocardial injury induced by two models, namely, myocardial infarction (MI) and I/R. First, we examined the role of TLR3 in MI. TLR3 deficient ($TLR3^{-/-}$) and wild type (WT) mice were subjected to MI induced by permanent ligation of the left anterior descending coronary artery (LAD) for 21 days. Cardiac function was measured by echocardiography. Next, we examined whether TLR3 contributes to myocardial I/R injury. $TLR3^{-/-}$ and WT mice were subjected to myocardial ischemia (45 min) followed by reperfusion for up to 3 days. Cardiac function and myocardial infarct size were examined. We also examined the effect of TLR3 deficiency on I/R-induced myocardial apoptosis and inflammatory cytokine production. $TLR3^{-/-}$ mice showed significant attenuation of cardiac dysfunction after MI or I/R. Myocardial infarct size and myocardial apoptosis induced by I/R injury were significantly attenuated in $TLR3^{-/-}$ mice. TLR3 deficiency increases Bcl2 levels and attenuates I/R-increased Fas, FasL, FADD, Bax and Bak levels in the myocardium. TLR3 deficiency also attenuates I/R-induced myocardial NF-κB binding activity, TNF-α and IL-1β production as well as I/R-induced infiltration of neutrophils and macrophages into the myocardium. TLR3 plays an important role in myocardial injury induced by MI or I/R. The mechanisms involve activation of apoptotic signaling and NF-κB binding activity. Modulation of TLR3 may be an effective approach for ameliorating heart injury in heart attack patients.

Introduction

Cardiovascular disease is the number one killer in the United States [27]. Each year, an estimated 785,000 Americans will have a new coronary attack, 470,000 will have a recurrent attack and 195,000 Americans will have silent myocardial infarctions [27]. Despite extensive investigation, the cellular and molecular mechanisms that are involved in the initiation and progress of myocardial injury in response to ischemia/reperfusion (I/R) are still unclear.

Innate immune and inflammatory responses mediated by Toll-like receptors (TLRs) have been demonstrated to be involved in the pathophysiology of myocardial I/R injury [3, 23]. TLRs are pattern recognition receptors that play an important role in the induction of innate immune and inflammatory responses [21, 42]. TLR-mediated signaling predominately activates nuclear factor KappaB (NF-κB) which is an important transcription factor regulating the expression of genes associated with innate immunity and inflammatory responses as well as cell growth, cell survival, and cell death [21,42]. We and others have reported that TLR4 deficiency or modulation of TLR4 mediated signaling decreases myocardial injury following I/R [1, 3, 4, 13, 17, 23].

TLR3 is located in intracellular endosomes and recognizes double-stranded RNA (dsRNA) and polyinosinic-polycytidylic acid (Poly I: C, a synthetic analog of dsRNA), resulting in induction of antiviral immune responses [20]. Recently, Cavassani et al. reported that TLR3 deficient ($TLR3^{-/-}$) mice showed an increased survival rate in cecal ligation and puncture induced sepsis [2]. We have shown that $TLR3^{-/-}$ mice exhibit protection against polymicrobial sepsis-induced cardiac dysfunction [10]. These data suggest that TLR3 plays an important role in cardiac function during sepsis. However, whether TLR3 contributes to myocardial injury induced by myocardial infarction or I/R has not been investigated. It is possible that TLR3 plays

a role in myocardial ischemic injury by recognition of endogenous ligands, i.e. damage-associated molecular patterns (DAMPs) that are released during myocardial I/R injury.
We hypothesized that TLR3 contributes to myocardial injury by recognition of DAMPs during myocardial I/R. To evaluate our hypothesis, we examined the role of TLR3 in myocardial injury induced by either permanent ligation-induced myocardial infarction (MI) or ischemia/reperfusion (I/R) using TLR3 deficient ($TLR3^{-/-}$) mice. We observed that TLR3 deficiency significantly attenuates myocardial dysfunction induced by models, i.e MI and I/R. TLR3 deficiency also reduces infarct size and myocardial apoptosis after I/R injury. Our data indicate that TLR3 plays an important role in myocardial ischemic and I/R injury.

Materials and Methods

Animals

TLR3 knockout mice ($TLR3^{-/-}$) and wild type (WT) genetic background control mice (C57BL/6) were obtained from Jackson Laboratory (Indianapolis, IN) [10]. The mice were maintained in the Division of Laboratory Animal Resources at East Tennessee State University (ETSU). The experiments outlined in this article conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication, 8th Edition, 2011). All aspects of the animal care and experimental protocols were approved by the ETSU Committee on Animal Care.

Models of Myocardial Infarction (MI) and Ischemia/Reperfusion (I/R) Injury

Myocardial infarction was induced by permanent ligation of the left anterior descending (LAD) coronary artery as described previously [18]. Myocardial I/R injury was induced as

described previously [11, 13, 17, 38]. Briefly, TLR3$^{-/-}$ and age-matched WT male mice (26-28 gram body weight) were anaesthetized by 5.0% isoflurane inhalation, intubated and ventilated with room air using a rodent ventilator. Anesthesia was maintained by inhalation of 1.5% isoflurane driven by 100% oxygen flow. Body temperature was regulated at 37^{o}C by surface water heating. Following the skin incision, the hearts were exposed through a left thoracotomy in the fourth intercostal space. For induction of MI, the LAD coronary was permanently ligated with 8-0 silk ligature [18]. For induction of I/R injury, the LAD coronary artery was ligated with 8-0 silk ligature that was tied using a 'shoestring knot' over a 1 mm polyethylene tube (PE-10). After completion of 45 min of occlusion, the coronary artery was reperfused by pulling on the exteriorized suture to release the knot. Cardiac function was measured by echocardiography [26, 38]. After completion of the experiments, the mice were euthanized by CO2 inhalation and the hearts were harvested.

Evaluation of Myocardial Infarct Size

Myocardial infarct size was evaluated by triphenyltetrazolium chloride (TTC, Sigma-Aldrich) staining as described previously [11, 13, 17, 38]. Briefly, the hearts were perfused with saline on a Langendorff system to wash blood from the coronary vasculature. The LAD coronary artery was re-ligated at the previous site of ligation prior to staining with 1% Evans Blue in order to assess area at risk. Each heart was then sliced horizontally to yield five slices. The slices were incubated in 1% TTC for 15 min at 37°C, fixed by immersion in 10% neutral buffered formalin. The area of infarction on both sides of each slice was determined by an image analyzer, corrected for the weight of each slice, and summed for each heart. Ratios of risk area vs. left

ventricle area (RA/LV) and infarct area vs. risk area (IA/RA) were calculated and expressed as a percentage.

Echocardiography

Transthoracic two-dimensional M-mode echocardiogram and pulsed wave Doppler spectral tracings were obtained using a Toshiba Aplio 80 Imaging System (Toshiba Medical Systems, Tochigi, Japan) equipped with a 12-MHz linear transducer as described previously [26, 38]. Percent fractional shortening (%FS) and percent ejection fraction (%EF) were calculated as described previously [26, 38]. All measurements were made by one observer who was blinded with respect to the identity of the tracings. All data were collected from 10 cardiac cycles.

Western Blot

Western blots were performed as described previously [11, 13, 17, 38]. Briefly, the cellular proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ). The membranes were incubated with appropriate primary antibody [anti-Fas (CD95), anti-FasL, anti-FADD, anti-vascular cell adhesion molecule-1 (VCAM-1), anti-intercellular adhesion molecule-1 (ICAM-1), anti-Bcl-2, anti-Bax, and anti-Bak, (Santa Cruz Biotech, Santa Cruz, CA), and anti-phospho-IκBα, (Cell Signaling Technology, Inc., Danvers, MA), respectively, followed by incubation with peroxidase-conjugated second antibodies (Cell Signaling Technology) and analysis by the ECL system (Amersham Pharmacia, Piscataway, NJ). To control for lane loading, the same membranes were probed with anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase,

Biodesign, Saco, Maine) after being washed with stripping buffer. The signals were quantified using the Syngene G: Box gel imaging system (Syngene, USA, Fredrick, MD).

In Situ Apoptosis Assay

Myocardial apoptosis was examined as described previously [11, 13, 17, 38] using the In Situ Cell Death Detection Kit, Fluorescein (Roche, USA). Briefly, hearts were harvested and slices cut horizontally. One slice was immersion-fixed in 4% buffered paraformaldehyde, embedded in paraffin and cut at 5 μm thick. The sections were incubated with the commercially prepared labeling mixture supplied by the manufacturer at 37oC for one hr. Nuclei of living and apoptotic cells were counterstained with Hoechst 33342 (Invitrogen). Three slides from each block were evaluated for percentage of apoptotic cells and four fields on each slide were examined at the border areas using a defined rectangular field area with 20x magnification. The numbers of apoptotic cardiac myocytes are presented as a percentage of total cells counted.

Caspase-Activity

Caspase-3/7 and -8 activities in heart tissues were measured as described previously [19] using a Caspase-Glo assay kit (Promega).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins were isolated from heart samples as previously described [11,13,17,38] and NF-κB binding activity was measured using a LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific, Waltham, MA) according to the instructions of manufacturer.

ELISA Quantification of Cytokines

The levels of inflammatory cytokines (TNF-α and IL-1β) in the serum were assessed by ELISA (PeproTech, Rocky Hill, NJ) according to the instructions provided by the manufacturer [10, 24, 38].

Immunohistochemistry Staining

Immunohistochemistry was performed as described previously [10, 38]. Briefly, heart tissues were immersion-fixed in 4% buffered paraformaldehyde, embedded in paraffin, and cut at 5 um sections. The sections were stained with specific goat anti-ICAM-1 (1:50 dilution, Santa Cruz Biotechnology) and rabbit anti-VCAM-1 (1:50 dilution, Santa Cruz Biotechnology), respectively, and treated with the ABC staining system (Santa Cruz Biotechnology) according to the instructions of the manufacturer. Three slides from each block were evaluated, counterstained with hematoxylin, and examined with brightfield microscopy. Four different areas of each section were evaluated.

Accumulation of Neutrophils and Macrophages

Neutrophil accumulation in the heart tissues was examined by staining with naphtol AS-D Chloroacetate Esterase (Sigma-Aldrich, St. Louis, MO) as described previously [10,38]. Macrophages in the myocardium were examined with the macrophage specific antibody F4/80 (1:50 dilution, Santa Cruz, CA). Three slides from each block were evaluated, counterstained with hematoxylin, and examined with brightfield microscopy. Four different areas of each section were evaluated. The results are expressed as the numbers of macrophages/field (40x).

Statistical Analysis

All data were expressed as mean ± SEM. Comparisons of data between groups were made using one-way analysis of variance (ANOVA) and Tukey's procedure for multiple range tests was performed. $P< 0.05$ was considered to be significant.

Results

TLR3 Deficiency Attenuates Cardiac Dysfunction Induced by Myocardial Infarction

To investigate whether TLR3 plays a role in cardiac dysfunction following myocardial infarction, we performed permanent ligation of LAD artery in $TLR3^{-/-}$ and WT mice and measured cardiac function before and 3, 7, 14 and 21 days after induction of myocardial infarction. As shown in Figure 4.1A, ejection fraction (EF%) and fractional shortening (FS%) values were significantly reduced, compared with baseline, in WT and $TLR3^{-/-}$ mice following permanent ligation. The EF% and FS% in $TLR3^{-/-}$ and WT mice decreased as a function of time. However, TLR3 deficiency resulted in significantly greater attenuation of cardiac dysfunction after myocardial infarction. There was no significant difference in EF% and FS% of baseline between $TLR3^{-/-}$ and WT mice. The data indicates that TLR3 contributes to cardiac dysfunction following induction of myocardial infarction.

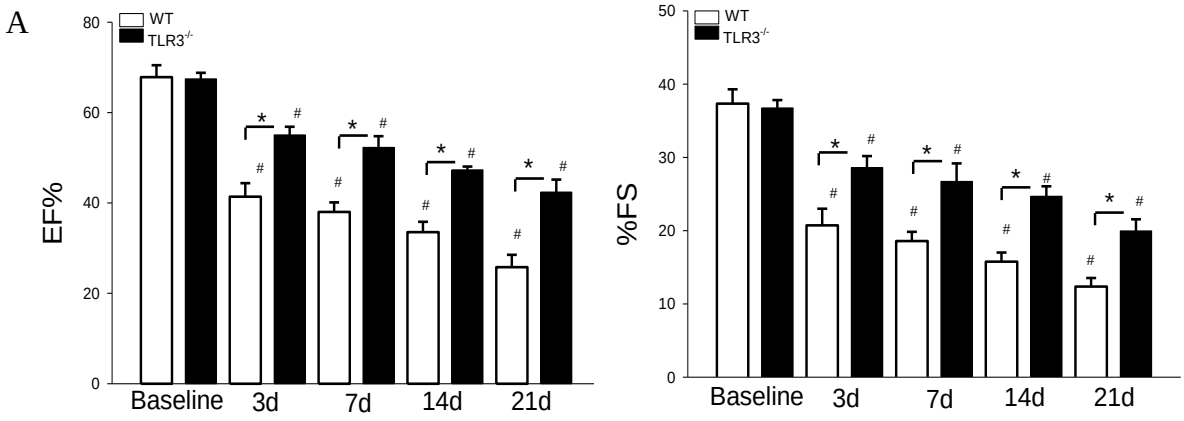
TLR3 Deficiency Attenuates Cardiac Dysfunction Following Transient Myocardial Ischemia Followed by Reperfusion

Next, we examined the role of TLR3 in cardiac function following myocardial I/R injury. $TLR3^{-/-}$ and WT mice were subjected to myocardial ischemia (45 min) followed by reperfusion for up to 3 days. Cardiac function was assessed by echocardiography before and after reperfusion

for 1 and 3 days. Figure 4.1B showed that EF% and FS% in WT and TLR3$^{-/-}$ mice were significantly decreased following myocardial I/R injury compared with the baseline. However, TLR3 deficiency markedly attenuated I/R-induced cardiac dysfunction. The EF% and FS% values in TLR3$^{-/-}$ mice were significantly greater on day 1 (45.1% and 52.7%) and on day 3 (39.7% and 42.3%) after reperfusion than in WT I/R mice, respectively. The data further confirm that TLR3 contributes to cardiac dysfunction in myocardial I/R injury.

TLR3 Deficiency Reduces Myocardial Infarct Size Following Myocardial I/R Injury

We also examined the role of TLR3 in myocardial infarct size following I/R. TLR3$^{-/-}$ and WT mice were subjected to myocardial ischemia (45 min) following by reperfusion (4 hrs). Hearts were harvested for the evaluation of infarct size. As shown in Figure 4.1C, ischemia followed by reperfusion induced significant myocardial injury as denoted by the infarct size in WT mice. In contrast, infarct size was significantly reduced (42.9%) in TLR3$^{-/-}$ mice following I/R, when compared with WT mice. There was no significant difference in the ratio of risk area/left ventricle (RA/LV), which reflects the position of the coronary artery ligation, between TLR3$^{-/-}$ and WT mice.



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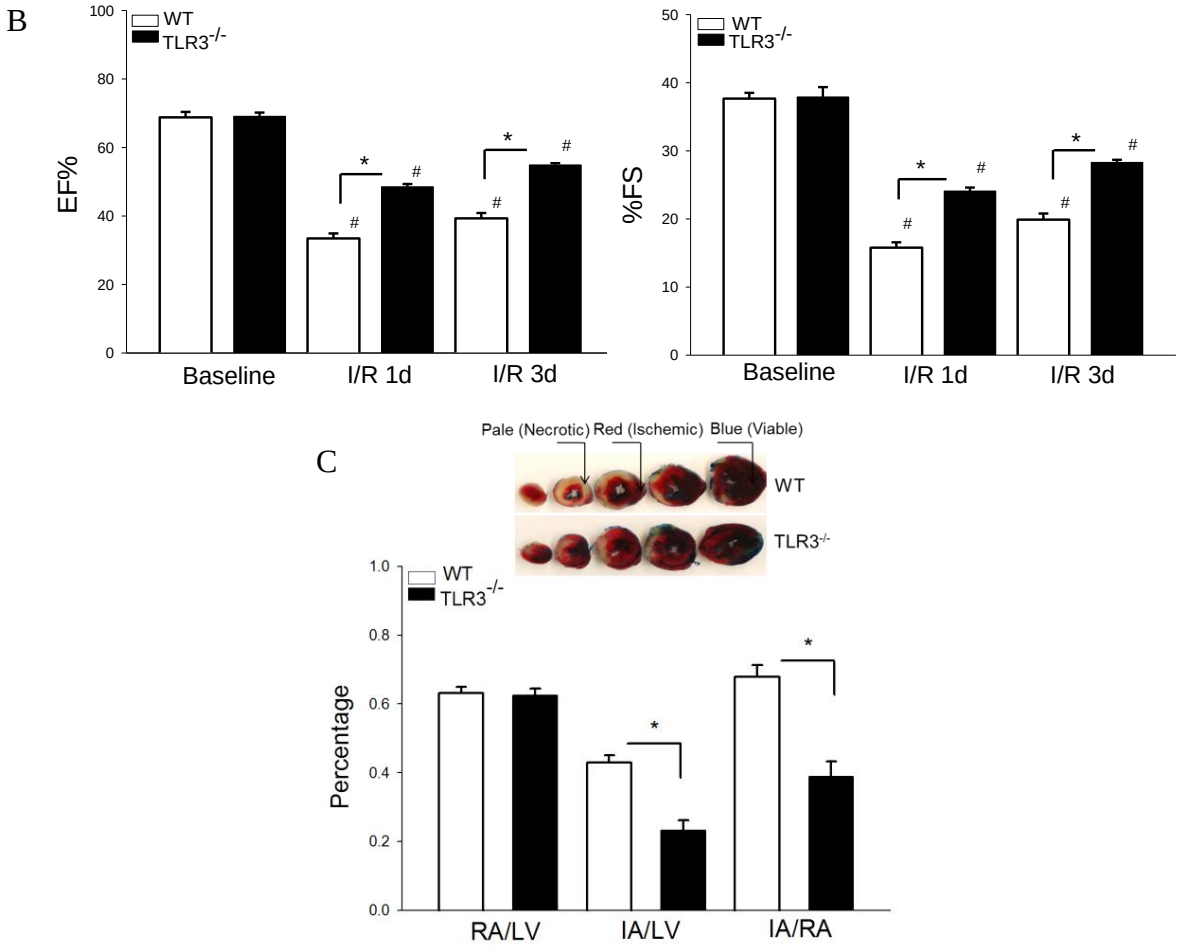
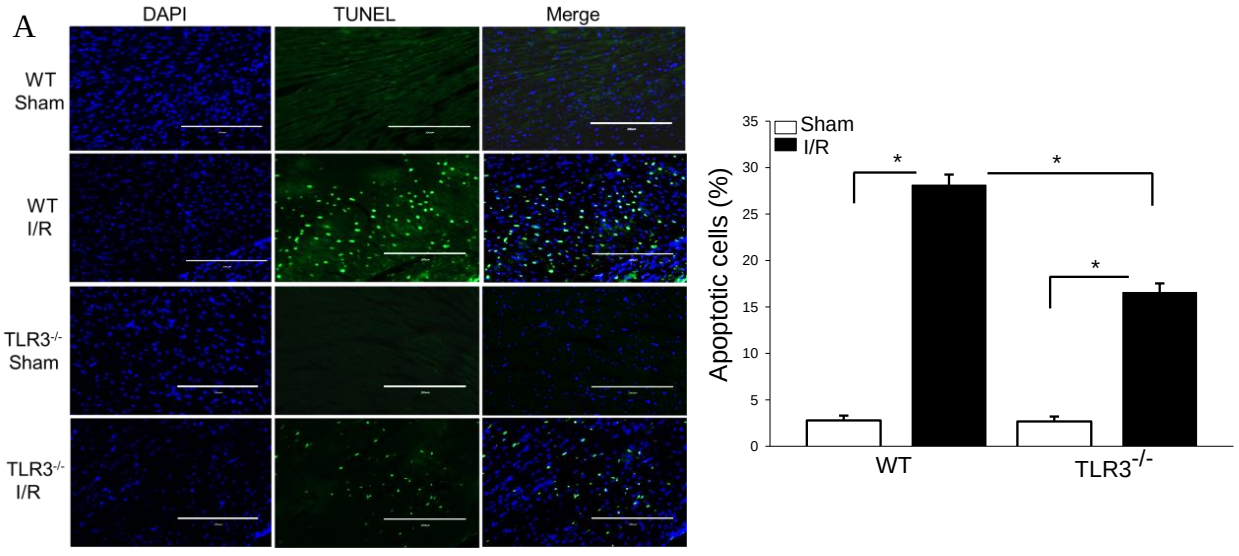


Figure 4.1 TLR3 deficiency attenuates cardiac dysfunction following myocardial infarction or I/R injury and decreases myocardial infarct size following myocardial I/R injury. TLR3-/- and age-matched WT mice were subjected to myocardial infarction (MI) by permanent ligation of LAD coronary artery (A) or myocardial ischemia (45 min) followed by reperfusion for up to 3 days (B). Cardiac function was measured by echocardiography before (baseline) and after ischemia/reperfusion. (C) TLR3 deficiency decreases myocardial infarct size. TLR3-/- and age-matched WT mice were subjected to myocardial ischemia (45 min) followed by reperfusion (4 hrs). Infarct size was determined by TTC staining. Blue color shows non-ischemic areas, red color indicates ischemic areas. Pale (white) indicates necrotic tissues. Ratios of risk area vs. left ventricle area (RA/LV) and infarct area vs. risk area (IA/RA) were calculated and are presented in the graphs. Photographs of representative heart sections are shown above. There were 8 mice in each group. *$p < 0.05$ compared with indicated groups. #$p < 0.05$ compared with respective baseline.

