THE ROLE OF LIPOCALIN-2 IN STROKE-REPERFUSION INJURY

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by

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<tr>
<td>ACA</td>
<td>anterior cerebral artery</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BOCT</td>
<td>brain type organic cation transporter</td>
</tr>
<tr>
<td>CXCL1</td>
<td>chemokine (C-X-C motif) ligand 1</td>
</tr>
<tr>
<td>ECA</td>
<td>external cerebral artery</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular-matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>Iba 1</td>
<td>Ionized calcium binding adaptor molecule 1</td>
</tr>
<tr>
<td>ICA</td>
<td>internal cerebral artery</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>JAM</td>
<td>junction adhesion molecules</td>
</tr>
<tr>
<td>LCN2</td>
<td>lipocalin-2</td>
</tr>
<tr>
<td>MAP2</td>
<td>microtubule-associated protein 2</td>
</tr>
<tr>
<td>MCA</td>
<td>middle cerebral artery</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Monocyte chemoattractant protein-1 alpha</td>
</tr>
<tr>
<td>MMPs</td>
<td>matrix-metalloproteinases</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NeuN</td>
<td>neuronal nuclear protein</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>OGD</td>
<td>oxygen glucose deprivation</td>
</tr>
<tr>
<td>PcomA</td>
<td>posterior communicating artery</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>pMCAO</td>
<td>permanent middle cerebral artery occlusion</td>
</tr>
<tr>
<td>rCBF</td>
<td>regional cerebral blood flow</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>TJ$s$</td>
<td>tight junctions</td>
</tr>
<tr>
<td>tMCAO</td>
<td>transient middle cerebral artery occlusion</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrotic factor</td>
</tr>
<tr>
<td>TNF-$\alpha$</td>
<td>tumor necrosis factor - alpha</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>TTC</td>
<td>2, 3, 5-triphenyltetrazolium chloride</td>
</tr>
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</table>
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CHAPTER 1

Introduction

1.1 Stroke and current treatment strategy

Stroke is the third leading cause of death and the leading cause of adult disability in United States. Every year more than 700,000 people have a stroke, and 140,000 people die from it (approximately one person every 3.3 minutes) (Hinkle and Guanci, 2007). However, only 10% stroke people recover almost completely, 90% stroke survivors suffer minor to severe residual impairments. Due to the causes, stroke is divided into ischemic stroke and hemorrhagic stroke. Ischemic stroke is caused by the blockage of blood vessels in brain. Hemorrhagic stroke is due to bleeding in brain. Approximately 87% stroke is caused by ischemia. Current acute treatment aims to restore blood supply by administering fibrinolytic agents such as tissue plasminogen activator (tPA) (Al-Khoury and Lyden, 2004). Unfortunately, less than 5% of stroke victims receive these drugs, since their efficacy is dependent upon delivery within the first 4.5 hours of stroke onset (Saver et al., 2013). Treatment beyond 4.5 hours can also increase the risk of hemorrhagic transformation and initiate reperfusion injury, which induces severe neurological deficits and increased mortality (Del Zoppo et al., 2009; Kidwell and Wintermark, 2008). However, only less than 3% stroke patients receive tPA treatment on time and recover.

The timely diagnosis of stroke is challenging. Neuroimaging techniques such as magnetic resonance imaging (MRI) and computed tomography scan (CT) are invaluable (Baird et al., 2001; Kidwell et al., 2004). However, small clinics and community hospitals are often
not equipped with specialized radiologic equipment. Thus, the availability of a rapid assay using blood biomarkers would greatly enhance the diagnosis, treatment and prognosis of patients with stroke, avoiding utilization of costly resources and providing cost effective care (Reynolds et al., 2003; Whiteley et al., 2009).

1.2 Ischemic stroke and reperfusion injury

Ischemic stroke initiates with the disruption of regional cerebral blood flow (rCBF). When rCBF decreased to less than 35% of normal, the cellular ionic equilibrium is disrupted (Garcia et al., 1977). Ischemia alters membrane potential, increases intracellular edema and destroys cytoskeletal organization of endothelia. It initiates brain inflammatory reaction, starting with the activation of local cells (microglial cells and vascular endothelial cells), and the expression of cytokines (IL-6, IL-1β, TNF-α), chemokines (MCP-1 and MIP-1α) and reactive oxygen species (ROS) from injured brain tissue (Jin et al., 2010). These cytokines and chemokines attract leukocyte (neutrophils and monocytes/macrophages), following by leukocyte infiltration, the mediators of blood brain barrier leakage and edema formation in both animal models and stroke patients (Amantea et al., 2009; Kriz, 2006; Price et al., 2004).

Although ischemia per se is responsible for the initiation of neuronal damage during ischemic stroke, partial or complete recanalization by tPA may promote secondary infarct growth and worsen functional outcomes (Iadecola and Anrather, 2011). The tissue damage caused by blood resupply after ischemia is reperfusion injury. Therefore, great interest exists in developing effective neuroprotective agents that may be administered alone or with thrombolytic agents to reduce reperfusion injury (Chou and Messing, 2005; Eltzschig and Eckle, 2011). Reperfusion of oxygenated blood is followed by generation of reactive oxygen
species (ROS) and a series of inflammatory events including activation and infiltration of circulating neutrophils, monocytes/macrophages, and T-cells into infarcted brain tissue (Iadecola and Anrather, 2011; Jin et al., 2010). Reperfusion injury is associated with microvascular dysfunction, which contains injured endothelium, increased infiltration of fluid into interstitium, leukocyte adhesion, and leukocytes and plasma protein extravasation (Carden and Granger, 2000). The impairment of endothelial cells is exacerbated by reperfusion (Kvietys and Granger, 1997). In the initial period of reperfusion, severe endothelium dysfunction is observed, including enhanced cell swelling, lifting of endothelium from basement membrane and activated neutrophil adhesion to endothelium (Carden and Granger, 2000). And the nitric oxide-superoxide imbalance caused by reperfusion (increased superoxide and less NO) enhances the platelet aggregation, thrombus formation and adhesive interaction between endothelia and leukocytes (Carden and Granger, 2000; Grisham et al., 1998). The accumulation of superoxide increases the generation of $\text{O}_2^-$ and $\text{H}_2\text{O}_2$, which rapidly enhance inflammatory response and leukocyte-endothelia adhesion by increasing expression of adhesive molecules.

Post-ischemic inflammation is necessary for repair, but may have deleterious effects (Ceulemans et al., 2010; Eltzschig and Eckle, 2011). Thus, identifying neuroprotective and neurotoxic components of post-ischemic inflammation is central to developing effective and balanced therapeutic approaches to counteract stroke-reperfusion injury.

1.3 The role of neutrophils in stroke-reperfusion injury

In stroke-reperfusion injury, cytokines and chemokines generated by injured endothelial cells attract leukocytes in circulation system. Neutrophil is one of the earliest cells responding to the
microvascular obstruction, its accumulation has been reported as early as 30 minutes after permanent middle cerebral artery occlusion (pMCAO) in rat (Dereski et al., 1993). The processes of tissue injury caused by neutrophil contain the generation of oxygen free radical, release of proteases and proinflammatory mediators. Neutrophils activated by proinflammatory mediators generate superoxide anions and hydrogen peroxide. Oxygen free radicals produced by neutrophil promote the release of proinflammatory mediators and the expression of adhesive molecules from endothelial cells and increase endothelial permeability (McIntyre et al., 1995; Svendsen, 1990, 1994; Svendsen and Bjerrum, 1992). Neutrophil degranulates to release proteases to degrade the components of endothelial basement membrane and the proteins maintaining tight junction function (Carden and Granger, 2000). In stroke-reperfusion injury, proinflammatory mediators released by neutrophils activate more neutrophils, amplify the inflammatory response and promote neutrophil recruitment. The accumulating neutrophils infiltrate BBB, following rolling, adhesion, tight binding, diapedesis and migration. Three classes of cell adhesion molecules, selectins, \( \beta_2 \)-integrins (CD11/CD18 complex) and proteins of the immunoglobulin superfamily, affect neutrophil migration and cell-cell interactions.

1.4 Blood brain barrier in stroke-reperfusion injury

BBB leakage is crucial for the formation of cerebral edema, a lethal complication in stroke-reperfusion injury. BBB is a highly selective permeability barrier separating brain tissue with the peripheral circulation. Composition of BBB includes vascular endothelial cells, pericytes, astrocytes, neurons and extracellular-matrix (ECM) (Figure 1). The tight junctions (TJs) between the vascular endothelial cells prevent the substances of blood from entering the brain. The function of TJs is governed by claudins, occludin and junction adhesion molecules (JAM)
(Figure 1). Under normal physiological condition, TJs does not allow molecules with a molecular weight higher than 180 Da to access (Mitic and Anderson, 1998).

However, under ischemic phase of stroke, the loss of oxygen and nutrients insult vascular endothelial cells and cause depletion of ATP, ionic imbalance (increased intracellular calcium leading to edema), increased acidosis, oxidative stress and inflammatory response in ischemic brain tissue. These events cause TJs dysregulation and disassembly. Later on, the induction of proteases (like matrix-metalloproteinases (MMPs) and tissue plasminogen activator (tPA) promotes the degradation of BBB ECM (Sandoval and Witt, 2008). But the observable changes in BBB TJs permeability requires hours of reduction in rCBF (Betz, 1995). In the reperfusion
phase, the increased BBB permeability is due to the increased oxidative stress, inflammation response and enzymatic degradation (Heo et al., 2005; Wang and Shuaib, 2007).

1.5 Lipocalin-2 (LCN2) and its receptor

LCN2, a 25-kDa secreted glycoprotein, also known as 24p3 or neutrophil gelatinase-associated lipocalin (NGAL), was originally identified as a 25 kDa protein secreted by activated neutrophils (Kjeldsen et al., 2000; Kjeldsen et al., 1993). It binds small molecular weight ligands and can form complex with mmp9 (Åkerstrom et al., 2000; Nickolas et al., 2012). LCN2 is expressed at a low level in blood, heart, artery, kidney, and skeletal muscle under non-ischemic condition (Ip et al., 2011; Kjeldsen et al., 2000). Using a chemical-genetics approach, LCN2 was identified as one of PKCδ phosphorylation substrates in neutrophils (Allen et al., 2007; Kumar et al., 2015; Weng et al., 2015). PKCδ directly phosphorylates LCN2 at Thr-115, and mediates the secretion of LCN2 from activated neutrophils in vitro and after cerebral ischemia in vivo (Weng et al., 2015).

Two cellular receptors for LCN2 have been reported, brain type organic cation transporter (BOCT) and megalin, in various cell types. Brain type organic cation transporter (BOCT), also named as 24p3R or solute carrier family 22 member 17 (SLC22A17) (Devireddy et al., 2005), is LCN2’s putative cell surface receptor, playing a key role in iron homeostasis. It is reported that BOCT mediates iron uptake and apoptosis through LCN2-siderophore complex (Devireddy et al., 2005). Megalin belongs to low-density lipoprotein receptor family, mainly expressed in reactive astrocytes in rat’s brain (Bi et al., 2013). Previous study reported that megalin binds human LCN2 with high affinity and it mediates LCN2’s endocytosis (Hvidberg et al., 2005).
Molecular and cellular studies suggest that LCN2 is able to induce apoptosis in mouse pro-B lymphocytic cell line FL5.12 (Devireddy et al., 2005; Devireddy et al., 2010). In the ischemia reperfusion injury of organ transplantation, LCN2 increases the movement and infiltration of neutrophil, which is an essential factor of the inflammatory response (Aigner et al., 2007). The induction of LCN2 is also involved in the enhancement of several proinflammatory chemokines and cytokines (Rathore et al., 2011). LCN2 gene is strongly up regulated after 24 hours of global ischemia-reperfusion injury in rat brain (Büttner et al., 2009). The patients with ischemic stroke are also reported increased plasma concentration of LCN2 (Chan et al., 2012). And stroke patients with higher LCN2 level have higher mortality rate and are associated with worse prognosis of stroke (Chan et al., 2012). Based on the evidences, LCN2 might be a detrimental mediator in the reperfusion injury.

1.6 Immunotherapy in stroke-reperfusion injury

Immunotherapy is an effective adjuvant therapy for stroke-reperfusion injury in animal research (Yu et al., 2013). Many signaling pathways are altered after stroke attack (Yu et al., 2013). Due to the specificity of immune response, monoclonal antibodies can block specific signaling pathways, which may delay or reduce brain tissue damage in mice and even extend the therapeutic window of tPA induced thrombolysis (Macrez et al., 2011; Yu et al., 2013). Moreover, the interaction of antibodies with their target cytotoxic molecules and receptors may delay cell death or rescue cells (Yu et al., 2013). In the current immunotherapy study of stroke, antibody was injected directly into animal. Many molecules have been used as target of monoclonal antibodies in immunotherapy for stroke-reperfusion injury (Liesz et al., 2011a; Macrez et al., 2011; Wiessner et al., 2003). These molecules are usually located on the cell
membrane or in the extracellular space, like secreted protein. For example, tPA binds to and cleaves N-methyl-D-aspartate (NMDA) receptor, which induces cell death (Macrez et al., 2011; Nicole et al., 2001). Antibody against the interaction of tPA with NMDA receptor attenuated the side effects (like cerebral edema and hemorrhage) of tPA. Inflammation is reported to be involved in stroke-reperfusion injury. Tumor necrotic factor (TNF), which contributes to systematic inflammation, interacts with TNF receptor 1 and induces cellular apoptosis. Humanized monoclonal antibodies neutralize TNF and reduce brain injury in both permanent and transient stroke (Lavine et al., 1998; Meistrell III et al., 1997).

Monoclonal antibody has been established as an important targeted therapy for stroke (Hansel et al., 2010; Yu et al., 2013). The advantages of choosing monoclonal antibodies in animal research of stroke include their high specificities, long half-lives, and safety. The high specificity of monoclonal antibody promotes precise action, which reduces the side effects. The long half-lives of monoclonal antibody allow it work longer and to be administered in infrequent dosing, unlike traditional drugs (Reichert and Dewitz, 2006). Moreover, the structure of monoclonal antibody can be fine designed for specific therapeutic actions to minimize immunogenicity and improve its risk–benefit ratio (Hale, 2006; Nissim and Chernajovsky, 2008; Presta, 2008).

The reported monoclonal antibodies were effective in the treatment of stroke-reperfusion injury in animal model (Lavine et al., 1998; Macrez et al., 2011; Meistrell III et al., 1997; Nicole et al., 2001; Yu et al., 2013). However, most of them failed in clinical trials due to their severe side effects (Yu et al., 2013). The common side effects contain transient low-grade fever, malaise, nausea, dyspnea, diarrhea, fatigue, hoarseness, palpitations, and headache (Hansel et al., 2010; Yu et al., 2013). Other severe side effects include myocardial infarction, pulmonary
embolism, venous thrombosis, or even secondary stroke.

1.7 Hypothesis

The overall purpose of this research is to study the function of LCN2 in stroke-reperfusion injury and develop a potential immunotherapy to reduce reperfusion injury after stroke. This project contains three parts:

(i) **Determine the expression pattern of LCN2 and BOCT before and after stroke-reperfusion injury**;

(ii) **Determine the role of LCN2 in stroke-reperfusion injury**;

(iii) **Identify a potential therapeutic agent to reduce LCN2 induction after stroke-reperfusion injury**.

The aim of the part one was to identify whether LCN2 and its receptor BOCT are expressed in uninjured mouse brains and whether their expression changes after ischemic stroke. I hypothesized that LCN2 is expressed in mouse brains and its expression increases after stroke-reperfusion injury. First, we determined the type of cells that express LCN2 and its receptor BOCT in cerebrum of uninjured mice by Western blotting and immunofluorescence staining. Subsequently, we identified the expression of LCN2 and BOCT after stroke-reperfusion injury by immunofluorescence staining and the expression pattern of LCN2 in mouse serum after stroke-reperfusion injury by ELISA and Western blotting analysis.

The aim of part two was to identify the function of LCN2 in stroke-reperfusion injury. I hypothesized that LCN2 works as a detrimental factor in stroke-reperfusion injury, which increases neutrophil infiltration and cerebral damage. To prove this, we focused on the difference between the LCN2 wild-type (WT) and LCN2 deficiency mice after stroke-
reperfusion injury. After we performed tMCAO on both LCN2 WT and LCN2 deficiency mice, mouse brain was sectioned to measure cerebral infarct and edema volume. Furthermore, we focused on determining the mechanism of brain damage induced by LCN2 in stroke-reperfusion injury.