TLR3 Deficiency Attenuates I/R-Induced Myocardial Apoptosis

Cardiac myocyte apoptosis contributes to myocardial I/R injury [36]. We examined whether TLR3 deficiency will attenuate myocardial apoptosis following I/R injury. Figure 4.2A shows that I/R significantly increased the TUNEL positive apoptotic cells by 9.1 fold in the WT myocardium compared with WT sham control. I/R also induced myocardial apoptosis in TLR3$^{-/-}$ mice by 5.2 fold, when compared with TLR3$^{-/-}$ sham control mice. However, myocardial apoptotic cells in TLR3$^{-/-}$ I/R mice were markedly reduced by 41.1% compared with WT I/R mice.

Caspase-3 and -8 activities are the specific markers for apoptosis. As shown in Figure 4.2B, I/R significantly induced caspase-3/7 activity by 51.1% and caspase-8 by 45% respectively, in the WT I/R myocardium, when compared with sham control. In contrast, TLR3$^{-/-}$ mice showed a significant attenuation of I/R-induced caspase-3/7 and caspase-8 activities following myocardial I/R injury. The levels of caspase-3/7 and caspase-8 activity in TLR3$^{-/-}$ I/R mice were reduced by16.4% and 16.6% ($p<0.05$) when compared with WT I/R mice.



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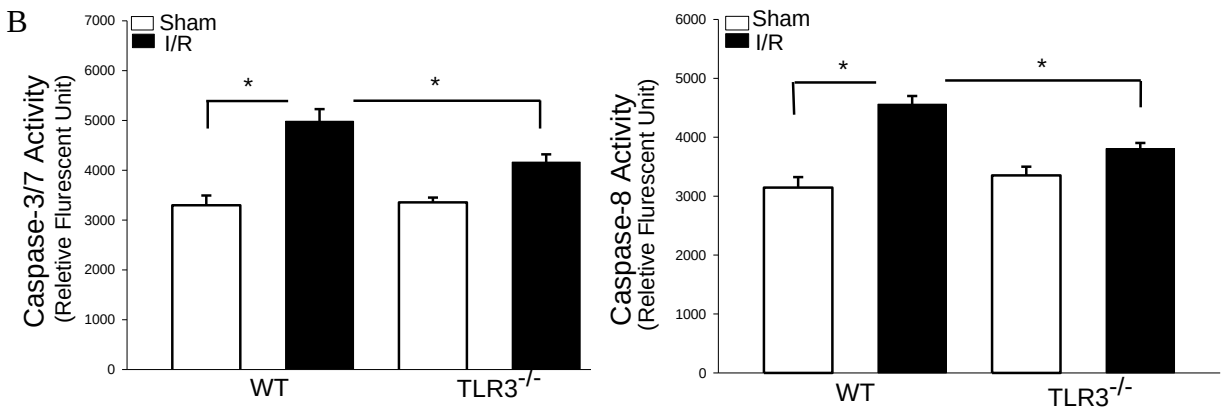


Figure 4.2 TLR3 deficiency attenuates I/R-induced myocardial apoptosis. TLR3$^{-/-}$ and WT mice were subjected to myocardial ischemia (45 min) followed by reperfusion (24 h). Sham surgical operation served as sham control. Myocardial apoptosis was examined by the TUNEL assay in the heart sections. (A) DAPI stained nuclei are blue color and TUNEL positive cells show green fluorescence. The bar graph shows the percent of apoptotic cells. n=3 in each group. (B) TLR3 deficiency prevents I/R-induced activity of caspase-3/7 and caspase-8 in the myocardium. There were 6 mice in each group. *$p < 0.05$ compared with indicated groups.

TLR3 Deficiency Attenuates up Regulation of Pro-Apoptotic Factors in the Myocardium

Activation of Fas/FasL mediated apoptotic signaling plays a role in I/R-induced myocardial apoptosis [36]. Bax and Bak1 are pro-apoptotic mediators [36]. We examined the effect of TLR3 deficiency on I/R-induced activation of Fas/FasL-mediated extrinsic apoptotic signaling in the myocardium. Figure 4.3A-C show that I/R significantly increased the levels of Fas, FasL and FADD, respectively in the WT myocardium when compared with WT sham control. In contrast, TLR3 deficiency markedly attenuated I/R-induced increases in the levels of Fas by 27.2% and prevented I/R-increased FasL and FADD, respectively, compared with WT I/R mice.

We also examined the effect of TLR3 deficiency on I/R-induced activation of intrinsic apoptotic signaling in the myocardium. In intrinsic apoptotic signaling, Bcl-2 is an important

anti-apoptotic factor while Bak and Bax are pro-apoptotic mediators [36]. Figure 4.3D-F shows that I/R markedly increased the levels of Bax (↑ 52.6%) and Bak (↑ 52.9%) and decreased the levels of Bcl2 in the myocardium compared with sham control. In contrast, TLR3 deficiency prevented I/R increases in the levels of Bax and Bak, when compared with WT I/R mice. Importantly, TLR3$^{-/-}$ completely prevented the deleterious effect of I/R on myocardial Bcl2 levels. In addition, the levels of Bcl2 in TLR3$^{-/-}$ sham mice were significantly greater than in WT sham mice.

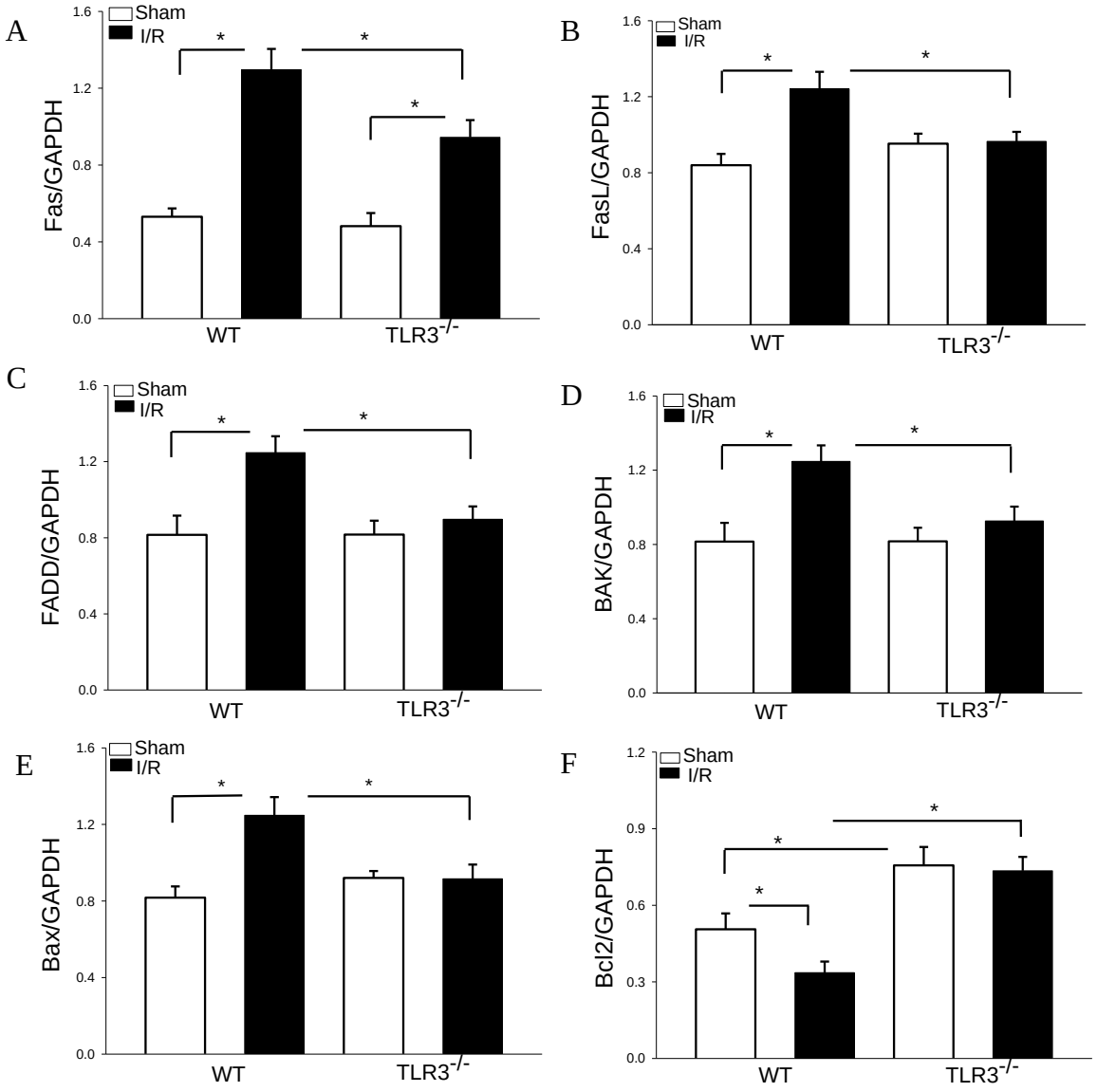


Figure 4.3 TLR3 deficiency attenuates I/R-increased FasL, Fas, FADD, Bak, and Bax expression, and prevents I/R-induced decreases in myocardial Bcl-2 levels. $TLR3^{-/-}$ and WT mice were subjected to myocardial ischemia (45 min) followed by reperfusion (24 h). Sham surgical operation serve as sham control. TLR3 deficiency attenuates I/R-increased the levels of Fas (A), FasL (B), FADD (C), Bak (D), and Bax (E) in the myocardium. (F) TLR3 deficiency increases the levels of Bcl2 in the myocardium following myocardial I/R. There were 6 mice in each group. *$p < 0.05$ compared with indicated groups.

TLR3 Deficiency Prevents I/R-Induced Myocardial NF-κB Binding Activity and Systemic TNF-α and IL-1β Production

We have previously reported that I/R significantly induced myocardial NF-κB binding activity [12, 13, 16, 17]. Inhibition of NF-κB activity has been shown to attenuate myocardial injury following I/R [22, 29]. We determine the whether TLR3 deficiency will attenuate I/R-induced myocardial NF-κB activity. Figure 4.4A shows that I/R significantly induced NF-κB binding activity by 1.76 fold in WT mice compared with sham control. TLR3 deficiency prevented I/R-induced NF-κB binding activity in the myocardium. Activation of NF-κB stimulates the expression of numerous genes, including pro-inflammatory cytokines [21, 42]. Figure 4.4B shows that circulating levels of TNFα and IL-1β were markedly increased by 1.2 fold and 2.6 fold, respectively, following myocardial I/R compared with sham control. However, TLR3 deficiency attenuated I/R-induced increases in the production of TNFα and IL-1β in the serum.

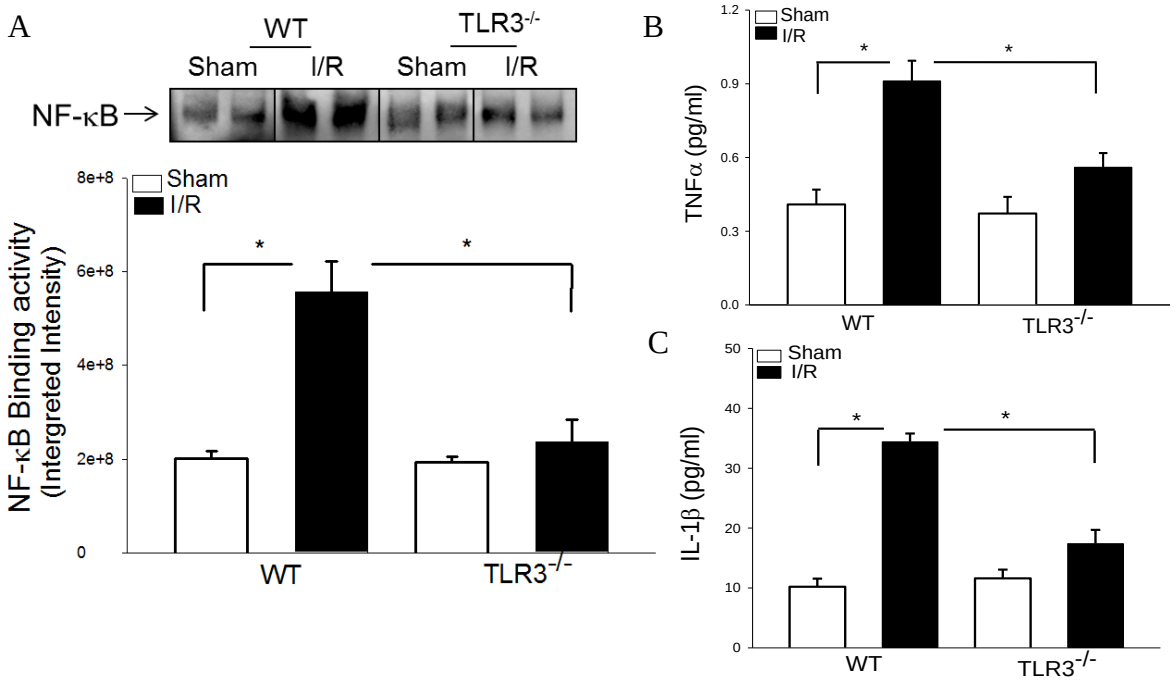


Figure 4.4 TLR3-deficiency prevents I/R-induced myocardial NF-κB binding activity and pro-inflammatory cytokine production in the circulation. TLR3$^{-/-}$ and WT mice were subjected to myocardial ischemia (45 min) followed by reperfusion (24 h). Sham surgical operation serve as sham control. Hears were harvested for the preparation of the nuclear and cytoplasmic proteins. TLR3 deficiency prevents I/R-induced NF-κB binding activity (A), TNF-α (B), and IL-1β (C) production. There were 6 mice in each group. *$p < 0.05$ compared with indicated groups.

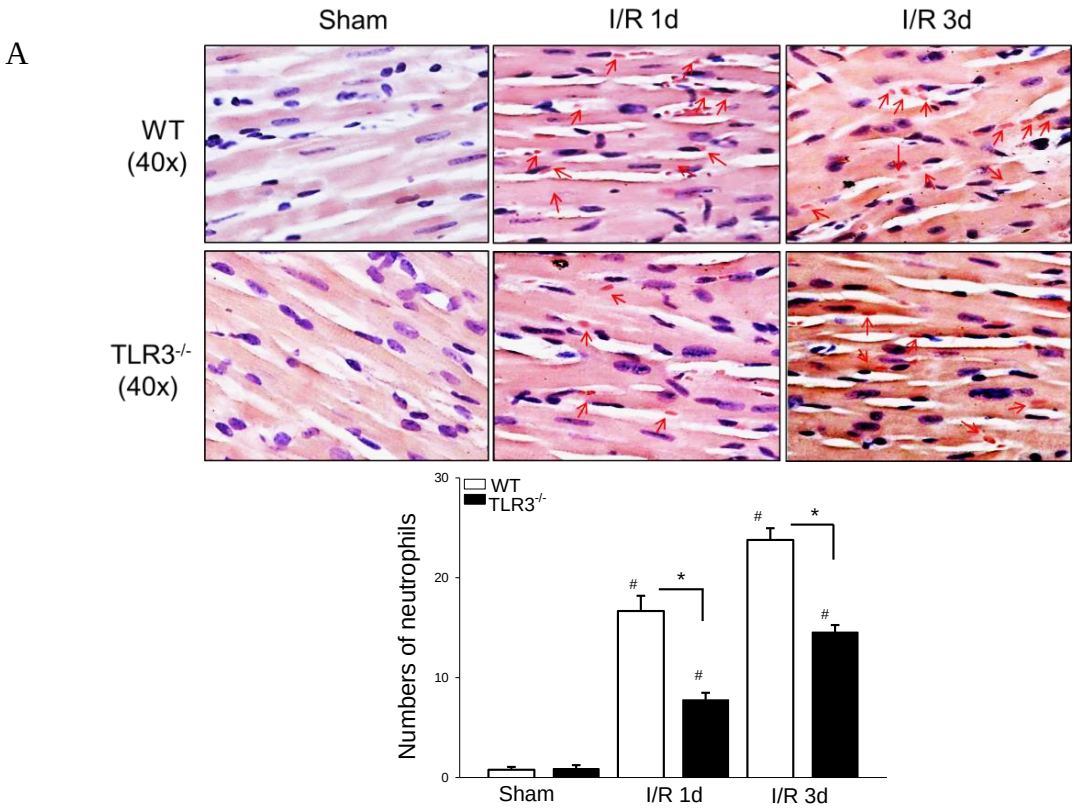
TLR3 Deficiency Attenuates I/R-Induced Infiltration of Neutrophils and Macrophages into the Myocardium

Infiltration of neutrophils and macrophages into the myocardium plays an important role in cardiac dysfunction and myocardial I/R injury [7, 14]. We examined the effect of TLR3 deficiency on the infiltration of neutrophils and macrophages into the myocardium following myocardial I/R injury. Figure 4.5A shows that I/R increased the number of neutrophils in the WT myocardium by 20 fold on day 1 and by 30 fold on day 3 after reperfusion, respectively, when compared with sham control. I/R also induced the infiltration of neutrophils into the myocardium

in TLR3$^{-/-}$ mice when compared with sham control. However, the numbers of neutrophils in the myocardium of TLR3$^{-/-}$ I/R mice were markedly reduced by 53.3% on day 1 and by 38.8% on day 3 after reperfusion, respectively compared with WT I/R mice. Figure 4.5B shows that the numbers of positively staining macrophages were markedly increased in the WT myocardium by 8.6 fold on day 1 and by 15 fold on day 3 after reperfusion compared with sham control. In contrast, I/R induced infiltration of macrophages into the myocardium in TLR3$^{-/-}$ mice was significantly reduced, when compared with WT I/R mice.

TLR3 Deficiency Attenuates I/R-Induced Expression of Adhesion Molecules in the Myocardium

Increased expression of adhesion molecules, such as VCAM-1 and ICAM-1, promotes neutrophil and macrophage infiltration into the myocardium during myocardial I/R injury [8, 9]. We examined the role of TLR3 deficiency in I/R-induced the expression of adhesion molecules in the myocardium. As shown in Figure 4.5C, I/R significantly increased the levels of VCAM-1 by 57.8% and ICAM-1 by 118.7% in the myocardium of WT mice compared with sham control. Immunohistochemistry also showed more positive staining of VCAM-1 and ICAM-1 in the heart tissues of WT I/R mice (Figure 4.5D). In contrast, TLR3 deficiency attenuated I/R-increased the levels of VCAM-1 by 40.7% and ICAM-1 by 31.4%, respectively compared with WT I/R mice. Immunohistochemistry showed that there was less staining of VACAM and ICAM-1 in the heart tissues of TLR3$^{-/-}$ I/R mice.



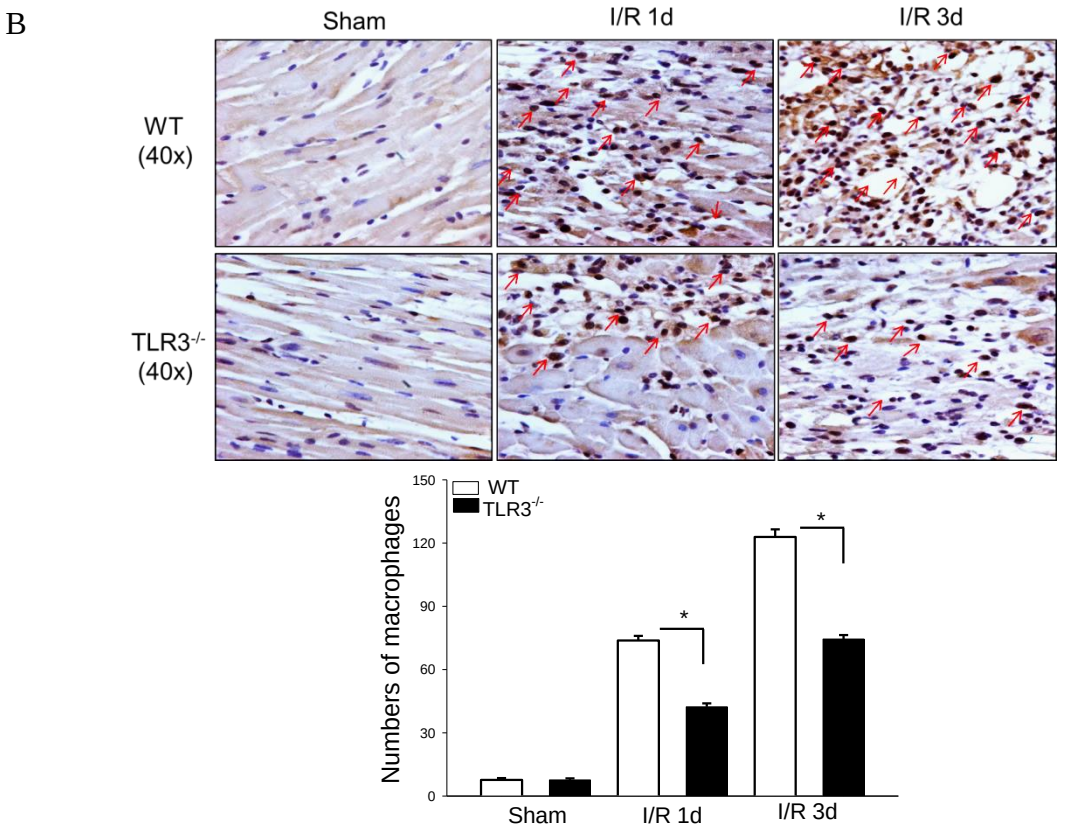


Figure 4.5 (continued on next page)

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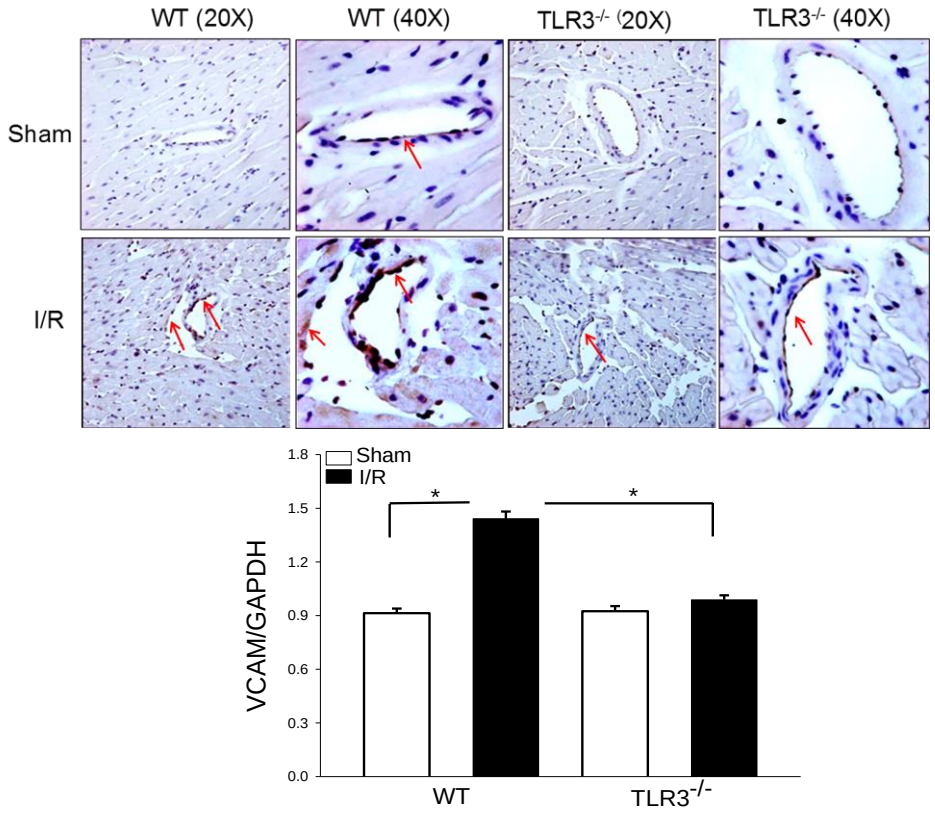
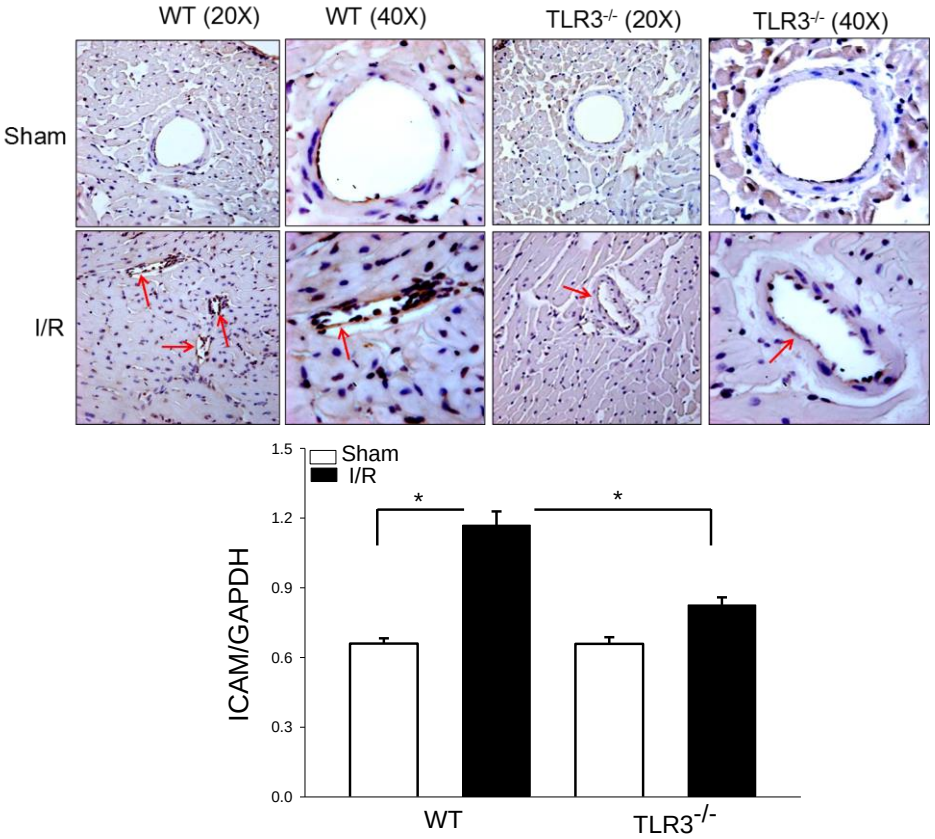

WT (20X)
WT (40X)
TLR3-/- (20X)
TLR3-/- (40X)
Sham
I/R
Sham
I/R
VCAM/GAPDH
1.8
1.5
1.2
0.9
0.6
0.3
0.0
*
*
WT
TLR3-/-

D


WT (20X)
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0.9
0.6
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*
WT
TLR3-/-

Figure 4.5 TLR3 deficiency attenuates I/R-induced infiltration of neutrophils and macrophages into the myocardium. $TLR3^{-/-}$ and WT mice were subjected to myocardial ischemia (45 min) followed by reperfusion for indicated time. Sham surgical operation served as sham control. (A) TLR3 deficiency decreases the infiltration of neutrophils (A) and macrophages (B) into the myocardium. The pink color indicates positive neutrophils in the myocardium (A). The dark brown color indicates positive macrophages (B). There were 3 mice in each group. TLR3 deficiency prevents I/R-induced increases in the expression of VCAM-1 (C) and ICAM-1 (D) in the myocardium. The dark brown color indicates positive staining of VCAM-1 or ICAM-1 in the myocardium. There were 6 mice in each group. *$p < 0.05$ compared with indicated groups.

Discussion

The present study demonstrates that TLR3 contributes to myocardial injury induced by either permanent ligation-induced myocardial infarction (MI) or myocardial I/R. Specifically, we observed that $TLR3^{-/-}$ mice showed significantly attenuated cardiac dysfunction following either permanent ligation of the LAD artery or transient ischemia followed by reperfusion. Myocardial I/R-induced infarct size observed in WT mice was also significantly reduced in $TLR3^{-/-}$ mice. In addition, TLR3 deficiency attenuated I/R-induced myocardial apoptosis, prevented I/R-induced NF-κB binding activity, and sequestration of inflammatory cells into the myocardium. The data suggest that TLR3 plays an important role in myocardial I/R injury through activation of apoptotic signaling, stimulation of inflammatory responses, and promotion of inflammatory cell infiltration into the myocardium. Thus, TLR3 may be an important target for the management and treatment of myocardial I/R injury.

TLR3 recognizes viral double-stranded RNA (dsRNA) and induces antiviral immune responses[20]. Our data show that TLR3 deficiency attenuates myocardial injury in response to I/R, suggesting that TLR3 may recognize endogenous ligands during myocardial I/R. At present we do not fully understand the nature of the endogenous ligands or DAMPs that activate TLR3

during myocardial I/R injury. Recently, Cavassani et al. [2] have demonstrated that TLR3 serves as an endogenous sensor that recognizes RNA released from necrotic cells, resulting in amplification of inflammation in experimental polymicrobial peritonitis and ischemic gut injury. Interestingly, recent studies have shown that TLRs serve as microRNA receptors [5,6]. MicroRNAs (miRs) are 21 to 23 nucleotide non-protein-coding RNA molecules, which have been identified as novel regulators of gene expression at the post-transcriptional level by binding to target messenger RNAs (mRNAs)[30,32]. Recent data indicates that several miRs are involved in ischemic heart disease [25,28,39]. Whether TLR3 is involved in the recognition of the increased miRs, resulting in amplification of inflammation during myocardial I/R has not been established.

We have observed that TLR3 deficiency significantly attenuated cardiac dysfunction following either myocardial infarction induced by permanent ligation of LAD artery or transient ischemia followed by reperfusion. Since transient ischemia followed by reperfusion injury is the most common clinical presentation among patients with heart attack, we employed the I/R model of myocardial injury for the subsequent mechanistic studies.

TLR3 mediates signaling via TIR/TRIF which interacts with either 1) RIP1/Peli1 to activate TRAF6, leading to NF-κB activation and nuclear translocation; 2) RIP1/RIP3 to stimulate FADD-dependent apoptotic signaling; and 3) TRAF3/TBK1 to activate IRF3/7, resulting in stimulating interferon production[41]. At the present, the role of IRF3-mediated signaling in myocardial I/R injury is unclear. Yang et al. reported that HMGB1 mediates brain I/R injury via TRIF adaptor independent TLR4 signaling[40]. Since TRIF is an important component of TLR3-mediated signaling[41], the data indicates that TLR3/TRIF signaling may contribute to brain ischemic injury. On the other hand, recent studies have shown that IRF3-

mediated signaling may contribute to preconditioning induced protection against cerebral I/R injury[34]. For example, Stevens et al. reported that multiple preconditioning paradigms converge on IRF3/7 dependent signaling to promote tolerance to ischemic brain injury[33].

TLR3 also mediates activation of NF-κB which is an important transcription factor controlling innate immune and inflammatory cytokine gene expression[21,42]. Activation of NF-κB contributes to myocardial I/R injury [22,29]. We have previously reported that I/R significantly increased the levels of TLR4-mediated MyD88-dependent NF-κB activation in the myocardium [12,13,16,17]. In the present study, we observed that I/R markedly induced NF-κB activation in WT mice but not in TLR3 deficient mice, suggesting that TLR3 plays a role in the induction of myocardial NF-κB nuclear translocation and binding activity during myocardial I/R injury. TLR3 is located in intracellular endosomes and recognizes double stranded RNA and poly (I:C), a synthetic analog of dsRNA and byproducts from apoptotic and necrotic cells[2,15,31]. The TLR3-mediated signaling pathway predominately activates IRF3 and NF-κB through TRIF-dependent pathways[21,42]. Activation of these pathways results in the expression of various inflammatory cytokines including TNFα, IL-1β and IL-6 as well as IFNs[21,42]. It is possible that I/R results in the production of endogenous ligands, i.e. disease associated molecular patterns which are recognized by TLR3, leading to NF-κB and subsequent inflammatory cytokine production. Indeed, we have observed that TLR3 deficiency markedly attenuated I/R-increased inflammatory cytokine production in the circulation.

The infiltration of neutrophils and macrophages into the myocardium plays an important role in mediating cardiac dysfunction following myocardial I/R injury [7,14]. Recent studies have shown that ischemia induces rapid recruitment of circulating macrophages into the myocardium[7,14]. These recruited inflammatory cells release inflammatory cytokines and

chemokines which further attract neutrophil infiltration and promote inflammatory responses [8,9]. We have observed in the present study that TLR3 deficiency significantly attenuated I/R-induced infiltration of macrophages and neutrophils into the myocardium which were observed in the myocardium of WT I/R mice. These results positively correlated with I/R-induced increases in the expression of adhesion molecules, such as ICAM-1 and VCAM-1. However, adhesion molecule expression in the myocardium was significantly attenuated by TLR3 deficiency. It has been well documented that activation of NF-κB regulates the expression of inflammatory cytokines and chemokines during myocardial I/R. Therefore, it is possible that TLR3 deficiency attenuated I/R-induced infiltration of inflammatory cells into the myocardium by preventing I/R-induced NF-κB binding activity.

It has been well demonstrated that cardiac myocyte apoptosis contributes to myocardial I/R injury[36]. We have observed that TLR3 deficiency significantly attenuated I/R-induced myocardial apoptosis. The mechanisms by which TLR3 deficiency attenuated I/R-induced myocardial apoptosis involve attenuation of Fas/FasL-mediated apoptotic signaling in the myocardium following I/R. In addition, TLR3 deficiency also prevented I/R-induced Bax and Bak expression and increased Bcl2 levels in the myocardium. Bax acts as an antagonist against anti-apoptotic Bcl2, while Bak-1 is the pro-apoptotic mitochondrial membrane protein. When Bcl2 is decreased and Bak-1 is oligomerized, it increases mitochondrial membrane permeability and promotes the release of cytochrome c [37,43]. Our data suggest that TLR3 contributes to I/R-induced myocardial apoptosis via activation of both extrinsic and intrinsic apoptotic signaling pathways. At present, we do not fully understand the mechanisms by which TLR3 activates apoptotic signaling during myocardial I/R injury. However, recent studies have shown that TLR3-mediated cell death is involved in the engagement of both extrinsic and intrinsic

apoptotic pathways[35]. Sun et al. reported that treatment of endothelial cells with the TLR3 agonist, poly (I:C) up-regulated the p53 family member,TAp63α, and initiated both intrinsic and extrinsic apoptotic pathways in a caspase-dependent manner leading to cell death[35]. The authors proposed that activation of TLR3 by either endogenous dsRNA or exogenous poly (I:C) induced the up-regulation of TAp63α, which translocated into the nucleus and bound to p53- or p63-responsive elements to up-regulate the expression of Noxa, the pro-apoptotic Bcl-2 family member and down-regulate anti-apoptotic Bcl-2[35]. TAp63α also up-regulated the expression of TRAIL and its receptors, DR4 and DR5. The interaction of TRAIL with DR4/5 activates caspase-8, resulting in the initiation of the extrinsic apoptotic signaling pathway [35]. Collectively, TLR3 may be a target for preventing I/R-induced myocardial apoptosis.

In summary, the present study demonstrated that TLR3 deficiency attenuates cardiac dysfunction induced by MI or I/R. $TLR3^{-/-}$ mice showed a significantly reduced myocardial infarct size following I/R injury. The mechanisms involve inhibition of I/R-activated apoptotic signaling and prevention of I/R-induced NF-κB binding activity, resulting in attenuation of I/R-induced infiltration of inflammatory cells into the myocardium. TLR3 may be an attractive target for the treatment and management of ischemic heart disease.

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CHAPTER 5

INCREASED EXPRESSION OF MICRORNA-130A IMPROVES CARDIAC FUNCTION FOLLOWING MYOCARDIAL INFARCTION VIA SUPPRESSION OF PTEN

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Keywords: Myocardial Infarction, MicroRNA-130a, Angiogenesis, PTEN, PI3K/Akt Signaling

Running head: Attenuation of Cardiac Dysfunction by MicroRNA-130a

Abstract

Background — Myocardial infarction (MI) is the main cause of death worldwide. MicroRNAs as a novel class of regulators mediate the pathogenesis of myocardial infarction.

Methods and Results —This study examined the role of microRNA-130a in cardiac function and remodeling following myocardial infarction. Lentiviral expressing miR-130 (LmiR-130a) was delivered into mice hearts. Lentiviral expressing control miR (LmiR-con) served as transfection control. Seven days after transfection, mice were subjected to myocardial infarction (MI). Cardiac function was assessed by echocardiography before and after MI. In LmiR-130a transfected MI group, EF% and FS% were significantly greater (~50%) than in LmiR-con and MI groups. LVESD value in LmiR-130a MI mice was markedly lower than in LmiR-con and MI groups. LmiR-130 transfection remarkably increased the number of capillaries and decreased fibrotic deposition in the myocardium. To examine the effect of miR-130a on infarct size, we induced myocardial ischemia (45 min) followed by reperfusion (24 h). Transfection of LmiR-130a significantly reduced myocardial infarct size by 61.6% compared with untransfected I/R group. Western blot showed LmiR-130a transfection significantly suppressed PTEN expression, increased the levels of phospho-Akt and phospho-GSK-3β, enhanced VEGF levels, and decreased HoxA5 levels. *In vitro* data showed that transfection of endothelial cells with miR-130a mimics promoted endothelial cell proliferation and migration. *In vivo* administration of LY294002, a PI3K inhibitor, completely abolished attenuation of cardiac dysfunction by miR-130a after myocardial infarction.