The aim of part three was to develop anti-LCN2 antibody as a new therapeutic agent for stroke-reperfusion injury. I hypothesized that anti-LCN2 antibody injection will reduce brain damage in stroke-reperfusion injury by attenuating neutrophil induced inflammation. To prove this, we focused on the cerebral damage and motor outcomes, the infiltration of neutrophil, and BBB disruption after anti-LCN2 antibody treatment.
Chapter 2

Expression pattern of LCN2 and BOCT before and after stroke-reperfusion injury

2.1 Introduction and rational

LCN2 is a 25 kDa protein secreted by activated neutrophils (Kjeldsen et al., 2000; Kjeldsen et al., 1993). The expression of LCN2 is detected in numerous organs in non-ischemic condition, including lung, spleen, uterus, stomach, salivary gland, colon, prostate and appendix, but not in brain (Ip et al., 2011; Kjeldsen et al., 2000). LCN2 has multiple functions, involved in diverse physiological processes, like regulation of cell death/survival, cell migration, molecular transportation and cell differentiation. In addition, LCN2 regulates glial cell death, reactive astrogliosis, migration and morphologic changes (Lee et al., 2007; Lee et al., 2009). In the central nervous system, LCN2 also works as chemokine inducer, involved in neuroinflammation (Lee et al., 2011). Furthermore, LCN2 is remarkably induced after ischemia reperfusion injury in kidney and heart (Cheng et al., 2015; Vinuesa et al., 2008). Hence, it appears that LCN2 is not expressed in brain under non-ischemia condition, but possibly induced in brain after stroke-reperfusion injury. Moreover, the level of serum LCN2 is increased in patients with stroke (Chan et al., 2012). Thus, it is possible that the level of LCN2 is also increased in serum in mice after stroke-reperfusion injury.

LCN2 is internalized by its putative cell surface receptor, BOCT, which is expressed at a high level in brain, primary neurons, brain endothelial cells, as well as liver (Bennett et al., 2011) (Devireddy et al., 2005; Ip et al., 2011). The expression of BOCT is significantly increased at 96
hours reperfusion after ischemia, but not at 24 hours reperfusion after ischemia in kidney (Vinuesa et al., 2008). Based on these studies, I hypothesized that the expression of BOCT is not increased in brain at 23 hours reperfusion after stroke.

In this chapter, first, we would like to confirm which type of cell express LCN2 and BOCT in brain under non-ischemia condition. Second, we would like to determine the expression changes of LCN2 in both brain and serum, as well as BOCT in brain after stroke-reperfusion injury.

**Specific aim.** Determine the expression pattern of LCN2 and BOCT before and after stroke-reperfusion injury.

1. Determine the expression pattern of LCN2 and BOCT in uninjured mice
2. Determine the expression pattern of LCN2 and BOCT after stroke-reperfusion injury
3. Determine the induction of LCN2 in mouse serum after stroke-reperfusion injury
2.2 Materials and methods

Animal
Male $Lcn2^{+/+}$ and $Lcn2^{-/-}$ mice on a C57BL/6 background between 3 and 5 months of age were used for all experiments (Flo et al., 2004). Mice were genotyped by PCR of tail DNA. All procedures were conducted in accordance with Institutional Animal Care and Use Committee policies.

Middle cerebral artery occlusion (MCAO)
Focal cerebral ischemia was induced by transient or permanent intraluminal monofilament occlusion of the right MCA (Chiang et al., 2011; Chou et al., 2004; Longa et al., 1989). Mice weighing 25-35 g were anesthetized with 1.5% isoflurane in 30% O$_2$ / 70% N$_2$ using the V-10 Anesthesia system (VetEquip, Inc., Pleasanton, CA). Rectal temperature was monitored and maintained at 37 ± 0.5 °C throughout the procedure by the TR-200 homoeothermic temperature system (Fine Science Tools Inc., Foster City, CA). A silicon-coated monofilament suture (Doccol, Sharon, MA) was inserted into the lumen of the external carotid artery and passed into the internal carotid artery (ICA) 9-10 mm past the bifurcation of the common carotid artery (CCA), resulting in occlusion of the MCA. Following one hour of MCAO, the suture was removed from the CCA to induce reperfusion for the tMCAO model. The occluding suture was left in place for 24 hours until sacrificing the mice for the pMCAO model.

Regional cerebral blood flow (rCBF)
During focal cerebral ischemia, rCBF was continuously monitored by Laser Doppler Flowmetry (PF5001, Perimed, Sweden) with a flexible 0.8-mm fiber optic extension probe (Probe 407, Perimed). The tip of the probe was affixed to the intact skull over the right cortex at 2 mm posterior to the bregma and 4 mm lateral to the midline. The rCBF was recorded 20 minutes before ischemia to obtain a steady-state baseline (100%) and continued until 15 minutes after the reperfusion. The percentage of the baseline rCBF (%) was calculated as the percentage relative to the baseline. The abrupt drop of Laser Doppler signal to ~20% of the baseline rCBF indicated the occlusion of MCA by the suture. Mice were excluded from further studies if sufficient occlusion (<30% of the baseline) and reperfusion (>80% of the baseline) was not achieved based on the Laser Doppler Flowmetry, if excessive bleeding occurred during surgery, or if hemorrhage was found in the brain slices or at the base of the circle of Willis during postmortem examination.

Collection of mouse serum

At different time points after ischemic stroke, mice were anesthetized with 5% isoflurane and euthanized by cervical dislocation. The blood was collected from the decapitated trunk and placed at room temperature for one hour. The blood was centrifuged at 2000 X g for 20 min at room temperature, and the supernatant was collected as blood serum for Western blotting and ELISA (Thavasu et al., 1992).

Immunofluorescence microscopy

Primary cultured neurons were fixed with 4% paraformaldehyde (PFA) in PBS and immunostained as described (Chou et al., 2010; Qi et al., 2007). Mice were perfused
intracardially with 4% PFA, and coronal sections (50 μm) of fixed brains were prepared using a Leica CM1950 Cryostat (Chou et al., 2004; Qi et al., 2007). Fixed neurons or brain sections were incubated overnight at 4 °C with primary antibodies using the following concentrations: goat anti-LCN2 (1:100, R&D, Cat# AF1857, RRID: AB_355022), goat anti-BOCT (1:100, ProSci, Cat# 46-899, RRID: AB_1948680), rat anti-mouse neutrophil mAb - clone 7/4 (1:200, Serotec, Cat# MCA771GA, RRID: AB_324243), rabbit anti-GFAP (1:100, Abcam, Cat# ab48050, RRID: AB_941765), rabbit anti-Iba1 (1:100, Wako Chemicals, Cat# 019-19741, RRID: AB_839504), mouse anti-MAP2 (1:200, Millipore, Cat# MAB3418, RRID: AB_11212326) and mouse anti-NeuN (1:100, Millipore, Cat# MAB377, RRID: AB_11210778) antibodies. After washing, the neurons and sections were stained with Alexa Fluor conjugated secondary antibodies (1:200, Jackson ImmunoResearch, Donkey anti-Goat IgG, Cat# 705-035-147, RRID: AB_2313587; Donkey anti-Rabbit IgG, Cat# 711-035-152, RRID: AB_10015282; Donkey anti-Mouse IgG, Cat# 715-035-150, RRID: AB_2313608) and mounted in media containing DAPI (4’, 6-diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA). The images were acquired using an Olympus FV500/IX81 confocal microscope (Olympus, Center Valley, PA, USA).

**Primary neuronal culture and oxygen glucose deprivation (OGD)**

Primary mixed neuronal-glial cultures were prepared from the cerebral cortex or hippocampus of postnatal day 1 (P1) to P3 mice as previously described (Chou et al., 2010; Goslin, 1998; Qi et al., 2007). Primary neuronal-glial cells were cultured in a humidified CO₂ incubator with 5% CO₂ and 95% air at 37 °C for 10-14 days in vitro (DIV 10-14). To initiate OGD, culture media were changed into Neurobasal-A media without glucose (Invitrogen) containing B27 Supplement
Minus AO (antioxidants), Glutamax, and sodium pyruvate (Jiang et al., 2004). The cultures were placed into a humidified Modular Incubator Chamber (Billups-Rothenberg Inc., Del Mar, CA) and flushed with 5% CO$_2$ and 95% N$_2$ for 5 min. The chamber was then sealed and incubated at 37 °C for one hour of OGD. To mimic reoxygenation, the culture was removed from the Modular Incubator Chamber and the media replaced with regular Neurobasal-A media with glucose and incubated with 5% CO$_2$ and 95% air at 37 °C for 23 hours.