Conclusion — MiR-130a improved cardiac function and reduced cardiac remodeling following MI. The mechanisms involved activation PI3K/Akt signaling via suppression of PTEN expression.

Introduction

Myocardial infarction is a leading cause of death worldwide. In the United States, cardiovascular disease is the number one killer. Each year, an estimated 785,000 Americans will have a new coronary attack, 470,000 have a recurrent attack and 195,000 Americans have "silent" myocardial infarction [1, 2].

Acute myocardial ischemia results in cellular hypoxia and the subsequent cascade of cellular events, including an increase in reactive oxygen species, activation of endothelial cells, production of proinflammatory cytokines and chemokines, and the infiltration of inflammatory cells to the infarcted region [3, 4]. The activation of deleterious cellular signaling triggers further release of oxidants and proteolytic enzymes, leading to infarct size extension, cardiomyocyte death, and endothelial capillary impairment. The loss of cardiac muscle promotes progressive remodeling of the remaining active myocardium. Furthermore, the left ventricular remodeling process leads to fibrosis, left ventricular dilatation, and heart failure. Importantly, insufficient myocardial capillary density after myocardial infarction has been identified as a critical event in the remodeling process.

Phosphatase and tensin homologue deleted on chromosome ten (PTEN) is a tumor suppressor gene that negatively regulates phosphatidylinositol 3-kinase (PI3K) pathway [5]. Akt is an important kinase downstream of PI3K [6]. PI3K/Akt signaling pathway plays an important role in multiple cellular functions, such as cell metabolism, proliferation, cell-cycle progression, and survival. Recent studies have demonstrated that PTEN/PI3K/Akt signaling plays a critical role in angiogenesis [7, 8]. In addition, PTEN modulates angiogenesis though PTEN/Akt/VEGF signaling [9]. Indeed, deficiency of PTEN promotes angiogenesis and increases VEGF expression in zebrafish [10].

MicroRNAs (miRs) are 21 to 23 nucleotide non-protein-coding RNA molecules, which have been identified as novel regulators of gene expression at the post-transcriptional level by binding to target messenger RNAs (mRNAs) [11-15]. Recently published data indicates that miRs, such as miR-1/106, miR-133, miR-21, miR-320, miR-494, and miR-92a, are involved in ischemic heart disease [16-20]. Thum et al. reported that miR-21 contributes to a profibrotic role in failing heart [17]. Van Rooij et al. demonstrated the direct impact of miR-29 expression for fibrotic scar formation in the failing heart [12].

MicroRNA-130a has been reported to be a regulator of the angiogenic phenotype of vascular endothelial cells [21]. Yun et al. reported that miR-130a down-regulates anti-angiogenic genes GAX and HoxA5 in endothelial cells, resulting in stimulating angiogenesis [21]. In contrast, down-regulation of miR-130a expression contributes to dysfunction of endothelial progenitor cells [22]. MicroRNA-130a has been predicated to down-regulate PTEN [23]. However, the role of miR-130a in myocardial infarction has not been elucidated.

In the present study, we examined the role of miR-130a in cardiac function following myocardial infarction. We observed that increased expression of miR-130a remarkably attenuated cardiac dysfunction after MI via a PI3K/Akt dependent mechanism.

Materials and Methods

Animals

C57BL/6 male mice were purchased from The Jackson Laboratory (Indianapolis, IN). The mice were maintained in the Division of Laboratory Animal Resources at East Tennessee State University (ETSU). The experiments described in this manuscript conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health

(Publication, 8th Edition, 2011). The animal care and experiments protocols were approved by the ETSU Committee on Animal Care.

qPCR Assay of MicroRNAs

MicroRNAs were isolated from heart tissues or cultured cells using the mirVanaTM miR isolation kit (Ambion) in accordance with the manufacturer's protocol as described previously [24, 25]. Quantitative real-time (qPCR) was conducted using a 4800 Real-time PCR machine (Bio-Rad). MicroRNA levels were quantified by qPCR using specific Taqman assays for miR (Applied Biosystems, USA) and Taqman Universal Master Mix (Applied Biosystems). Specific primers for miR-130a were obtained from Applied Biosystems [primer identification numbers: 000454 for hsa-miR-130a and 001973 for U6 small nucleolar RNA (snRU6)]. MicroRNA-130a levels were quantified with the $2(-\Delta\Delta ct)$ relative quantification method that was normalized to the snRU6.

Construction of MiR-130a into Lentiviral Expression System

MicroRNA-130a was constructed into the lentivirus expression vector using a lentivirus expressing system (Invitrogen corporation) as described previously [24, 25]. Briefly, the oligonucleotides for miR-130a were synthesized at Integrated DNA Technologies, annealed and ligated into pcDNATM6.2-GW/EmGFP-miR. The pcDNATM6.2-GW/EmGFP-miR cassette was subsequently transferred to pDONR221TM and final pLenti6/V5-DEST by two sequential Gateway BP and LR recombination. The lentiviral control vector contains a non-sense miR sequence that allows formation of a pre-miRNA hairpin predicted not to target any known

vertebrate gene (Invitrogen Corporation). The viral particles were produced by third-generation packaging in 293FT cells and Lentiviral stocks were concentrated using ultracentrifugation [24, 25].

In Vitro Experiments

Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial basal medium-2 (EBM-2) supplemented with EGM-2 SingleQuots Kit (Lonza) and 20% fetal bovine serum (FBS). H9C2 cardiomyoblasts were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and penicillin (Gibco) [24]. When ECs and H9C2 cells reached 70-80% confluence, they were transfected with miR-130a mimics (80 nmol; invitrogen), scramble-miR mimics (80 nmol; invitrogen), and anti-miR-130a mimics (80 nmol; invitrogen) using Lipfectamine RNAiMAX reagent (invitrogen). Scramble-miR mimics served as control negative miR. After 48 hours of transfection, the HUVECS and H9C2 cells were harvested for isolation of microRNAs. The expression of miR-130a in HUVECs and H9C2 cells was examined by qPCR.

After 24 hours of transfection, the HUVECs and H9C2 cells first were incubated with hypoxic medium at 37˚C with 5% CO2 and 0.1% O2 in a hypoxia chamber (Pro-Ox Model C21, BioSpherix Ltd., Redfield NY) for 4 hours, and then the HUVECs and H9C2 cells were exposed to reoxygenation with normal medium for 24 hours in an incubator at 37˚C with 5% CO2 [24]. The cells (HUVECs and H9C2) that were not subjected to hypoxia/reoxygenation (H/R) were incubated at 37˚C with 5% CO2 and severed as control (normoxia). The cellular proteins were prepared for Western blot.

The endothelial cells migration capacity was determined by scratch assay after 24 hours of transfection. HUVECs were scratched by 200 μl tips, and cells were photographed at 0, 12, 24

hours after injury. The percent closure of the wound was analyzed by an image analyzer (Image J, NIH).

In separate experiments, H9C2 cells were treated with JSH-23 (30μM) or LY294002 (20μM) for 30 minutes and followed by treatment of Pam3CSK4 (1μg/ml) for 5 h. In addition, H9C2 cells were treated with Pam3CSK4, JSH-23, or LY294002 for 5 h. MicroRNAs were isolated from H9C2 cells and miR-130a expression was examined by qPCR.

In Vivo Treatment of Pam3CSK4 or CpG-ODN

Pam3CSK4 (Catalog number: tlrl-pms, InvivoGen; 2mg/Kg body weight) or CpG-ODN (Catalog number: ODN 1826, InvivoGen; 0.4mg/Kg body weight) was dissolved in sterile endotoxin-free water and injected intraperitoneally immediately after permanent ligation of LAD coronary. Twenty-four hours after LAD coronary artery ligation, ischemic areas were collected for isolation of microRNAs. MiR-130 levels were measured by qPCR.

In Vivo Transfection of Lentiviral Expressing MiR-130a into Mouse Hearts

Mice were intubated and anaesthetized with mechanical ventilation using 5% isoflurane. Anesthesia was maintained by inhalation of 1.5-2% isoflurane in 100% oxygen. Body temperature was maintained at 37˚C by heating pad. Following the skin incision, the right common carotid artery (CCA) and the right external carotid artery (ECA) were carefully exposed. Microvascular aneurysm clips were applied to the right CCA and right ECA. A micro-catheter was introduced into the right external carotid artery and positioned into the aortic root. One hundred microliters of LmiR-130a (1×10^8 PFU) or LmiR-control was injected through the micro-catheter[25, 26]. The micro-catheter was gently removed and the external carotid artery was

tightened before the skin was closed. Seven days after transfection, the hearts were harvested for isolation of microRNAs. The expression of miR-130a in the heart tissues were examined by qPCR.

Induction of Myocardial Infarction

Myocardial infarction was induced as previously described [27]. Briefly, mice (28-30g) were anaesthetized by 5.0% isoflurane, intubated, and ventilated with room air using a rodent ventilator. Anesthesia was maintained by inhalation of 1.5-2% isoflurane driven by 100% oxygen flow. Body temperature was regulated at 37˚C by heating pad. Following the skin incision, the hearts were exposed through a left thoracotomy in the fourth intercostal space. The left anterior descending (LAD) coronary artery was permanently ligated with an 8-0 silk ligature. The skin was closed, anesthesia was discontinued, and the animals were allowed to recover in pre-warmed cages.

Myocardial ischemia/reperfusion (I/R) injury was induced as described previously [28-30]. In brief, mice were anaesthetized by 1.5-2% isoflurane with 100% oxygen flow. The hearts were exposed and the left anterior descending (LAD) coronary artery was ligated with an 8-0 silk ligature over a 1mm polyethylene tube (PE-10). After completion of 60 min of occlusion, the coronary artery was reperfused by pulling on the exteriorized suture to release the knot. The skin was closed, anesthesia was disconnected, and the animals were allowed to recover in the pre-warmed cages.

Echocardiography

The mice were transfected with lentiviral expressing miR-130a (LmiR-130a) or lentivial expression control-miR (LmiR-con). Seven days after transfection, the mice were subjected to permanent ligation of LAD coronary artery (MI). Cardiac function was examined by echocardiography before (Baseline) and after MI (1, 3, 7, 14, and 21 days). M-mode tracings were used to measure LV wall thickness, LV end-systolic diameter, and LV end-diastolic diameter. Percent fractional shortening (FS %) and ejection fraction (EF %) were calculated as described previously [29-31].

Examination of Cardiac Fibrosis

Cardiac fibrosis was determined by masson's trichrome stain. In brief, mice were transfected with LmiR-130a or LmiR-control 7 days before myocardial infarction. After 21 days of MI, hearts were harvested and slices cut horizontally. One slice below the ligation site was immersion-fixed in 4% buffered paraformaldehyde, embedded in paraffin, and cut at a 5 mm thickness. The sections were stained by trichrome stain (Masson) kit (Sigma-Aldrich) according to the manufacturer's protocol, as described previously [32]. The stained-sections were examined using x12.5 magnification macroscope and analyzed by an image analyzer (Image J, NIH).

Determination of Myocardial Infarct Size

Infarct size was evaluated by TTC (triphenyltetrazolium chloride, Sigma-Aldrich) staining as described previously [29]. Briefly, the hearts were perfused with saline on a Langendorff system to wash blood from the coronary vasculature. The LAD coronary artery was religated at the previous site of ligation prior to staining with 1% Evans Blue in order to assess

area at risk. Each heart was then sliced horizontally to yield five slices. The slices were incubated in 1% TTC for 15 min at 37˚C and fixed by immersion in 10% neutral buffered formalin. The area of infarction on both sides of each slice was determined by an image analyzer, corrected for the weight of each slice, and summed for each heart. Ratios of risk area (RA) to LV area and infarct area to RA were calculated and expressed as a percentage.

In Situ Apoptosis Assay

Myocardial apoptosis was examined as described previously using the in situ cell death detection kit, fluorescein (Roche, USA) [28-30, 33]. Briefly, hearts were harvested and slices cut horizontally. One slice below the ligation site was immersion-fixed in 4% buffered paraformaldehyde, embedded in paraffin, and cut at a 5 mm thickness. The sections were incubated at 37˚C for 1 hour with the commercially prepared labeling mixture supplied by the manufacturer. The nuclei of living and apoptotic cells were stained with ProLong Gold Antifade Reagent with DAPI (Invitrogen). Fields of the border areas were randomly evaluated for the percentage of apoptotic cells. The images were viewed on an EVOS-fl digital inverted fluorescent microscopy (Advanced Microscopy Group, Bothell, WA). Total cells were counted in each field, and apoptotic cells are presented as the percentage of total cells counted.

Western Blot

Western blot was performed as described previously [31, 34-36]. Briefly, the cellular proteins were extracted from ischemic hearts and cells. The protein concentrations were determined by BCA protein assay kit (Thermo Scientific). The cellular proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred onto Hybond ECL membranes (Amersham

Pharmacia, Piscataway, NJ, USA). The ECL membranes were incubated with the appropriate primary antibody, including anti-PTEN, anti-phospho-Akt, anti-phospho-GSK-3β, anti-Bax, (Cell Signaling Technology, Inc, Danvers, MA), anti-Akt, anti-GSK-3β, anti-Bcl-2,anti-VEGF, anti-HOXA5 (Santa Cruz Biotechnology), respectively, followed by incubation with peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.) and analysis by the ECL system (Amersham Pharmacia, Piscataway). To control for lane loading, the same membrane were probed with anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase, Biodesign, Saco, Maine). The signals were quantified using the G: Box gel imaging system by Syngen (Syngene, USA, Fredrick, MD, USA).

Immunohistochemistry Staining

Immunohistochemistry was performed as described previously [30, 37, 38]. Briefly, one slice below the ligation site was immersion-fixed in 4% buffered paraformaldehyde, embedded in paraffin, and cut at 5 um sections. The sections were stained with specific anti-CD31 antibody (1:50 dilution, abcam, ab28364) and treated with the ABC staining system (Santa Cruz Biotechnology). Three slides from each block were evaluated, counterstained with hematoxylin, and examined with bright-field microscopy. Four fields on each slide were examined at the infarct areas using a defined rectangular field area with × 40 magnification.

Caspase-3/7 and Caspase-8 Activities Assay

Caspase-3/7 and caspase-8 activities were measured using a Caspase-Glo assay kit (Promega) according to the manufacturer's protocol as described previously [30].

Statistical Analysis

The data are expressed as mean ± SEM. Comparisons of data between groups were performed using either student t-test or analysis of variance (ANOVA), and the least significant difference procedure for multiple-range tests was performed. $P< 0.05$ was considered to be significant.

Results

Myocardial Infarction Decreases the Expression of MiR-130a in the Myocardium

We have previously reported that TLR2 ligand (Pam3CSK4) or CpG-ODN, TLR9 ligand, induced protection against myocardial ischemic injury via activation of PI3K/Akt signaling [30,39]. Figure 5.1A shows that administration of Pam3CSK4 or CpG-ODN significantly increased the levels of miR-130a in the myocardium, indicating that miR-130a may play a protective role in myocardial ischemic injury. Figure 5.1B shows that the levels of miR-130a in the myocardium were significantly reduced at 3 days after myocardial infarction, when compared with sham control. The data indicates that miR-130a is required for the protection against myocardial ischemic injury.

PI3K/Akt Signaling and NF-κB Regulate MiR-130a Expression in H9C2 Cells

Toll-like receptor mediated signaling predominately activates NF-κB [33]. There is a cross talk between TLRs and PI3K/Akt signaling [30, 39]. To investigate whether PI3K/Akt signaling and NF-κB activation involved in regulation of miR-130a expression, we treated H9C2 cells with a NF-κB inhibitor (JSH-23) or PI3K inhibitor (LY294002) in the presence or absence of Pam3CSK4. We observed that either inhibition of NF-κB activation or PI3K/Akt signaling

completely prevented Pam3CSK4-induced increases in the levels of miR-130a in H9C2 cells (Figure 5.1C). The data suggests that miR-130a expression is regulated by PI3K/Akt and NF-κB activation.

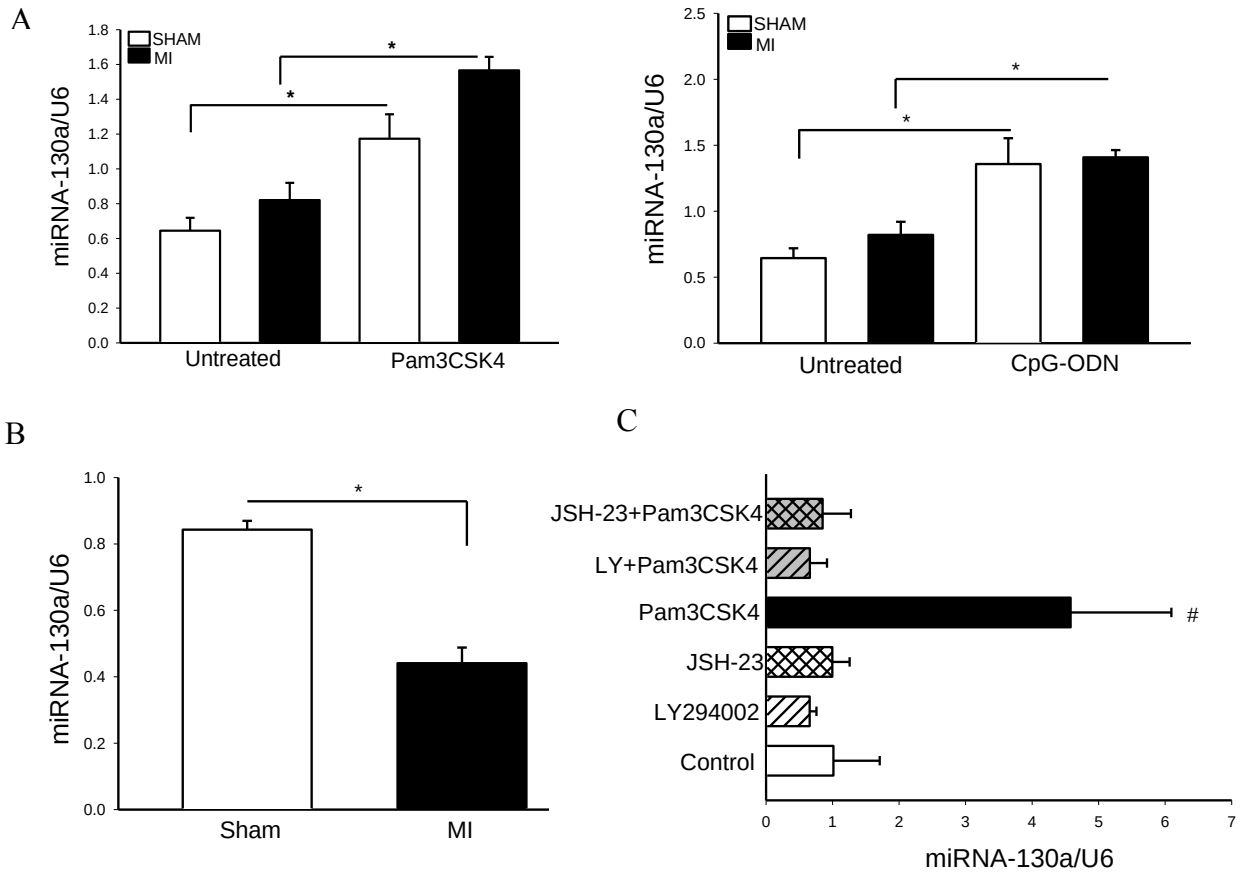


Figure 5.1 TLR ligands induces an increase in the expression of miR-130a in H9C2 cardiaomyoblasts. (A) Mice were treated with and without Pam3CSK (50µg/25 g body weight) or CpG-ODN (10µg/25 g body weight) immediately after permanent ligation of LAD coronary artery (myocardial infarction). Sham surgical-operated mice treated with or without Pam3CSK4 or CpG-ODN served as sham controls. Twenty-four hours after LAD coronary artery ligation, hearts were harvested for microRNA isolation. MiR-130 levels were measured by qPCR. (B) Myocardial infarction decreased the expression of miR-130a in the myocardium. Mice were subjected to permanent ligation of LAD coronary artery (MI). Three days after myocardial infarction, hearts were harvested for microRNAs isolation. MiR-130a levels were measured by qPCR. (C) PI3K/Akt signaling and NF-κB regulate miR-130a expression in H9C2 cells. H9C2

cells were treated with NF-κB inhibitor (JSH23) or PI3K inhibitor (LY294002) in the presence and absence of Pam3CSK4. Five hours after treatment, the cells were harvested for isolation of microRNAs. MiR-130a levels were measured by qPCR (n=4). n=3 for each sham group. n=4 for each MI group. *$p<0.05$ compared with indicated group. # $p<0.05$ compared with all other groups.