**ELISA**

The level of LCN2 protein in mouse sera and brain homogenates was quantified following the manufacturer's protocol for mouse lipocalin-2/NGAL Quantikine ELISA kit (R&D).
2.3 Results

2.3.1. Expression pattern of LCN2 and BOCT in uninjured mice

We first assessed the expression of LCN2 and its putative receptor BOCT in neutrophils and neurons under non-ischemic condition. LCN2 protein was enriched in neutrophils, but undetectable in neuronal cells by Western blot analysis (Figure 2A). In contrast, BOCT protein was detected in cultured neurons and brain tissues, but not neutrophils. Immunofluorescence staining revealed a dense, punctate pattern of BOCT expression in microtubule-associated protein 2 (MAP2) - positive cultured neurons (Figure 2B) as well as neuronal nuclear protein (NeuN) - positive neurons in cerebral cortex (Figure 2C). NeuN and MAP2 are two specific markers for mature neurons.
Figure 2. Expression of LCN2 and BOCT under non-stimulated conditions. (A) A representative Western blot shows the expression of LCN2 (~25 kDa) and BOCT (~65 kDa) in cultured cortical neurons (neurons), neutrophils and brain homogenates, respectively. Samples were normalized to β-Actin. Confocal images show the expression pattern of BOCT in cultured cortical neurons (B) and brain sections (C). Neurons were stained for BOCT (red) and neuronal marker MAP2 or NeuN (green). Nuclei were stained with DAPI (blue). Scale bars, 10 µm.
2.3.2. Expression pattern of LCN2 and BOCT after stroke-reperfusion injury

To determine whether LCN2 is induced in brain tissues after ischemic stroke, we analyzed brain homogenates of WT and Lcn2−/− mice after one hour of tMCAO and 23 hours of reperfusion (Figure 3A). The hemispheres of mice without tMCAO were used as controls (con). LCN2 protein was undetectable in the uninjured brains of WT and Lcn2−/− mice, but elevated in the ipsilateral (I), and faintly in the contralateral (C) hemispheres 23 hours after tMCAO (Figure 3A). The LCN2 level measured by ELISA was 3.9 ± 1.3 pmol/g (picomoles per gram of brain proteins) in the control brain tissues and reached 307.3 ± 81.7 pmol/g in the ipsilateral brain tissues 23 hours after tMCAO (Figure 3B), representing a 80-fold increase in expression. The expression level of BOCT receptor in brain homogenates was unchanged after tMCAO, and in the absence of LCN2 (Figure 3A).
Figure 3. Detection of LCN2 in the ipsilateral hemisphere of mouse after tMCAO. (A) The ipsilateral (I) and contralateral (C) hemispheres of Lcn2+/+ and Lcn2−/− mice were isolated after 1 hr of tMCAO and 23 hrs of reperfusion. The hemispheres of mice without tMCAO were used as controls (con). The brain homogenates were analyzed by Western blotting using anti-LCN2 and anti-BOCT antibodies. β-Actin was used as a loading control. (B) The levels of LCN2 in the brain homogenates of ipsilateral hemispheres at 23 hrs after tMCAO were determined by ELISA. The ipsilateral hemispheres of mice without tMCAO were prepared as controls (con). *P < 0.05 compared with control (two-tailed, unpaired t-test).
We next assessed the cellular localization of LCN2 in the ipsilateral hemispheres by double immunofluorescence staining. Brain sections isolated 23 hours after tMCAO were stained with antibodies recognizing LCN2 and specific markers for neutrophils (7/4), neurons (NeuN), microglia (Iba1), or astrocytes (GFAP). LCN2 was detected in the 7/4-positive neutrophils surrounding a striatal lesion (peri-infarct or penumbra area) in the ipsilateral hemispheres (Figure 4A). It is likely that these neutrophils are derived from the peripheral blood and emigrated into ischemic brain tissue during reperfusion. LCN2 expression was also detected in a subset of GFAP-positive astrocytes in the ipsilateral hemispheres (Figure 4B and 4C), but not in NeuN-positive neurons or Iba1-positive microglia (data not shown). To confirm the cellular distribution of LCN2, we subjected the primary mixed neuronal-glial cultures to oxygen-glucose deprivation (OGD), an in vitro model of ischemic stroke. After one hour of OGD and 23 hours of reoxygenation, LCN2 was detected in a subset of the GFAP-positive reactive astrocytes, but absent in MAP2 positive neurons or Iba1 positive microglia (Figure 5). The LCN2-positive astrocytes displayed morphological characteristics of reactive astrocytes with enlarged nuclei and hypertrophy of cell bodies and processes (Figure 4C and 5B) (Kimelberg, 2005; Takano et al., 2009)
Figure 4. Detection of LCN2 in infiltrating neutrophils and astrocytes in the ipsilateral hemisphere after tMCAO. (A) Immunoreactivities of LCN2 (red) and neutrophil-specific marker 7/4 (green) in the ipsilateral hemispheres at 23 hrs after tMCAO were detected using confocal microscopy. (B and C) Confocal images of LCN2 (red) and GFAP-positive astrocytes (green) in the ipsilateral hemispheres at 23 hrs after tMCAO. Amplified images of LCN2- and GFAP- positive astrocytes are shown in the bottom panels. The arrows indicate the enlarged and eccentric nuclei of GFAP-positive astrocytes. Merged images indicate colocalization of LCN2 with 7/4 or GFAP in yellow. Scale bars, 50 μm (A and B panel) and 12.5 μm (C panel).
Figure 5. Expression of LCN2 in the subset of astrocytes after the OGD. (A and B) Confocal images show the expression of LCN2 (red) and GFAP (green) in cultured cortical neurons at 23 hrs after 1 hr of OGD. Merged images indicate colocalization of LCN2 with GFAP in yellow. Images of LCN2-positive cells and MAP2-positive neurons (C) and Iba1-positive microglia (D) after the OGD demonstrating no colocalization. Scale bars, 50 μm.
2.3.3. Induction of LCN2 in mouse serum after stroke-reperfusion injury

Previous studies reported the elevated levels of LCN2 in human plasma 1-3 days after ischemic stroke (Anwaar et al., 1998; Elneihoum et al., 1996; Falke et al., 2000). To assess the time course of LCN2 induction after ischemic stroke, we occluded the middle cerebral artery (MCA) for one hour and collected sera of wild-type (WT) and Lcn2−/− mice at 23 hours after reperfusion (Chiang et al., 2011; Chou et al., 2004; Longa et al., 1989). A major immunoreactive band of LCN2 (~25 kDa) was detected in the sera of WT, but not Lcn2−/− mice by Western blot analysis (Figure 6A). The expression of LCN2 was barely detectable in the control sera of WT mice not subjected to tMCAO. To more carefully examine the temporal profile of LCN2 induction, we analyzed sera collected at 1, 4, 23, 48 and 72 hours after tMCAO (Figure 6B). Increased LCN2 levels were observed one hour after tMCAO, continued to raise between 4 and 23 hours, and were diminished by 48 to 72 hours (Figure 6B). The level of LCN2 protein measured by ELISA was 133.1 ± 18.3 ng/ml (6.4 nM) in control mouse sera, which is comparable with previous studies (Flo et al., 2004; Slater et al., 2013). LCN2 protein increased 41-fold to 5509 ± 539.5 ng/ml (263.904 nM) 23 hours after tMCAO (Figure 6C).
Figure 6. Induction of LCN2 in mouse serum after ischemic stroke. (A) Mouse sera collected from Lcn2+/+ and Lcn2−/− mice after one hour of tMCAO and 23 hours of reperfusion were analyzed by Western blotting using anti-LCN2 antibody. The serum of mice without tMCAO was collected as a control (con). (B) Mouse sera collected at different time points after one hour of tMCAO were analyzed by Western blotting using anti-LCN2 antibody. The top panel is a representative Western blot. The LCN2 protein bands were quantified by densitometry in the bottom panel (n = 3-6). There was a significant induction of LCN2 at 1 hour (*P < 0.05), 4 and 23 hours (***P < 0.0005), and 48 hours (**P < 0.005) after tMCAO compared with control (two-tailed, unpaired t test). (C) Levels of LCN2 in control mouse sera and at 23 hours after tMCAO were determined by ELISA (n = 7). ***P < 0.0001 compared with control (two-tailed, unpaired t test).
2.4. Conclusion

My result indicated that the expression of LCN2 is not detected in the brain tissue of uninjured mice. However, LCN2 is acutely induced after transient middle cerebral artery occlusion in mice. Induced LCN2 protein is identified in a subset of reactivated astrocytes and infiltrated neutrophils after tMCAO. Moreover, LCN2 appears in mouse sera as early as one hour, peaks at 23 hours, and diminishes by 48 to 72 hours after tMCAO.

The expression of BOCT, LCN2 receptor, is not changed in mouse brain after tMCAO. Due to the short time window for effective thrombolytic therapy, it is of great interest to diagnose stroke early and reduce the risk of cerebral hemorrhage. The early induction of LCN2 suggests the possibility of using LCN2 as an early blood biomarker to detect stroke.
Chapter 3
The role of LCN2 in ischemic stroke

3.1 Introduction and rational

The plasma level of LCN2 is increased in patients with stroke-reperfusion injury (Anwaar et al., 1998; Elneihoum et al., 1996; Falke et al., 2000). And stroke patients with worse outcomes have higher serum LCN2 concentrations (Anwaar et al., 1998; Elneihoum et al., 1996; Falke et al., 2000). Furthermore, my previous results indicated that LCN2 in mice serum is markedly induced since 1 hour of reperfusion. However, the role of LCN2 in acute stroke-reperfusion injury is still unknown.

Reperfusion injury includes a series of inflammatory events with activation and infiltration of circulating neutrophils, macrophages, and T-cells into infarcted brain tissue (Chou et al., 2004; Eltzschig and Eckle, 2011). LCN2 is originally identified as a secreted protein from neutrophils and enhances proinflammatory response (Kjeldsen et al., 2000). As a proinflammatory mediator, LCN2 mediates neutrophil recruitment by promoting the production of circulating neutrophils chemoattractant chemokine (C-X-C motif) ligand 1 (CXCL1) and enhances infiltration in ischemia and reperfusion in heart, kidney and liver diseases (Aigner et al., 2007; Guglani et al., 2012; Perco et al., 2007; Sickinger et al., 2013). During stroke-reperfusion injury, peripheral blood immune cells (neutrophil and macrophage) infiltrate ischemic brain tissue and cause more injury (Chou et al., 2004; Iadecola and Anrather, 2011). Thus, it is possible that LCN2 is induced to promote the infiltration of immune cells in acute
stroke-reperfusion injury and LCN2 deficiency reduces the immune cell infiltration during stroke-reperfusion injury. We identify the infiltrating neutrophils and macrophages in mouse brain by anti-myeloperoxidase (MPO) antibody, the marker for neutrophil and macrophage. Therefore, if LCN2 improves the infiltration of immune cells (neutrophils and macrophages) in stroke-reperfusion injury, we should detect more MPO positive immune cells in ipsilateral hemisphere of $Lcn2^{+/+}$ mice after stroke compared to $Lcn2^{-/-}$ mice.

Previous study reported that LCN2 released by astrocytes promotes neuronal death in mice after stroke-reperfusion injury (Jin et al., 2014). LCN2 is also reported to be able to induce apoptosis in mouse pro-B lymphocytic cell line FL5.12 (Devireddy et al., 2005; Devireddy et al., 2010). Moreover, LCN2 may contribute to cellular apoptosis by regulating intracellular iron (Devireddy et al., 2005; Devireddy et al., 2010). LCN2 cannot interact with iron directly. Instead, it binds iron through siderophore, an iron-chelating compound (Devireddy et al., 2010; Richardson, 2005). Apo-LCN2 enters cell through endocytosis and binds to putative endogenous siderophore-iron complex, resulting in reduction of intracellular iron. The decreased intracellular iron contributes to the induction of Bim, which stimulate cellular apoptosis (Devireddy et al., 2005; Devireddy et al., 2010; Richardson, 2005). Based on these studies, I hypothesized that the induction of LCN2 causes neuronal apoptosis in stroke-reperfusion injury in $Lcn2^{+/+}$ mice.

Furthermore, if the induction of LCN2 facilitates infiltration of immune cells and contributes to neuronal apoptosis in $Lcn2^{+/+}$ mice after stroke-reperfusion injury, $Lcn2^{+/+}$ mice will induce bigger infarct volume in ipsilateral hemisphere and more severe neurological impairments than $Lcn2^{-/-}$ mice after stroke-reperfusion injury.
Specific aim. Determine the role of LCN2 in ischemic stroke.

1. Determine the role of LCN2 in brain injury and motor behavior after ischemic stroke
2. Determine the role of LCN2 in immune cells infiltration after ischemic stroke
3. Determine the role of LCN2 in neuronal death after stroke-reperfusion injury
3.2 Materials and methods

Ischemic stroke model

Male Lcn2\(^{+/+}\) and Lcn2\(^{-/-}\) mice on a C57BL/6 background between 3 and 5 months of age were used for experiments (Flo et al., 2004). Focal cerebral ischemia was induced by transient occlusion of the right MCA for one hour with a silicon-coated monofilament suture (Doccol) (Chou et al., 2004). Twenty-three hours after the tMCAO, we determined edema-corrected infarct volumes and brain swelling from coronal brain slices stained with 2,3,5-triphenyltetrazolium chloride (TTC, Sigma). All procedures were conducted in accordance with Institutional Animal Care and Use Committee policies.

Regional cerebral blood flow (rCBF)

During focal cerebral ischemia, rCBF was continuously monitored by Laser Doppler Flowmetry (Perimed). Mice were excluded from further studies if sufficient occlusion (<30% of the baseline) and reperfusion (>80% of the baseline) was not achieved, if excessive bleeding occurred during surgery, or if hemorrhage was found in the brain slices or at the base of the circle of Willis during postmortem examination.

Neurological deficit scores and corner tests

Twenty-three hours after the initiation of ischemic stroke, mice were evaluated for neurological deficits using a four-tiered grading system (Chou et al., 2004; Prestigiacomo et al., 1999). Score 0 indicates no observed neurological deficit; score 1, inability to walk straight; score 2, circling toward the paretic side; score 3, falling on the paretic side; and score 4, loss of the righting
reflex. For the corner test, mice were placed between two vertical boards attached at a 30° angle (Zhang et al., 2002). The number of right (ipsilateral) turns when the mouse reached the wedge of the corner were recorded in ten trials.

**Determination of infarct volume and brain swelling**

Twenty-three hours after one hour of tMCAO, mice were anesthetized with 5% isoflurane and euthanized by cervical dislocation. The brain was removed and sliced into 1-mm-thick coronal sections using a brain matrix (Braintree Scientific) on ice. The brain sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) in 1X PBS at room temperature for 20 minutes and fixed in 10% formalin (Sigma-Aldrich) at 4 °C until imaging. Both sides of sections were photographed using a Leica EZ4HD stereomicroscope with integrated High Definition digital camera. The areas of cerebral infarction and of hemispheres were measured using the NIH ImageJ by an investigator blinded to the treatment conditions. The total infarct volumes were calculated using the following equations to consider the effect of edema: infarct area on one side of brain section = area of contralateral hemisphere – (area of ipsilateral hemisphere – area of infarct area) (Lin et al., 1993; Swanson et al., 1990). Total infarct volume = (front infarct area of a section + rear infarct area of the same section) / 2 × thickness of the section × total numbers of sections. Brain swelling (the amount of edema formation) = [(ipsilateral hemisphere volume – contralateral hemisphere volume) / contralateral hemisphere volume] × 100.

**Western blot analysis**

Ipsilateral (I, right) and contralateral (C, left) hemispheres were isolated and homogenized using a Teflon-glass homogenizer as described (Chou et al., 2010; Olive et al., 2005; Qi et al., 2007).
Neutrophils were isolated from mouse bone marrow by Percoll density gradient centrifugation (Chou et al., 2004; Lowell and Berton, 1998). Proteins within brain homogenates, cell lysates and blood sera were separated by NuPAGE 4–12% Bis-Tris gels (Invitrogen), then transferred to PVDF membranes and analyzed by Western blotting using goat anti-LCN2 (1:1000, R&D Systems, Cat# AF1857, RRID: AB_355022), goat anti-BOCT (1:500, ProSci Incorporated, Cat# 46-899, RRID: AB_1948680), mouse anti-Actin (1:2000, Sigma, Cat# A4700, RRID: AB_476730), rabbit anti-cleaved caspase-3 (Asp175) (1:1000, Cell Signaling, Cat# #9661, RRID: AB_331440), and goat anti-myeloperoxidase (MPO) heavy chain (1:400, R&D, Cat# AF3667, RRID: AB_2250866) antibodies. Immunoreactive bands were detected using enhanced chemiluminescence (ECL) (Pierce), imaged using a Luminescent Image Analyzer LAS-3000 (Fujifilm), and quantified by NIH ImageJ.