Transfection of Lentiviral Expressing MiR-130a Attenuates Myocardial Infarction- Induced Cardiac Dysfunction

To investigate whether miR-130a plays a protective role in cardiac function following myocardial infarction, we constructed lentiviral expressing miR-130a (LmiR-130a) and transfected it into the myocardium. Lentiviral expressing scramble miR served as control (LmiR-control). We observed that transfection of LmiR-130a significantly increased the levels of miR-130a by 1.3 fold in sham control and by 2.1 fold in myocardial infarcted hearts, respectively, compared with LmiR-Con respective controls (Figure 5.2A). Figure 5.2BC shows that increased expression of miR-130a expression by transfection of LmiR-130a into the myocardium significantly attenuated MI-induced cardiac dysfunction. Myocardial infarction decreased the values of ejection fraction (EF%) (Figure 5.2A) and fractional shortening (FS%) (Figure 5.2B) by 36.8% and 43.8% at day 1, by 42.9% and 49.8% at day 3, by 45.6% and 52.2% at day 7, by 49.6% and 56.0% at day 14, and by 48.8% and 55.0% at day 21 in untransfected hearts, when compared with the baseline. Myocardial infarction also significantly increased the levels of left ventricular end-systolic diameter (LVESD) compared with baseline (Figure 5.2D). In contrast, the values of EF% and FS% in LmiR-130a transfected hearts were significantly greater than in untransfected group at all the time points measured after MI. Increased expression of miR-130a prevented MI-induced the increase in LVESD value. Transfection of LmiR-control did not prevent MI-induced the decrease in EF% and FS% values, as well as the increase in LVESD

value. There were no significant differences in the baseline of cardiac function between untransfected, LmiR-Control transfected, and LmiR-130a transfected hearts.

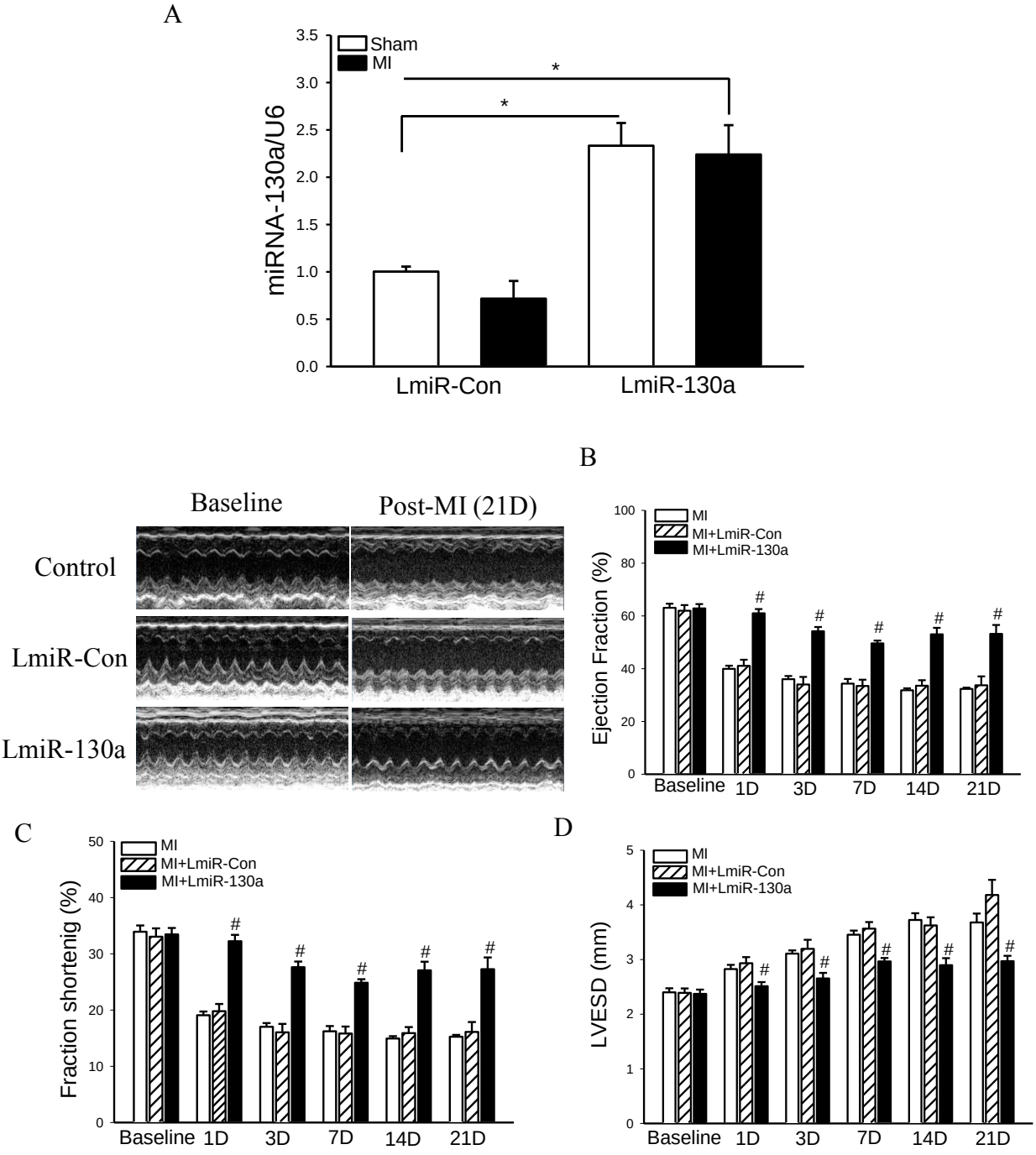


Figure 5.2 Transfection of lentiviral expressing miR-130a attenuates myocardial infarction-induced cardiac dysfunction. The mice were transfected with lentivirus expressing miR-130a

(LmiR-130a) or lentivirus expression control-miR (LmiR-con). Seven days after transfection, the mice were subjected to permanent ligation of LAD coronary artery (MI). Cardiac function was examined by echocardiography before (Baseline), 1, 3, 7, 14, and 21 days after myocardial infarction. (A). Transfection of LmiR-130a increased expression of miR-130a in the myocardium. Hearts were harvested at 3 days after LAD coronary artery ligation. Ischemic areas were collected for isolation of microRNAs. The levels of miR-130a were measured by qPCR (n=3/group). (B-D) LmiR-130 transfection significantly improved cardiac function after myocardial infarction. Transfection of LmiR-130a increased the levels of ejection fraction (EF%) (B), fractional shortening (FS%) (C), and decreased in the left ventricular end-systolic diameter (LVESD) (D). Representative M-mode images from untransfected, LmiR-130a transfected, and LmiR-control transfected mice before and after myocardial infarction (21 days) were shown. n=24 in untransfected group. n=11 in LmiR-Control transfected group. n=9 in LmiR-130a transfected group. #$p<0.05$ compared to all other groups at the same time point. *$p<0.05$ compared with indicated group. $^{\#}$ $p<0.05$ compared with all other groups at the same time.

Increased Expression of MiR-130a Decreases Fibrotic Deposition in the Myocardium

It has been well known that myocardial infarction induces fibrotic deposition in the myocardium and thus contributes to cardiac dysfunction [40]. Figure 5.3 shows that, 21 days after myocardial infarction, fibrosis appeared in the infarction area as evidenced by Masson's Trichrome staining that showed positive staining of fibrosis in the heart tissue sections. In LmiR-130a transfected hearts, the percentage of fibrosis area was significantly reduced by 46.2% compared with untransfected group. Transfection of LmiR-control did not alter myocardial infarction-induced fibrosis deposition.

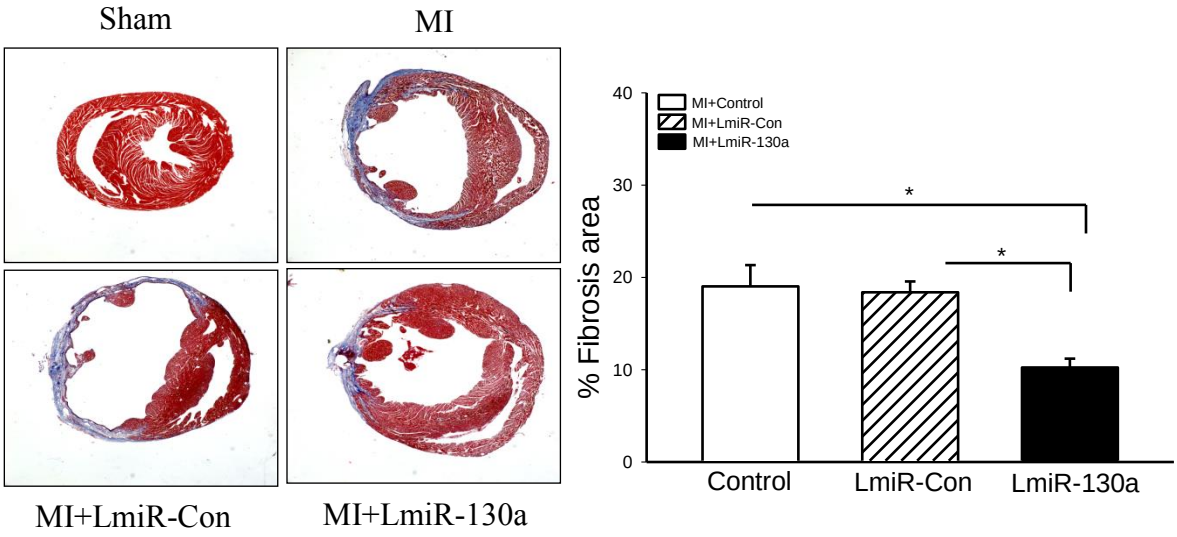


Figure 5.3 Increased expression of miR-130a decreases fibrotic deposition in the myocardium. Mice were transfected with LmiR-130a or LmiR-control 7 days before myocardial infarction. Three weeks after myocardial infarction, hearts were harvested for analysis of cardiac fibrosis. (A) Representative images of heart sections with masson's trichrome staining from untransfected and transfected mice with LmiR-130a and LmiR-Control. Quantitative data showed a significant decrease in the percentage of fibrosis area in LmiR-130a transfected heart compared with untranfected and LmiR-control transfected heart. n=3 in untransfeted and LmiR-Control transfected group. n=5 in LmiR-130a transfected group. *$p<0.05$ compared with indicated group.

Increased Expression of MiR-130a Decreases Infarct Size Following Myocardial Ischemic Injury

We examined the effect of increased expression of miR-130a on myocardial infarct size. LmiR-130a was transfected into the myocardium 7 days before the hearts were subjected to ischemia (45 min) followed by reperfusion (24 h). The infarct size was evaluated by TTC staining. As shown in Figure 5.4, ischemia followed by reperfusion induced myocardial infarct size in untransfected mice. However, the infarct size in LmiR-130a transfected hearts was markedly reduced by 61.6%, when compared with untransfected group. Transfection of LmiR-Control did not affect I/R-induced infarct size. There was no significant difference in the ratio of

risk area/left ventricle (RA/LV), which reflects the position of the coronary artery ligation, among three groups.

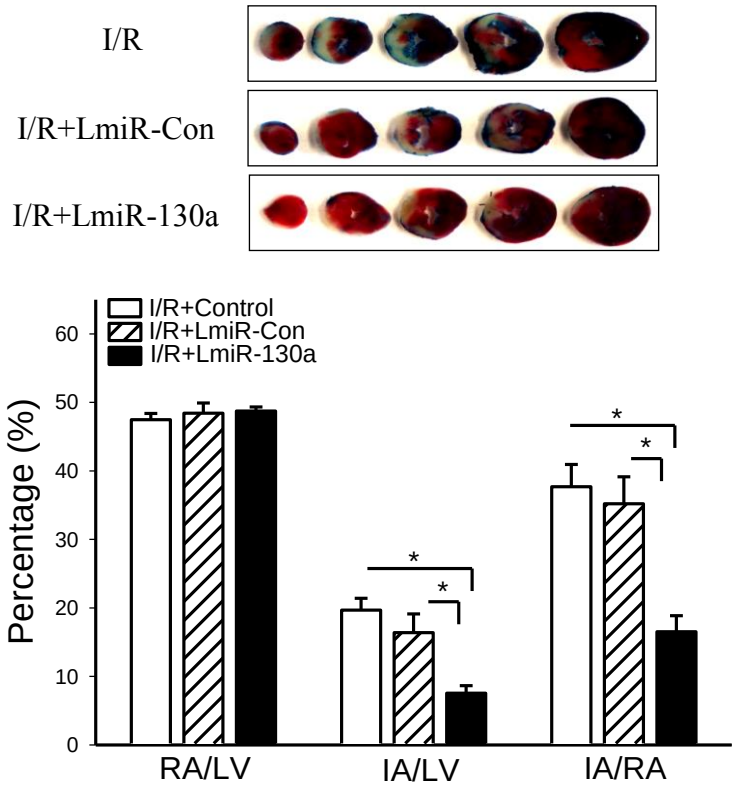


Figure 5.4 LmiR-130a transfection decreases infarct size following myocardial ischemia/reperfusion injury. The mice were transfected with lentivirus expressing miR-130a or lentivirus expression control-miR. 7 days after transfection, the mice were subjected to myocardial ischemia (45 minutes) and followed by reperfusion (24 hours). The hearts were harvested for evaluation of infarct size by TTC staining. The infarct area (white) and the area at risk (red + white) from each section were measured using an image analyzer. Ratios of risk area vs. left ventricle area (RA/LV) and infarct area vs. risk area (IA/RA) were calculated and are presented in the graphs. Photographs of representative heart sections were shown above. n=7 in untransfected group. n=7 in LmiR-Control transfected group. n=8 in LmiR-130a transfected group. *$p<0.05$ compared with indicated group.

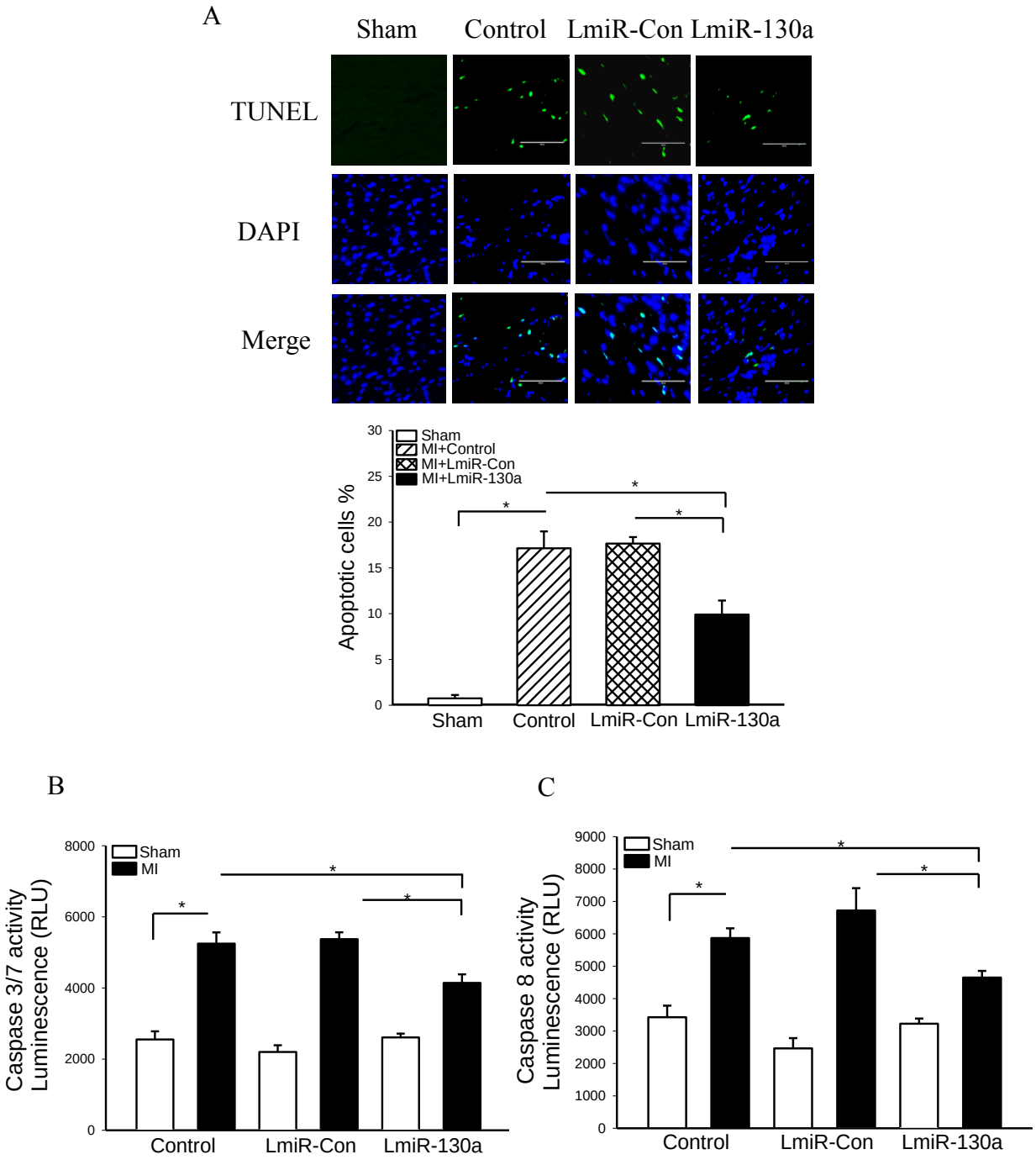
Increased Expression of MiR-130a Attenuates Myocardial Apoptosis Following Myocardial Infarction

It has been well documented that myocardial apoptosis contributes to cardiac dysfunction following myocardial infarction [41]. Figure 5.5A shows that myocardial infarction significantly induced myocardial apoptosis by 17.1% as evidenced by TUNEL positive staining in untransfected hearts. Myocardial infarction also induced caspase-3/7 and caspase-8 activities by 105.6% and 71.3%, respectively, when compared with the respective sham control (Figure 5.5B&C). In LmiR-130a transfected hearts, however, the TUNEL positive apoptotic cells were markedly reduced by 42.2%; caspase-3/7 and caspase-8 activities were reduced by 21.0% and by 20.7%, when compared with untransfected group (Figure 5.5B&C). Transfection of LmiR-control did not alter myocardial infarction-induced apoptosis and caspase-3/7 and -8 activities (Figure 5.5A-C).