Primary neuronal culture, oxygen glucose deprivation (OGD)

Primary mixed neuronal-glial cultures were prepared from the cerebral cortex or hippocampus of postnatal day 1 (P1) to P3 mice as previously described (Chou et al., 2010). The culture was subjected to one hour of OGD and 23 hours of reoxygenation (Jiang et al., 2004).

MTT assays

The viability of cells after treatment with different concentrations of recombinant human LCN2 protein (R&D Systems) was analyzed by MTT assays according to the protocols for the Vybrant® MTT Cell Proliferation Assay Kit (Molecular Probes). Briefly, cells were incubated with 1.2 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in a humidified CO2 incubator at 37 °C for 4 hours. Cells were then lysed in SDS-HCl solution and
incubated at 37 °C for 16 hours. The concentration of MTT formazan was measured by optical density (OD) at 570 nm. Cell viability was calculated by dividing the OD of treated group by the mean OD of untreated controls.

**Visualization of cerebral vessels**

The cerebral vessels were mapped as described (Panahian and Maines, 2000). Higgins Black Magic waterproof ink (200–250 μl; Sanford Corp.) was injected into the left ventricle using a 26-gauge needle, and the right atrium opened to release the effluent. Mice were decapitated and the heads fixed in 10% neutral buffered formalin (Sigma-Aldrich) for 7 days. The brains were removed carefully from the skulls and imaged using a Leica EZ4HD stereomicroscope with integrated High Definition digital camera.

Posterior communicating artery (PcomA) was analyzed in Lcn2+/+ and Lcn2−/− mice. Mice were perfused with 30% Higgins Black Magic waterproof ink -70% saline solution from the left ventricle for 5 minutes using a 26-gauge needle and blood was drained from right atrium (Murakami et al., 1998). Mice were decapitated and the heads fixed in 10% neutral buffered formalin (Sigma-Aldrich) for 7 days. The brains were removed carefully from the skulls and imaged using a Leica EZ4HD stereomicroscope with integrated High Definition digital camera. The development of PcomA was graded on a qualitative scale of 0 to 3 (Murakami et al., 1997). Score 0 indicates no PcomA between anterior and posterior circulation; score 1 indicates PcomA in capillary phase; score 2 indicates small truncal PcomA; score 3 indicates truncal PcomA. In dorsal surface of mouse brain, distances from the line of anastomoses to the midline were measured at coronal planes 2, 4 and 6 mm from the frontal pole.
3.3 Results

3.3.1. Role of LCN2 in brain injury and motor behavior after ischemic stroke

To assess the effect of stroke-induced LCN2 in vivo, we subjected WT and Lcn2−/− mice to tMCAO with 1 hour of ischemia followed by 23 hours of reperfusion (Figure 7). Infarct volume was reduced by 56% in Lcn2−/− mice compared with WT mice (62.05 ± 7.88 mm3 for WT versus 27.47 ± 10.29 mm3 for Lcn2−/−; *P* < 0.05; Figure 8A). Likewise, brain swelling in the ipsilateral hemispheres was reduced by 60% in Lcn2−/− mice (6.2% ± 1.4% for WT versus 2.5% ± 0.8% for Lcn2−/−; *P* < 0.05; Figure 8B). Reduction in cerebral infarct size and edema in Lcn2−/− mice was reflected in their functional outcome. Lcn2−/− mice showed improved neurological outcome (*P* < 0.05; Figure 8C) and reduced sensorimotor asymmetry in corner tests (*P* < 0.05; Figure 8D), compared to WT controls.
Figure 7. Brain injury in Lcn2+/+ and Lcn2−/− mice after stroke. Representative images of TTC-stained brain slices from Lcn2+/+ (left) and Lcn2−/− (right) mice after 1 hour of middle cerebral artery occlusion and 23 hours of reperfusion. Viable tissue is stained in red color, while the infarcted area in white color.
Figure 8. Stroke injury is reduced in LCN2 null mice after tMCAO. Total Infarct volume (A), brain swelling (B), neurological deficit scores (C) and corner tests (D) were determined after 1 hour of tMCAO and 23 hours of reperfusion from Lcn2+/+ (n = 8) and Lcn2−/− (n = 8) mice. *P < 0.05 compared with WT mice (two-tailed, unpaired t-test). †P < 0.05 compared with WT mice (one-tailed, unpaired t-test).
At baseline, \textit{Lcn2}^{−/−} mice display normal motor and sensory function and coordination (Flo et al., 2004; Rathore et al., 2011) and comparison of brains from WT and \textit{Lcn2}^{−/−} mice showed no gross differences in size or anatomy (Figure 9). To determine whether the attenuated brain damage in \textit{Lcn2}^{−/−} mice was because of changes in cerebral vasculature, we examined the distribution of cerebral vessels by ink perfusion. No difference was observed in the origins of the MCA or other major blood vessels of the circle of Willis (Figure 9A), the degree of posterior communicating artery (PcomA) patency (Figure 9A and 9B) and the size of MCA territory (Figure 9C and 9D). In addition, the rCBF in the MCA area monitored by Laser Doppler Flowmetry (Figure 10A) was similar between WT and \textit{Lcn2}^{−/−} mice before and during ischemia, and after reperfusion (Figure 10B).
Figure 9. Assessment of cerebrovascular anatomy and determination of regional cerebral blood flow (rCBF).  

(A) Shown are representative images of the brains from Lcn2<sup>+/+</sup> and Lcn2<sup>−/−</sup> mice perfused with black ink. The major arteries in the circle of Willis (upper panels) and PcomA (indicated by arrowheads in lower panels) were identified.  

(B) Shown are the scores of PcomA plasticity in Lcn2<sup>+/+</sup> (n = 13) and Lcn2<sup>−/−</sup> mice (n = 13).  

(C) Shown are the dorsal images of the brains from Lcn2<sup>+/+</sup> and Lcn2<sup>−/−</sup> mice perfused with black ink. The points of anastomoses were circled and connected to form the line of anastomoses.  

(D) Distances from the line of anastomoses to the midline in Lcn2<sup>+/+</sup> (n = 3) and Lcn2<sup>−/−</sup> (n = 3) mice were measured at coronal planes 2, 4, and 6 mm from the frontal pole.
Figure 10. Determination of regional cerebral blood flow (rCBF). (A) Shown is a continuous tracing of Laser Doppler Flowmetry monitoring rCBF during one hour of tMCAO and reperfusion. Laser Doppler signal drops to ~12% of the baseline rCBF when the suture is advanced to the origin of MCA (arrow head), and returns back to the baseline when the suture is 6 withdrawn (arrow). (B) The rCBF during tMCAO was measured continuously in Lcn2+/+ (n = 8) and Lcn2-/- (n = 8) mice. Steady-state rCBF before the MCAO were used as baseline (100%), and the subsequent changes after the onset of ischemia were shown as the percentage relative to the baseline. Time zero indicates the point of MCA occlusion. There were no significant differences in rCBF between Lcn2+/+ and Lcn2-/- during tMCAO.
Since LCN2 was induced in both blood and brain tissues after tMCAO, we evaluated the contribution of LCN2 from ischemic brain by minimizing the input of circulating blood using a permanent MCAO (pMCAO) model in which the MCA was occluded for 24 hours without reperfusion (Figure 11) (Iadecola and Anrather, 2011; Jin et al., 2010). Infarct volume (55.58 ± 9.69 mm³ for WT versus 66.95 ± 13.06 mm³ for Lcn2^−/−; P > 0.05; Figure 11A and 11B) and brain swelling of the ipsilateral hemispheres (5.95% ± 1.06% for WT versus 7.56% ± 1.99% for Lcn2^−/−; P > 0.05; Figure 11C) was marginally increased in Lcn2^−/− mice after pMCAO. The neurological scores after pMCAO were slightly worse in Lcn2^−/− mice (P > 0.05; Figure 11D). The reduced stroke injury in LCN2 null mice observed in the tMCAO, but not in the pMCAO model, suggests that LCN2 is an essential factor mediating reperfusion injury after ischemic stroke.
Figure 11. Infarct size is similar in WT and LCN2 null mice after pMCAO. (A) Shown are representative images of TTC-stained brain slices after 24 hours of pMCAO from Lcn2+/+ and Lcn2−/− mice. Total infarct volume (B), brain swelling (C), and neurological deficit scores (D) of Lcn2+/+ (n = 8) and Lcn2−/− (n = 8) mice were measured after 24 hours of pMCAO.
3.3.2. Role of LCN2 in immune cells infiltration after ischemic stroke

Peripheral blood neutrophils infiltrate ischemic brain tissue during reperfusion and exacerbate brain injury (Chou et al., 2004; Iadecola and Anrather, 2011). Since LCN2 was detected in infiltrating neutrophils after tMCAO (Figure 4), we determined whether LCN2 deficiency affects the infiltration of circulating immune cells. The presence of infiltrating immune cells in brain homogenates of WT and *Lcn2*−/− mice were detected by Western blotting using anti-myeloperoxidase (MPO) antibody. MPO is a key enzyme expressed in blood-borne neutrophils, macrophages and monocytes, and has been used as a marker for inflammation after stroke (Breckwoldt et al., 2008). Significantly increased amounts of MPO were present after tMCAO in the ipsilateral hemispheres of WT, but not *Lcn2*−/− mice (Figure 12), demonstrating that LCN2 deficiency limited the infiltration of immune cells into ischemic brain. The stroke-induced MPO (Figure 13A) and LCN2 (Figure 13B) was minimally detected in the pMCAO model and significantly lower than the tMCAO model, showing that the infiltration of immune cells in pMCAO was not as pronounced as in tMCAO.
Figure 12. The effect of LCN2 on the infiltration of peripheral immune cells after tMCAO. Immune cells infiltration was analyzed by the levels of MPO in brain homogenates. The ipsilateral (I) and contralateral (C) hemispheres of Lcn2+/+ (n = 4) and Lcn2−/− (n = 4) mice were collected after tMCAO. MPO immunoreactivity normalized to β-Actin (MPO/Actin) was compared between means using one-way ANOVA with Newman–Keuls post hoc tests. There was a significant induction of MPO after tMCAO (***P < 0.0001) compared with controls, and a significant reduction of MPO induction in the ipsilateral hemispheres of Lcn2−/− mice (**P < 0.01).
Figure 13. The infiltration of peripheral immune cells and LCN2 induction in mice after tMCAO and pMCAO. Immune cell infiltration (A) and LCN2 induction (B) in brains after tMCAO and pMCAO were analyzed by Western blotting using antibodies against MPO and LCN2, respectively. MPO or LCN2 immunoreactivity normalized to β-Actin (MPO/Actin or LCN2/Actin) was compared between means using one-way ANOVA with Newman–Keuls post hoc tests. There was a significant reduction of MPO (*\(P < 0.05\)) and LCN2 (***\(P < 0.0001\)) levels in the ipsilateral hemispheres after pMCAO compared with tMCAO.
3.3.3. Role of LCN2 in neuronal death after stroke-reperfusion injury

Infiltrating immune cells (e.g. neutrophils) release free radicals and proteins that aggravate brain injury after reperfusion (Chou et al., 2004; Iadecola and Anrather, 2011). LCN2 was originally identified as a secreted protein from neutrophils (Kjeldsen et al., 2000), thus it is possible that LCN2 promotes neuronal injury in the tMCAO model. To determine whether LCN2 directly mediates neuronal cell death, we treated cultured neurons with increasing concentrations of recombinant LCN2. Addition of LCN2 reduced cell viability (Figure 14A) and induced apoptotic cell death (Figure 14B) in a concentration-dependent manner.
Figure 14. The effect of LCN2 on the survival of neuronal cells. (A) and (B) Primary neurons were incubated with different concentrations of recombinant human LCN2 protein at 37 °C for 24 hours (n = 3). (A) The viability of neurons after the LCN2 treatments was determined by MTT assays. *P < 0.05 compared with treatments with PBS vehicle (two-tailed, unpaired t test). (B) Induction of apoptosis after the LCN2 treatments was determined by Western blotting using antibodies against cleaved caspase-3 (Asp175) (19 kDa). β-Actin was used as a loading control.
3.4 Conclusion

Cerebral infarction, brain swelling, neurological deficits and infiltration of immune cells are significantly reduced in LCN2 null mice after transient MCAO (tMCAO), but not after permanent MCAO (pMCAO). Our finding that the infiltration of MPO-positive immune cells is reduced in LCN2 null mice after tMCAO is consistent with recent reports focused on LCN2 function in other models. For example, immune cell recruitment to the site of injury in LCN2 null mice is diminished in mouse models of spinal cord injury (Rathore et al., 2011) and heterotopic heart transplantation (Aigner et al., 2007). Neutrophils isolated from LCN2 null mice display defective chemotaxis in vitro (Liu et al., 2013). These results suggest that LCN2 is a paracrine chemoattractant for immune cells during ischemia-reperfusion injury.

A recent study (Bi et al., 2013) supports our finding that recombinant LCN2 induced cell death in primary neurons (Figure 14), but not in astrocytes, microglia and oligodendrocytes. The results suggest that LCN2 is selectively toxic to neurons, but not neuroglia. These results suggest that LCN2 is a proinflammatory mediator during the acute stage of ischemic stroke. Therefore, LCN2 inhibitors or anti-LCN2 antibodies may be useful to reduce post-stroke inflammation and brain injury.
Chapter 4

Identify a potential therapeutic agent to reduce brain damage in stroke-reperfusion injury

4.1 Introduction and rational

Recent studies demonstrated that LCN2 is a detrimental factor in stroke-reperfusion injury (Jin et al., 2014; Wang et al., 2015). The induction of LCN2 promotes the recruitment and infiltration of peripheral circulating immune cells (neutrophils and macrophages) and mediates neuronal apoptosis (Jin et al., 2014; Wang et al., 2015). The intact BBB in uninjured brains is restricted from the infiltration of immune cells. However, in stroke-reperfusion injury, the loss of oxygen and nutrients in stroke and ROS produced in reperfusion contribute to the damage of vascular endothelial cells and tight junction proteins disassembly (Heo et al., 2005; Wang and Shuaib, 2007). The impaired BBB facilitates the infiltration of immune cells into ipsilateral hemisphere of mice after stroke-reperfusion injury (Jin et al., 2014; Wang et al., 2015). Moreover, immune cell infiltration further enhances the disruption of BBB and inflammatory response (Carden and Granger, 2000; McIntyre et al., 1995; Svendsen, 1990, 1994; Svendsen and Bjerrum, 1992). The structure disruption of BBB increases its permeability and causes vasogenic edema, a most life-threatening event in stroke-reperfusion injury (Hjort et al., 2008; Nguyen et al., 2013; Sandoval and Witt, 2008). LCN2 deficiency reduces breakdown of junction adhesion molecules (claudin-5 and β-catenin), BBB leakage (Jin et al., 2014) and cerebral edema (Figure 8B) after tMCAO. Based on these studies, LCN2 is involved in the degradation of BBB junction adhesion molecules and promotes disruption and leakage of BBB in stroke-reperfusion injury. Thus, if the
expression and release of LCN2 during tMCAO is reduced, or LCN2 cannot be taken up by cells, it is likely that the infiltration of immune cells and BBB leakage will be reduced and the following cerebral damage will also be attenuated.

tPA is the only approved drug by Food and Drug Administration (FDA) in stroke treatment in human (Al-Khoury and Lyden, 2004). However, few patients receive the drug on time due to its limited working time window. Increased attention has to be paid to the new therapeutic methods for stroke-reperfusion injury, such as immunotherapy. Monoclonal antibody against LCN2 has been used to inhibit LCN2 function with an aim to improve the outcomes of several diseases (Cheng et al., 2015; Leng et al., 2009; Shashidharamurthy et al., 2013). Previous findings have indicated that inhibition of LCN2 by intravenous injection of anti-LCN2 antibody reduced the invasion and migration of breast cancer cells (Leng et al., 2009) and inflammatory cell migration of autoimmune diseases (Shashidharamurthy et al., 2013). Anti-LCN2 antibody injection reduced the level of serum LCN2 and the recruitment of neutrophils and macrophages in ischemia-reperfusion injury of cardiac transplantation (Cheng et al., 2015). Based on these studies it appears that anti-LCN2 antibody could reduce the serum LCN2 concentration, reducing the recruitment of neutrophils and macrophages in stroke-reperfusion injury.

Monoclonal antibody (like anti-ICAM-1 antibody and anti-TNF antibody, among others) injection depleted specific inflammatory factors and reduced the brain injury after stroke in rat and mouse (Justicia et al., 2006; Liesz et al., 2011b; Wiessner et al., 2003). My previous results suggested that LCN2 also works as an inflammatory factor to facilitate the recruitment and infiltration of immune cells (neutrophils and macrophages). Therefore, if anti-LCN2 antibody could reduce or even deplete serum LCN2 in mice, it may attenuate the inflammatory response to
reduce stroke-reperfusion injury. My results also indicated that LCN2 could cause neuronal apoptosis. One important question is whether the antibody can cross the BBB and neutralize LCN2 in mouse brain. It has been reported that immunoglobulin transports through BBB within 24 hours and has a long half-life in murine brain (St-Amour et al., 2013). Based on these studies, I hypothesize that anti-LCN2 antibody neutralizes LCN2 protein to reduce cerebral damage induced by stroke-reperfusion injury.