Increased Expression of MiR-130a Increases Bcl2 Levels and Prevents MI-Induced Increases in Bax in the Myocardium

Bcl2 is an anti-apoptotic protein while Bax is pro-apoptotic protein [42]. Figure 5.5D shows the levels of Bcl2 were increased by 135.3% after myocardial infarction compared with sham control in untransfected group. Transfection of LmiR-130a into the myocardium further increased levels of Bcl2 by 55.8% in the myocardium, when compared with untransfected MI hearts. Figure 5.5E shows that the levels of Bax in untransfected group were significantly increased by 118.6% after myocardial infarction compared with sham control. In contrast, transfection of LmiR-130a significantly decreased the levels of Bax by 56.3% compared with

untransfected hearts. Transfection of LmiR-control did not alter myocardial infarction-increased the levels of Bax and decreased Bcl2 levels (Figure 5.5D&E).



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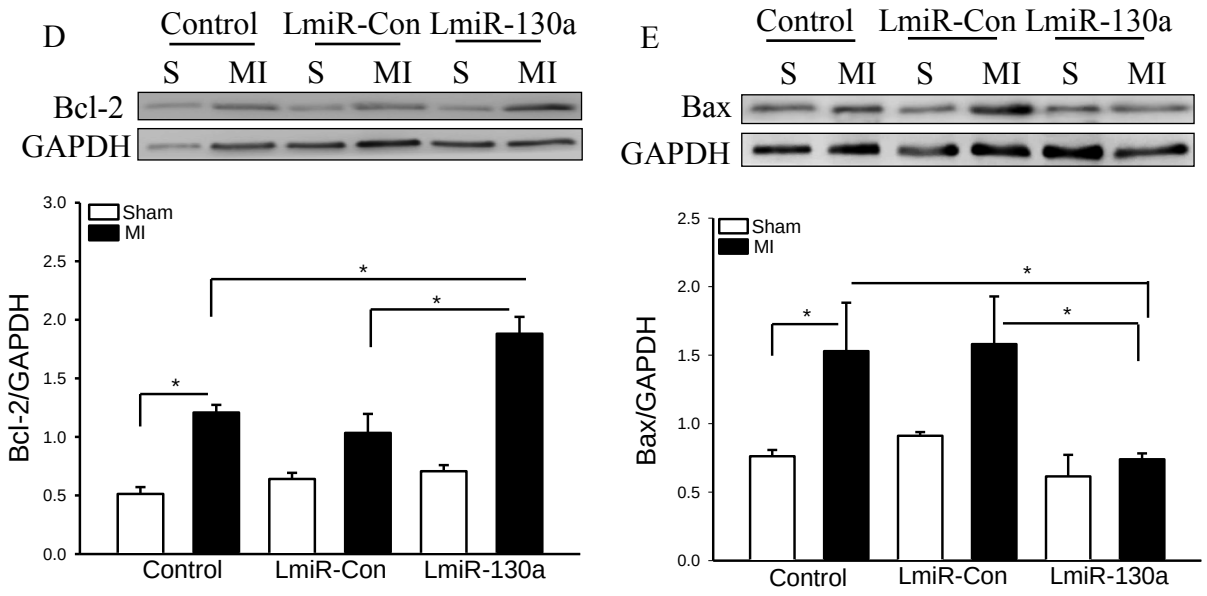


Figure 5.5 Increased expression of miR-130a attenuates myocardial apoptosis following myocardial infarction. Mice were transfected with LmiR-130a or LmiR-control 7 days. Hearts were subjected to myocardial infarction. Three days after myocardial infarction, hearts were harvested and sectioned. Myocardial apoptosis was examined by TUNEL assay. (A) TUNEL staining positive apoptotic cells (green fluorescence). DAPI staining nucleus (blue color). Qualitative data showed the percent apoptotic cells. (BC) Increased expression of miR-130a attenuated MI-induced caspase-3/7 and -8 activities in the myocardium. (D) Increased expression of miR-130a increased Bcl2 and decreased Bax levels in the myocardium after myocardial infarction. n=4 in each sham control group. n=6 in each MI group. *$p<0.05$ compared with indicated group.

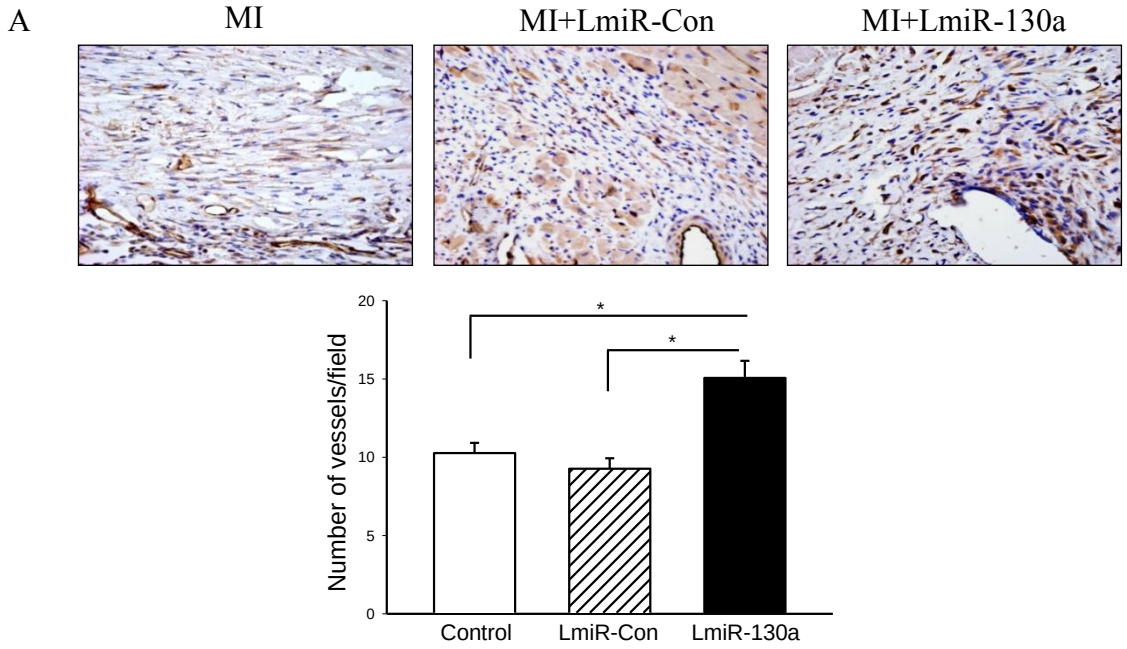
Transfection of LmiR-130a Increases Microvascular Density in the Myocardium Following Myocardial Infarction

Angiogenesis is an important factor for improvement of cardiac function and attenuation of cardiac remodeling after myocardial infarction [43]. We examined the effect of increased expression of miR-130a on angiogenesis by staining of microvascular density with anti-CD31 antibody. As shown in Figure 5.6A, at 21 days after MI, in LmiR-130a transfected hearts, the

numbers of microvascular densities were significantly greater by 46.8% than in untransfected group. There was no significant difference in the microvascular densities between untransfected and transfected LmiR-control groups. The data indicates that transfection of LmiR-130a promoted angiogenesis after myocardial infarction.

Transfection of MiR-130a Increases VEGF Levels and Suppresses HoxA5 Expression

Vascular endothelial growth factor (VEGF) is an important pro-angiogenic growth factor [44] while homeobox protein HoxA5 is an antiangiogenic factor [45]. As shown in Figure 5.6BC, myocardial infarction induced increases in the levels of VEGF by 142.1% and HoxA5 by 105.2%, respectively, compared with sham control. In LmiR-130a transfected hearts, however, the levels of VEGF were significantly increased by 83.1% (Figure 5.6B) and HoxA5 levels were decreased by 49.7% (Figure 5.6C), when compared with untransfected MI group (Figure 5.6BC). There was no significant difference in the levels of VEGF and HoxA5 between LmiR-control and untransfected groups following myocardial infarction.



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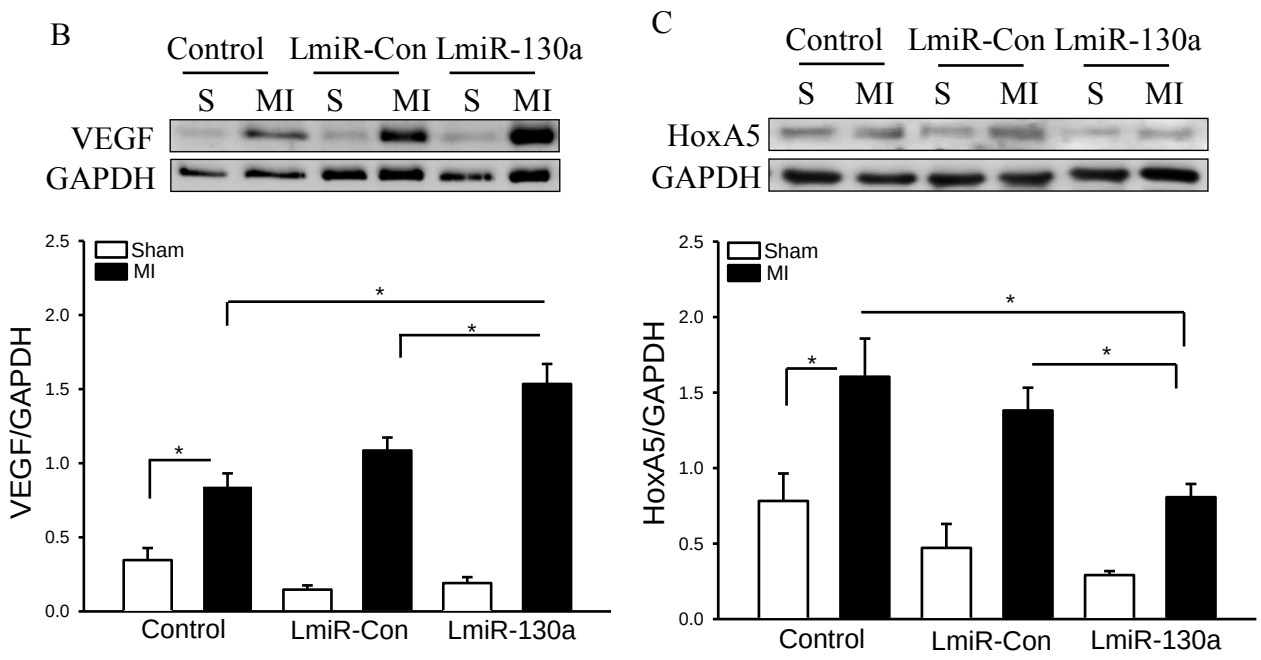


Figure 5.6 Transfection of LmiR-130a increases small vascular density following myocardial infarction. Mice were transfected with LmiR-130a or LmiR-control 7 days before the hearts were subjected to myocardial infarction. Hearts were harvested and sectioned at 21 days after myocardial infarction. Heart sections were stained with anti-CD31 antibody. (A) Representative images of heart sections stained with anti-CD31 antibody to visualize the vascular density from untransfected and transfected mice with LmiR-130a and LmiR-Control. Quantification of immunohistochemistry staining with anti-CD31 antibody in the ischemic zone showed increased numbers of small vascular densities by transfection of LmiR-130a. (B) Western blots showed that increased expression of miR-130a significantly increased the levels of VEGF and decreased HoxA5 expression in the myocardium. n=4 in each sham control group. n=6 in each MI group. n=3-5 for immunohistochemistry staining. *$p<0.05$ compared with indicated group.

Increased MiR-130a Expression Promotes Endothelial Cells Migration

We performed *in vitro* experiments using human umbilical vein endothelial cells (HUVECs) to examine the effect of miR-130a on the migration of HUVECs into the wound area. First, we transfected HUVECs with miR-130a mimics, scramble-miR mimics, and anti-miR-

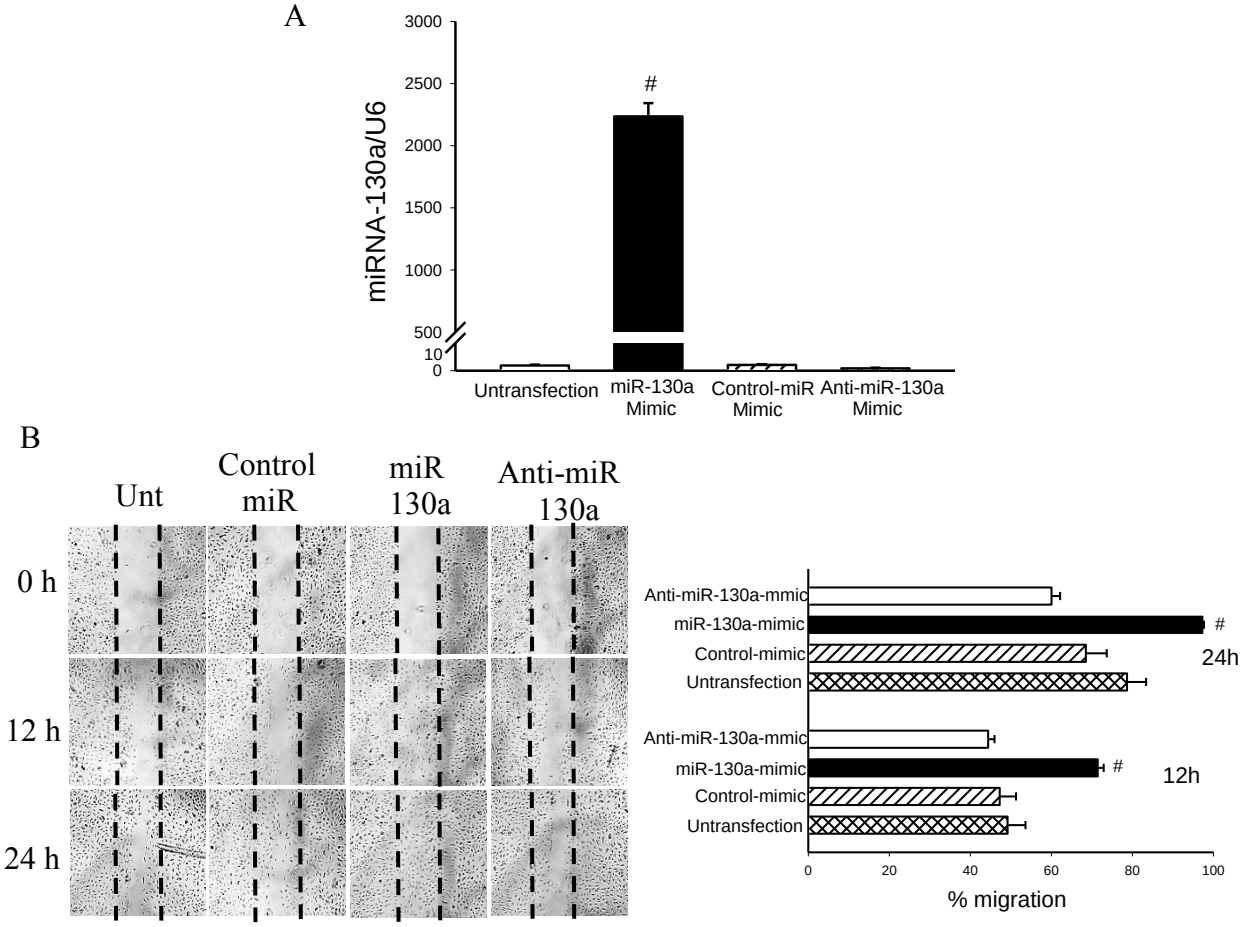
130a mimics, respectively. Scramble-miR mimics served as miR-control. Figure 5.7A shows that the levels of miR-130a were significantly increased (more than 1,000 fold) after transfection compared with untransfected control. Transfection of scramble-miR mimics and anti-miR-130a mimics did not alter the levels of miR-130a in the endothelial cells.

Next, we examined the role of miR-130a in HUVECs migration by scratch wound assays. Figure 5.7B shows that endothelial cells filled the wound area by 49.2% at 12 h and by 78.7% at 24 h in untransfected HUVECs after the scratch. However, transfection of miR-130a promoted HUVECs migration to the wound area by 71.4% at 12 h and 97.4% at 24 h, respectively, after the scratch. Transfection of miR-control or anti-miR-130a mimics did not promote the migration of HUVECs into the wound area.

MiR-130a Suppresses PTEN Expression and Increases Akt Phosphorylation in Endothelial Cells and H9C2 Cardiomyoblasts

Increased Akt activity has been shown to stimulate HUVECs migration and proliferation [46]. We examined whether miR-130a induces Akt phosphorylation in HUVECs. Figure 5.7C-D shows that transfection of miR-130a significantly increased the levels of Akt phosphorylation by 148.8% and suppressed PTEN expression by 50.9% in HUVECs that were cultured under normoxia condition, when compared with untransfected normal ECs. Transfection of miR-130a also suppressed PTEN expression by 59.4% and increased the phosphorylated Akt levels by 179.2% after H/R, when compared with untransfected H/R cells. Transfection of LmiR-control or anti-miR-130a did not alter the levels of Akt phosphorylation and PETN expression in HUVECs under normoxia or H/R condition.

Similar results were observed in H9C2 cardiomyoblasts. MiR-130a mimics transfection increased the levels of miR-130a (148 fold) compared with untransfected H9C2 cells (Figure 5.7E). Transfection of miR-130a suppressed PTEN expression by 53.9% and increased Akt phosphorylation by 4.3 fold compared with untranfected group under normoxia condition (Figure 5.7F). Following H/R, the levels of PTEN were lower and phospho-Akt levels were greater in miR-130a transfected cells than in untransfected cells (Figure 5.7G). Transfection of LmiR-control or anti-miR-130a did not alter the levels of Akt phosphorylation and PETN expression in H9C2 cells under normoxia or H/R condition.



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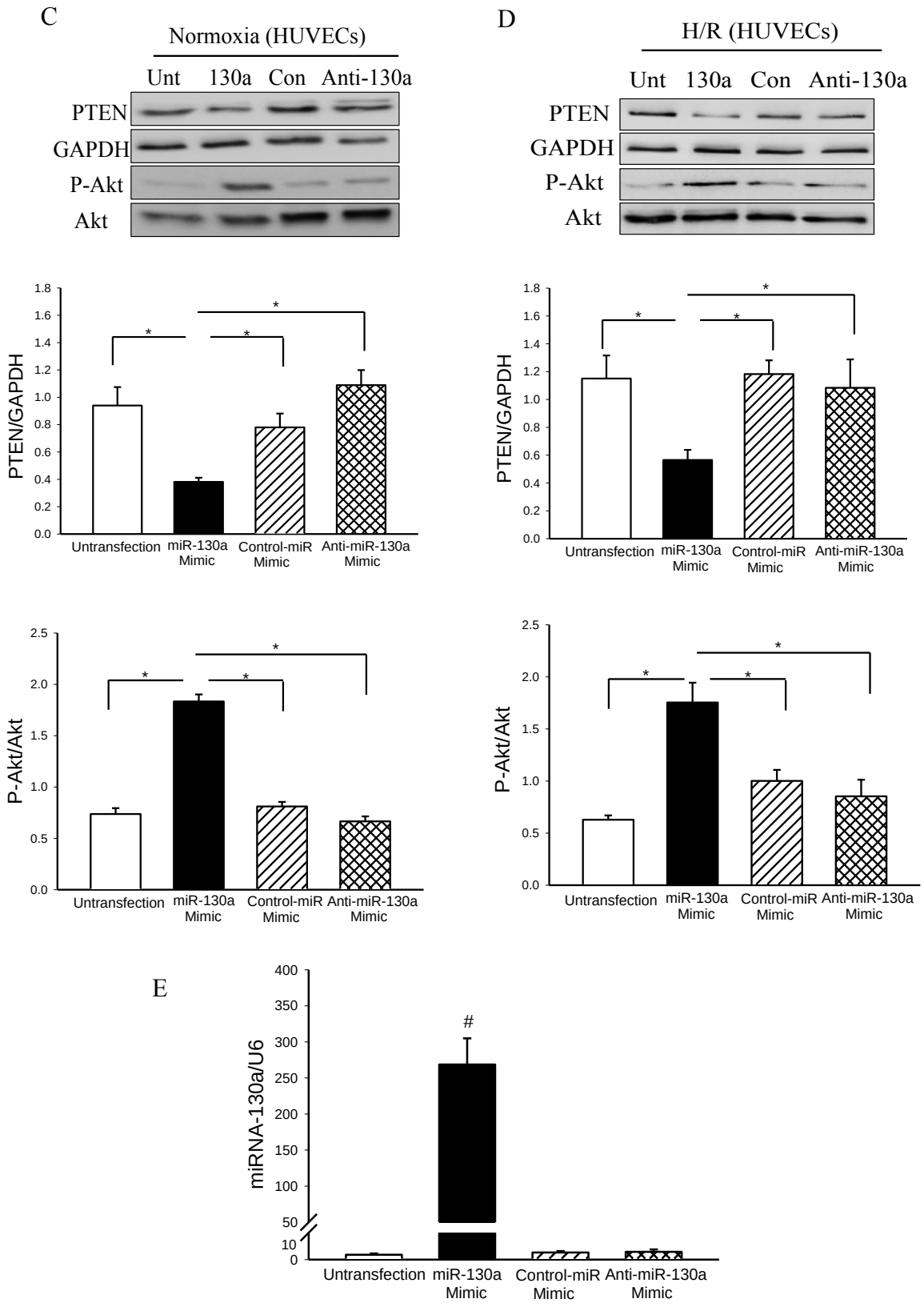

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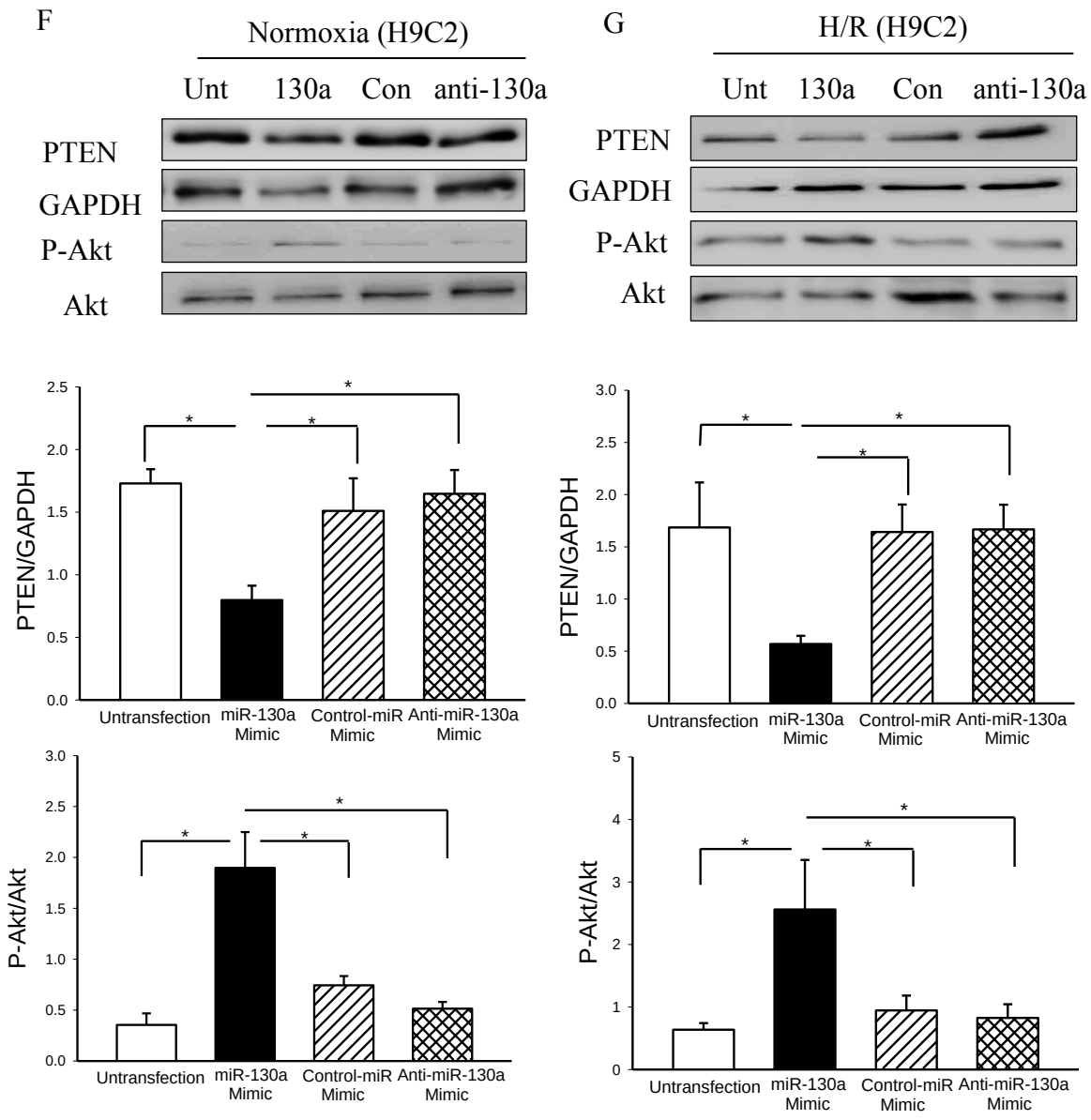


Figure 5.7 Increased miR-130a expression promotes endothelial cells migration. HUVECs or H9C2 cells were transfected with miR-130a mimics, scramble contro-miR mimics, and anti-miR-130a mimics, respectively. (A) Increased expression of miR-130a in HUVECs. 48 hours after transfection, cells were harvested for measurement of miR-130a levels by qPCR (n=3/group). (B) Scratch assay showed that transfection of miR-130a mimics promoted HUVECs migration to the wound area. Monolayers of HUVECs were scratched with a micropipette tip (200 µl) and photographed under light microscopy. Dashed line indicates the width of gap. Representative images were taken before and at 12 and 24 hours after injury (x4 magnification). Twenty four hours after transfection, HUVECs or H9C2 cells were subjected to

hypoxia (4 h) followed by reoxygenation (24 h). The cells cultured under normal condition served as control (normoxia). The cells were harvested and cellular proteins were isolated for Western blot. Transfection of miR-130a mimics suppressed PTEN expression (C) and increased Akt phosphorylation (D) in HUVECs. (E) Increased expression of miR-130a levels after transfection of miR-130a mimics in H9C2 cardiomyoblasts (n=3/group). (F.G) Increased expression of miR-130a suppressed PTEN expression and increased Akt phosphorylation under normoxia (F) and under H/R (G) conditions in H9C2 cells. n=4 in each group for Western blot. *$p<0.05$ compared with indicated group. $^{\#}p<0.05$ compared to all other groups.

Transfection of LmiR-130a Suppresses PTEN Expression and Increases the Levels of Phosphorylation of Akt and GSK-3β in the Myocardium

Activation of PI3K/Akt signaling protects cells from ischemia-induced injury [47]and apoptosis [48], as well as promotes angiogenesis [7]. Activation of PI3K/Akt signaling also improves cardiac function following myocardial ischemic injury [49]. We examined the effect of miR-130a on activation of PI3K/Akt signaling. PTEN is a negative suppressor of PI3K activity [9]. Interestingly, increased expression of miR-130a significantly suppressed the expression of PTEN (Figure 5.8A). The levels of PTEN in LmiR-130a transfected hearts were significantly reduced by 70.0% in sham control and by 63.8% in myocardial infarcted hearts, respectively, when compared with untransfected respective groups. Transfection of LmiR-130a also significantly increased the levels of phosphorylated Akt by 135.9% (B) and phosphorylated GSK-3β (Figure 5.8C) by 58.9% after myocardial infarction, when compared with untransfected MI hearts (Figure 5.8B&C). Transfection of LmiR-control did not alter the levels of PTEN as well as phosphorylated Akt and phosphorylated GSK-3β (Figure 5.8A-C)

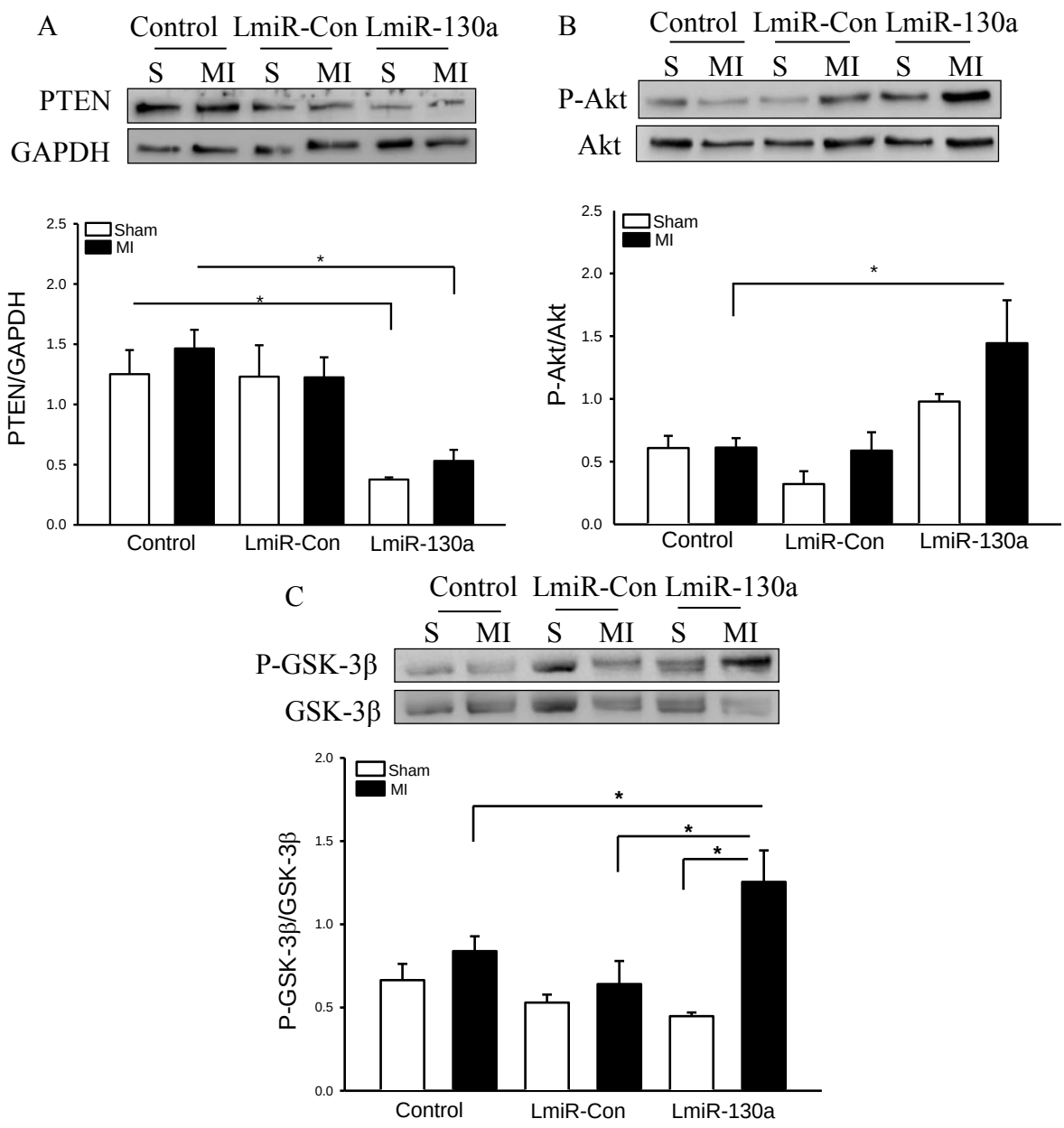
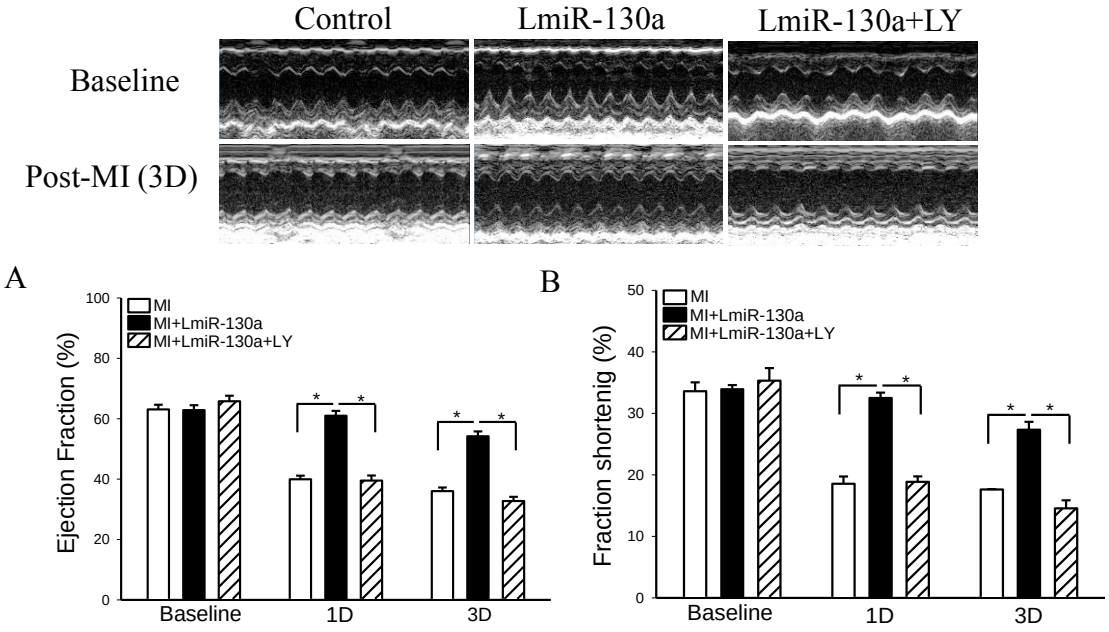


Figure 5.8 Transfection of LmiR-130a suppresses PTEN expression and increases the levels of phosphorylated Akt and GSK-3β in the myocardium. Mice were transfected with LmiR-130a or LmiR-control. Seven days after transfection, mice were subjected to myocardial infarction for 3 days. The hearts were harvested for isolation of cytoplasmic proteins. Western blots were performed for analysis of the levels of PTEN (A), phosphorylated Akt (B), and phosphorylated GSK-3β (C) in the myocardium. n=4 in each sham control group. n=6 in each MI group. *p<0.05 compared with indicated group.

PI3K Inhibition Abolishes MiR-130a-Induced Attenuation of Cardiac Dysfunction Following Myocardial Infarction

To determine whether activation of PI3K/Akt signaling plays a critical role in miR-130a-mediated protection against myocardial infarction, we administered a specific PI3K inhibitor LY294002 to LmiR-130a transfected mice immediately after permanent ligation of LAD coronary artery. Cardiac function was measured by echocardiography at 1 day and 3 days after myocardial infarction. Figure 5.9 showed that LY294002 administration completely abolished transfection of LmiR-130a-induced attenuation of cardiac dysfunction following MI. The EF% (Figure 5.9 A) and FS% (Figure 5.9 B) values in LY294002 treated MI hearts were significantly decreased on day 1 (35.2% and 39.6%) and on day 3 (41.5% and 45.1%), respectively, when compared with in LmiR-130a transfected MI hearts. LY294002 administration also significantly prevented miR-130a-induced increases in the levels of Akt phosphorylation (Figure 5.9C). The data indicates that increased expression of miR-130a-induced protection after MI is mediated by a PI3K/Akt dependent mechanism.



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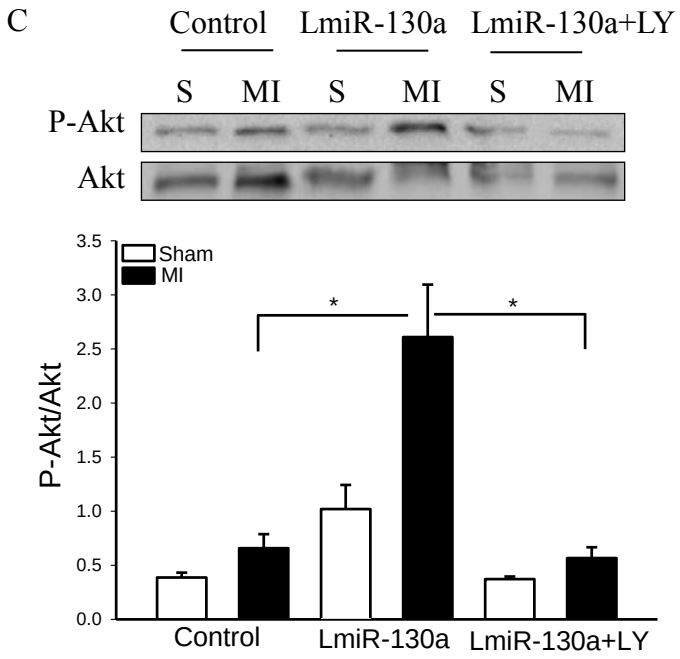

Figure 5.9 PI3K inhibition abolishes miR-130a-attenuated cardiac dysfunction following myocardial infarction. Mice were transfected with LmiR-130a. Seven days after transfection, mice were treated with or without LY294002 (1mg/25 gram body weight) immediately after induction of myocardial infarction. Cardiac function was measured by echocardiography at 1 day and 3 days following myocardial infarction. LY294002 administration completely abolished LmiR-130a-induced attenuation of cardiac dysfunction in EF% (A) and FS% (B) following MI. Representative M-mode images before and after MI at 3 days was shown above. n=24 in untransfected group. n=9 in LmiR-130a transfected group. n=9 in LmiR-130a transfected group with LY294002 administration. (C) PI3K inhibition by LY294002 abolished LmiR-130a-induced an increase in the levels of phosphorylated Akt. n=5 in each sham control group. n=6 in each MI group. * $p<0.05$ compared with indicated group.

Discussion

The present study demonstrated that miR-130a plays an important role in the protection against myocardial infarction. Specifically, we observed that increased expression of miR-130a by transfection of lentiviral expressing miR-130a (LmiR-130a) into the myocardium

significantly attenuated cardiac dysfunction and improved remodeling following myocardial infarction. The mechanisms involved suppression of PTEN expression, resulting in activation of PI3K/Akt signaling in the myocardium. Importantly, PI3K inhibition abolished the attenuation of cardiac dysfunction by LmiR-130a in myocardial infarcted hearts. The data suggests that miR-130a serves a protective role in myocardial ischemic injury. Increased expression of miR-130a may be a new therapeutic approach for heart attack patients.

We have previously reported that administration of Pam3CSK4 (TLR2 ligand) protected the myocardium from ischemia/reperfusion injury through a PI3K/Akt dependent mechanism [30]. We observed in the present study that Pam3CSK4 administration significantly increased the levels of miR-130a in the myocardium. In contrast, myocardial infarction markedly decreased the levels of miR-130a in the myocardium. Collectively, the data indicates that miR-130a may serve a protective role in myocardial ischemic injury. To evaluate our hypothesis, we constructed lentiviral expressing miR-130a (LmiR-130a) and transfected the myocardium with LmiR-130a 7 days before induction of myocardial infarction. We observed that increased expression of miR-130a significantly attenuated cardiac dysfunction, decreased myocardial apoptosis, attenuated fibrotic deposition, and enhanced angiogenesis following myocardial infarction.

Myocardial apoptosis has been demonstrated to contribute to cardiac dysfunction following myocardial infarction and myocardial I/R injury [41]. Myocardial infarction induced apoptosis in the myocardium of human [50] and experimental animals [51]. Inhibition of myocardial apoptosis has been shown to attenuate cardiac dysfunction and remodeling after MI [52-54]. We have observed in the present study that transfection of LmiR-130a markedly decreased myocardial apoptosis in infarcted hearts, which was positively correlated with the data that increased expression of miR-130a significantly improved cardiac function following MI. We

have shown that transfection of LmiR-130a suppressed MI-induced increases in Bax levels and increased in Bcl2 levels in the myocardium. It is well known that Bcl2 is anti-apoptotic protein while Bax acts as an antagonist of anti-apoptotic Bcl2 [42]. Bcl2 further prevented the release of cytochrome c and thus activation of caspases [55]. We found that LmiR-130a transfection markedly decreased the activities of caspase-3/7 and caspase-8 in the myocardium after MI. Therefore, inhibition of myocardial apoptosis by miR-130 could be one of the mechanisms for attenuation of cardiac dysfunction and remodeling after MI.

Promotion of angiogenesis is an important approach for improvement of remodeling and cardiac function after myocardial infarction [56, 57]. Stimulation of angiogenesis reduced the progression of myocardial infarction, decreased cardiac fibrosis, improved cardiac function, and decreased the risk of cardiac rupture following myocardial infarction [56, 57]. We observed that increased expression of miR-130a significantly increased the numbers of small vessels in the ischemic area, indicating that miR-130a promoted angiogenesis. Indeed, we found that the levels of VEGF in LmiR-130a transfected hearts were greater than in untransfected hearts after myocardial infarction. However, at present we do not understand the mechanism by which miR-130a stimulates VEGF expression in the myocardium following MI. Yun et al. reported that miR-130a suppressed the expression of anti-angiogenic gene GAX and HoxA5 in endothelial cells [21]. Homeobox (Hox) genes, including HoxA5, are transcriptional regulators which modulate embryonic morphogenesis and pathological tissue remodeling in adults via regulation of genes associated with cell-cell or cell extracellular matrix (ECM) interactions [45]. HoxA5 has been demonstrated to block angiogenesis [45]. Sustained expression of HoxA5 resulted in downregulation of many pro-angiogenic genes, including VEGFR2, ephrin A1, Hif1α, and COX-2 [45]. In addition, HoxA5 upregulated expression of anti-angiogenic genes including

Thrombospondin-2 [45]. We observed that increased expression of miR-130a significantly suppressed the expression of HoxA5 in the myocardium. Yun et al. reported that miR-130a targeted site in the 3'-UTR of the antiangiogenic homeobox gene HoxA5 [21]. Decreased expression of miR-130a resulted in dysfunction of endothelial progenitor cells [22]. In addition, the levels of miR-130a was reduced in the endothelial progenitor cells from patients with coronary artery disease [58]. Collectively, the data indicate that miR-130a may play a role in regulating endothelial cell proliferation and migration. Indeed, we observed that transfection of endothelial cells with miR-130a mimics stimulated endothelial cell proliferation and migration into wound area after scratched cultured ECs. The mechanisms of miR-130a stimulated angiogenesis involved suppression of HoxA5 and upregulation of VEGF expression.

Activation of PI3K/Akt signaling plays a critical role in the regulation of cell growth, cell proliferation, and survival [59]. PI3K/Akt signaling also plays an important role in the progression of angiogenesis [60]. PI3K/Akt signaling has implicated in the regulation of cardiac growth and coronary angiogenesis [61]. Moreover, PI3K/Akt signaling was involved in angiogenesis after myocardial ischemic injury [62]. We have previously reported that activation of PI3K/Akt signaling improved cardiac function, reduced infarct size, and decreased myocardial apoptosis in the myocardium following myocardial ischemic injury [29, 30, 39]. In the present study, we found that increased expression of miR-130a significantly increased the levels of Akt and GSK-3β phosphorylation in the myocardium. It is well known that PTEN is a negative regulator of PI3K activity [9]. Suppression of PTEN expression has reported to decrease myocardial ischemic injury via activation of PI3K/Akt signaling [63]. Recent studies have shown that several miRs, such as miR-21 [64], miR-486 [65], miR-214 [66], and miR-29 [67], suppressed PTEN expression, resulting in activation of the PI3K/Akt signaling. We demonstrated in the present study that increased

expression of LmiR-130a suppressed PTEN expression. *In vivo* data showed that transfection of the myocardium with LmiR-130a significantly decreased the levels of PTEN in the myocardium in the presence or absence of MI. *In vitro* data showed that transfection of endothelial cells with miR-130a mimics suppressed PTEN expression in the presence or absence H/R. Suppression of PTEN expression resulted in increases in the levels of Akt and GSK-3β phosphorylation, indicating activation of PI3K/Akt signaling, which were positively correlated with the improvement of cardiac function, stimulation of microvascular densities in the myocardium, and attenuation of remodeling following myocardial infarction. Moreover, we have demonstrated that PI3K inhibition by a PI3K specific inhibitor, LY294002, abolished miR-130a-induced attenuation of cardiac dysfunction after myocardial infarction.

In summary, we demonstrated that miR-130a served a protective role in attenuation of cardiac dysfunction and remodeling after myocardial infarction. The mechanisms involved suppression of PTEN expression, resulting in activation of PI3K/Akt signaling, which promoted angiogenesis, attenuated anti-apoptosis, and decreased cardiac fibrosis in the myocardium.

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CHAPTER 6

CONCLUSIONS

Ischemia/reperfusion injury has important clinical relevance in several human diseases, including but not limited to, heart attack, ischemic stroke, ischemic hepatitis, and ischemic renal disease as well as cardiovascular transplant surgeries. A hallmark of ischemia/reperfusion injury is the excessive inflammatory responses (Libby and others 2002; Danton and Dietrich 2003; Ren and others 2003; Onai and others 2004;). Toll-like receptors are critical factors mediating the innate immune and inflammatory responses through recognition of exogenous pathogens and endogenous damage-associated molecules (Kopp and Medzhitov 1999; Aderem and Ulevitch 2000; Bowie and Haga 2005; Kawai and Akira 2010). Therefore, evidence suggests that TLRs play an important role in ischemia/reperfusion injury. Indeed, evidence has shown that TLR4 plays a deleterious role in myocardial and cerebral I/R injury (Caso and others 2007). We have demonstrated that I/R injury rapidly increases myocardial IκB-α phosphorylation and NF-κB binding activity, suggesting that the TLR4/NF-κB-mediated signaling pathway plays a role in the inflammatory responses to myocardial I/R injury (Ha and others 2011; Hua, Ha, and others 2007). We found that NF-κB binding activity was significantly decreased, whereas the levels of phosphorylated Akt were increased in the myocardium or brain tissues of the TLR4-deleted mice subjected to I/R injury (Hua, Ma, and others 2007; Hua, Ha, and others 2007). This observation suggests that there may be a cross talk between the TLR/NF-κB-mediated signaling pathway and PI3K/Akt signaling. We speculated that inhibition of TLR/NF-κB-mediated signaling pathway

and activation of the PI3K/Akt signaling may be mechanisms of protection in response to I/R injury.

In Chapter 2 we demonstrated that modulation of TLR2 by its ligand, Pam3CSK4 induced protection against cerebral I/R injury through a TLR2/PI3K/Akt-dependent mechanism. 1) Pretreatment of mice with Pam3CSK4 significantly reduced infarct size following cerebral I/R injury. 2) Therapeutic administration of Pam3CSK4 also significantly reduced infarct size. 3) Pam3CSK4 administration improved morphology of neurons in the hippocampus. 4) Pam3CSK4 administration decreased I/R-activated apoptotic signaling and increased levels of Hsp27, Hsp70, and Bcl2 in the brain tissues. 5) Pam3CSK4 administration significantly attenuated I/R-induced NF-κB binding activity. 6) Pam3CSK4 treatment significantly increased the levels of Akt and GSK-3β phosphorylation in the brain tissues. 7) TLR2 deficiency completely abolished Pam3CSK4 induced protection against cerebral I/R injury. 8) PI3K inhibition by LY294002 completely abolished the protection by Pam3CSK4 in cerebral I/R injury.

In Chapter 3 we provided evidence that TLR9 ligand, CpG-ODN induced protection against cerebral I/R injury via activation of PI3K/Akt signaling. 1) Pretreatment of mice with CpG-ODN significantly reduced infarct size following cerebral I/R injury. Therapeutic administration of CpG-ODN also decreased I/R-induced infarct size. 2) CpG-ODN administration improved neuronal morphology in the ischemic hippocampus. 3) CpG-ODN treatment increased Bcl2 levels and decreased the expression of proapoptotic factors in ischemic brain tissues. 4) CpG-ODN administration increased the levels of Akt and GSK-3β phosphorylation. 5) PI3K inhibition by LY294002 abolished CpG-ODN induced protection against cerebral I/R injury.

In Chapter 4 we observed for the first time that TLR3 deficiency attenuated cardiac dysfunction after myocardial I/R injury and myocardial infarction. 1) TLR3 deficiency reduced myocardial infarct size following myocardial I/R injury. 2) TLR3 deficiency attenuated I/R-induced myocardial apoptosis and upregulation of proapoptotic factors in the myocardium. 3) TLR3 deficiency attenuated I/R-induced Fas-mediated apoptotic signaling in the myocardium. 4) TLR3 deficiency prevented I/R-induced myocardial NF-κB binding activity and the production of proinflammatory cytokine. 5) TLR3 deficiency attenuated I/R-induced infiltration of neutrophils and macrophages into the myocardium.

Recently microRNAs as small noncoding single stranded RNAs have been investigated for their biological functions. MicroRNAs can cleave their target mRNAs and inhibit the translation of their target mRNAs (Kim, 2005). MicroRNAs are implicated to influence the pathophysiological processes in myocardial ischemic injury. The levels of miRNAs are altered in ischemic hearts. Interesting, microRNAs have shown to regulate several cellular signaling pathways, such as TLR/NF-κB-mediated signaling and PI3K/Akt signaling (O'Neill and others 2011; Xu and Mo 2012) that play an important role in the pathogenesis of myocardial ischemic injury.

In Chapter 5 we provided the first evidence that increased expression of miR-130a significantly improved cardiac function and promoted angiogenesis after myocardial infarction. 1) Increased expression of miR-130a attenuated myocardial infarction-induced cardiac dysfunction and cardiac fibrosis. 2) Increased expression of miR-130a reduced infarct size in myocardial I/R injury model. 3) Increased expression of miR-130a attenuated myocardial infarction-induced apoptosis. 4) Increased expression of miR-1300a promoted angiogenesis via suppression of

PTEN expression and activation of PI3K/Akt signaling. 5) PI3K inhibition by LY294002 abolished the cardioprotoective effect of miR-130a on myocardial infarction.

In summary, this dissertation shows that Toll-like receptors and microRNAs play important roles in ischemic stroke and heart attack. This research described in this dissertation may provide novel directions for protecting the brain and heart against ischemic injury. The findings shown in this dissertation may provide important basic science insights into the molecular mechanisms and serve as evidence for further studies that will evaluate feasibility, efficacy, and safety of Pam3CSK4, CpG-ODN, a chemical drug inhibiting TLR3, and miR-130a in clinical application for cardiovascular disease. The working model for the major conclusions is shown in Figure 6.1. Taken together, the findings in this dissertation indicate that therapy using a TLR2 specific agonist (Pam3CSK4) or TLR9 (CpG-ODN) specific agonist may be effective in reducing ischemic stroke injury. Specially, evidence has reported that CpG-ODN is reasonably safe in clinical trials for cancer and infectious disease when administered as vaccine adjuvants (Bode and others 2011). Thus, it strongly indicates that CpG-ODN has significantly clinical application for ischemic stroke patients. For heart attack patients TLR3 may be an important target for the management and treatment of heart attack patients. Moreover, increased expression of miR-130a also may be a new therapeutic approach for patients with myocardial infarction.

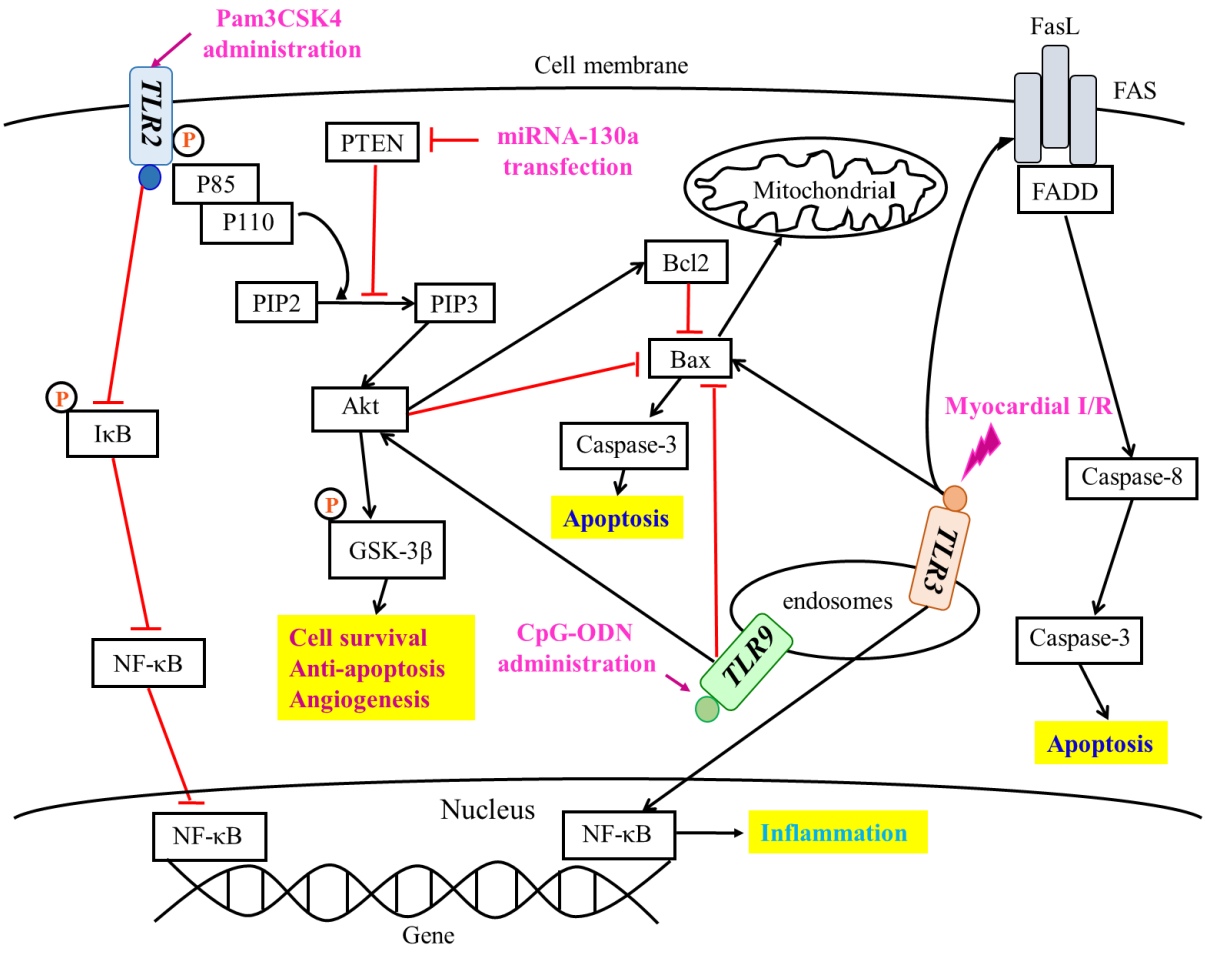

Figure 6.1. A working model for the major conclusions in this dissertation. Pretreatment with TLR2 ligand (Pam3CSK4) or TLR9 ligand (CpG-ODN) induces activation of PI3K/Akt signaling that further inhibits cell apoptosis following cerebral I/R injury. Stimulation of TLR2 or TLR9 also attenuates NF-κB binding activity following cerebral I/R injury. Stimulation of TLR2 leads to tyrosine phosphorylation of TLR2. Subsequently, the phosphorylated TLR2 associates with the p85 subunit of PI3K, resulting in PI3K activation. Following myocardial I/R injury, TLR3 contributes to activate Fas-death receptor-mediated signaling, increase NF-κB binding activity, as well as increase the levels of Bax and the production of proinflammatory cytokines. Transfection of miR-130a suppresses PTEN expression, resulting in activation of PI3K/Akt signaling that leads to promote cell survival and angiogenesis as well as inhibit apoptosis following myocardial infarction.

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APPENDICES

ABBREVIATIONS

ANOVA	-Analysis of variance
AP-1	-Activator protein-1
CpG DNA	-unmethylated CpG dinucleotides
DAMPS	-Damage associated molecular patterns
dsRNA	-Double stranded RNA
EF	-Ejection fraction
EMSA	-Electrophoretic mobility shift assay
ER	-Endoplasmic reticulum
FBS	-Fetal bovine serum
FS	-Fraction shortening
GAPDH	-Glyceraldehyde 3-phosphate dehydrogenase
HF	-Hippocampal formation
HMGB1	-High-mobility group protein B1
H/R	-Hypoxia/Reoxygenation
HSP60	-Heat shock protein 60
HSP70	-Heat shock protein 70
HUVECs	-Human umbilical vein endothelial cells
I/R	-Ischemia/reperfusion
IA	-Ischemic area
ICAM-1	-Intercellular adhesion molecule-1
IFN	-Interferon
IHC	-Immunohistochemistry
IKK	-IκB kinase
IL-1β	-Interleukin-1β
IRAK	-Interleukin-1 receptor-associated kinase
IRF	-Interferon regulatory factor

LAD	-Left anterior descending
LPS	-Lipopolysaccharide
LRR	-Leucine-rich repeat
LV	-Left ventricle
MCAO	-Middle cerebral artery occlusion
MI	-Myocardial infarction
MiRNAs	-MicroRNAs
mPTP	-Mitochondrial permeability transition pore
MyD88	-Myeloid differentiation primary response gene (88)
NF-κB	-Nuclear factor kappa-B
PAMPS	-Pathogen associated molecular patterns
PBS	-Phosphate buffered saline
PI3K	-Phosphoinositide 3-kinase
PIP2	-Phosphatidylinositol 4, 5-bisphosphate or PtdIns(4,5)P2
PIP3	-Phosphatidylinositol (3, 4, 5)-triphosphate
Poly I:C	-Polyinosine-polycytidylic acid
PRRS	-Pattern recognition receptors
PTEN	-Phosphatase and tensin homolog
PGN	-Peptidoglycan
RA	-Risk area
RIP-1	-Receptor-interacting protein-1
ROS	-Reactive oxygen species
SEM	-Standard error of the mean
ssRNA	-Single stranded RNA
TBST	-Tris buffered saline tween 20
TIR domain	-Toll/interleukin-1receptor homology domain
TLRs	-Toll-like receptors
TNF-α	-Tumor necrosis factor-α
TRAF6	-TNF receptor associated factor
TRIF	-TIR-domain-containing adapter-inducing interferon-β
TTC	-Triphenyl tetrazolium chloride

TUNEL	-Terminal deoxynucleotidyl transferase dUTP nick end labeling
Unt	-Untreated
UTR	-Untranslated region
VCAM-1	-Vascular cell adhesion molecule-1
mg	-Milligram
ml	-Milliliter
pg	-Picogram
µg	-Microgram
µl	-Microliter
µM	-Micromolar

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