**Specific aim. Identify a potential therapeutic agent to reduce LCN2 induction after stroke-reperfusion injury.**

1. Determine the effect of anti-LCN2 antibody treatment on brain injury and motor behavior after stroke-reperfusion injury

2. Determine the effect of anti-LCN2 antibody treatment on the BBB leakage after stroke-reperfusion injury
4.2 Materials and methods

Ischemic stroke model

Male \( Lcn2^{+/+} \) and \( Lcn2^{-/-} \) mice on a C57BL/6 background between 2 and 4 months were used for experiments (Flo et al., 2004). Focal cerebral ischemic stroke was induced by transient occlusion of the right middle cerebral artery (tMCAO) for one hour following by blood reperfusion, as described previously (Wang et al., 2015). Four hours after reperfusion, 100 \( \mu \)g anti-LCN2 antibody was injected intraperitoneally to \( Lcn2^{+/+} \) mice. Mouse serum was collected 23 hours after reperfusion. \( Lcn2^{+/+} \) mice with 100\( \mu \)g control IgG intraperitoneal injection were used as control. All procedures were conducted in accordance with Institutional Animal Care and Use Committee policies.

Regional cerebral blood flow (rCBF)

During focal cerebral ischemic stroke, rCBF was recorded 20 minutes before ischemia until 15 minutes after the reperfusion by Laser Doppler Flowmetry (PF5001, Perimed, Sweden). The monitor was attached to the right side of intact skull. Mice with insufficient occlusion and reperfusion were excluded, as described previously (Wang et al., 2015).

Collection of mouse serum

At 23 hours after tMCAO, mice were sacrificed by cervical dislocation, and blood serum and brain were collected. Mice blood was placed at 4\(^\circ\)C for one hour and centrifuged at 2000 \( X \) g for 20 min at room temperature. The supernatant was collected as blood serum for Western blotting.
Mice brain was homogenized in RIPA buffer and centrifuged at 2000 X g for 20 min at 4°C. The supernatant was collected for Western blotting.

**Immunoprecipitation**

Magnetic Dynabeads were washed by 500µl 1 X RIPA for three times. Twenty µl Dynabeads were mixed with 0.5, 2.5 and 5 µg anti-LCN2 antibody in 200 µl 1 X RIPA buffer at 4°C separately. Five µg recombinant mouse LCN2 protein was mixed with Dynabeads- anti-LCN2 antibody solution and rotated for 16 hours at 4°C. The next day, Dynabeads were precipitated by magnetic rack and supernatant was removed for Western blotting analysis. Dynabeads pellet was washed by 500 µl 1 X RIPA for three times. After washing, Dynabeads pellet was collected for Western blotting analysis.

**Determination of infarct volume and brain swelling**

At 23 hours after tMCAO, mouse brain was sliced coronally into 1-mm-thick sections and stained with 2% 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma) in 1X PBS at room temperature for 20 minutes. Unstained areas were infarction. The areas of cerebral infarction and uninjured hemispheres were measured using the NIH ImageJ by an investigator blinded to the treatment conditions. The total infarct volumes were calculated as described previously (Wang et al., 2015).

**Determination of blood brain barrier leakage**

2% Evans Blue in 0.9% sterilized saline was injected into right jugular vein (3ml/kg of body weight immediately after reperfusion. Twenty-three hours after reperfusion, mice were perfused
with saline through left ventricle and right atrium was cut to drain the blood. Mouse brains were removed and homogenized in 1ml 1X PBS. The homogenate was centrifuged at 15,000 rpm for 30 minutes. The supernatant was mixed with the same volume of trichloroacetic acid, incubated at 4 °C overnight, and centrifuged at 15,000 rpm for 30 minutes. The supernatant absorbance was measured at 615 nm using a spectrophotometer. The level of Evans Blue in mouse brains was calculated using a standard graph with serial dilutions. Evans Blue extravasation was normalized to brain weight (µg/g) as described previously (Yenari MA et al., 2006 Stroke).

**Neurological deficit scores and corner tests**

Twenty-three hours after the initiation of ischemic stroke, mice were evaluated for neurological deficits using a four-tiered grading system, as described previously (Chou et al., 2004; Prestigiacomo et al., 1999). Meanwhile, mouse behavior outcomes were also evaluated by corner test, as described previously (Chou et al., 2004; Prestigiacomo et al., 1999; Zhang et al., 2002).

**Western blot analysis**

Proteins within blood sera and brain lysate were separated by NuPAGE 4–12% Bis-Tris gels (Invitrogen), transferred to PVDF membranes and analyzed by Western blotting using goat anti-LCN2 (1:1000, R&D Systems, Cat# AF1857, RRID: AB_355022), donkey anti-mouse-IgG (1:2000, Jackson ImmunoResearch, Cat# 715035150), mouse anti-ß-Actin (1:2000, Sigma, Cat# A4700), goat anti-ICAM-1 (1:500, R&D Systems, Cat# AF796), goat anti-myeloperoxidase (MPO) heavy chain (1:400, R&D Systems, Cat# AF3667), goat anti-ß-Catenin (1:1000, R&D Systems, AF1329) and rabbit anti-Claudin 5 (1:1000, Abcam, ab15106) antibodies.
Immunoreactive bands were detected using enhanced ECL (Pierce), imaged using a Luminescent Image Analyzer LAS-3000 (Fujifilm), and quantified by NIH ImageJ.
4.3 Results

4.3.1. Effect of anti-LCN2 antibody on brain injury after stroke-reperfusion injury

To determine whether anti-LCN2 antibody can deplete LCN2 protein from serum and assess the effective working concentrations of anti-LCN2 antibody, we incubated 0.5 µg mouse recombinant LCN2 proteins and magnetic Dynabeads with 0.5, 2.5 and 5 µg anti-LCN2 antibody for 16 hours. LCN2 left in the sample was analyzed by Western blotting analysis. Mouse recombinant LCN2 protein mixed with magnetic Dynabeads only was used as control. The results indicated that anti-LCN2 antibody was able to bind and precipitate mouse recombinant LCN2 protein in vitro (Figure 15). The depletion of recombinant LCN2 was obvious, when anti-LCN2 antibody was incubated with ten times as much as recombinant LCN2, suggesting that 10 fold anti-LCN2 antibody injection might be effective to deplete serum LCN2 in mice (Figure 15). My finding showed that anti-LCN2 antibody might reduce the level of sera LCN2 in stroke-reperfusion injury.
Figure 15. Anti-LCN2 antibody precipitated mouse recombinant LCN2 protein.

Magnetic Dynabeads were mixed with 0.5, 2.5 and 5 µg anti-LCN2 antibody separately. Five µg mouse recombinant LCN2 proteins were incubated with Magnetic Dynabeads and anti-LCN2 antibody complex for 16 hours. LCN2 protein was removed from solution by the Magnetic Dynabeads- anti-LCN2 antibody complex. Mouse recombinant LCN2 proteins mixed with Dynabeads were loaded as control (con). Representative image showed the amount of anti-LCN2 antibody in the complex (top). LCN2 left in the solution was reduced (bottom).
To determine the effect of anti-LCN2 antibody on stroke-induced brain injury, I injected 100 µg LCN2 antibody intraperitoneally 4 hours after one hour of tMCAO into WT mice. The same amount of IgG was injected as control. After 23-hour reperfusion, mice were sacrificed, and mouse sera and brains were collected. Twenty-three hours after tMCAO, cerebral infarct area was detected by TTC staining (Figure 16). Infarct volume of LCN2 antibody injection mice was reduced to 25% of IgG injection mice (49.01 ± 5.580 mm³ in IgG injection mice vs 12.25 ± 8.105 mm³ in LCN2 injection mice; $P < 0.05$; Figure 17A). Meanwhile, we detected similar brain edema reduction. Brain edema volume was reduced to 1.244 ± 0.6332 mm³ in LCN2 injection mice, which is 20% of IgG injection mice (5.467 ± 0.91366 mm³; $P < 0.01$; Figure 17B). Cerebral infarct and edema volume also reflected the motor impairments. The neurological deficit score (Figure 17C) and corner test (Figure 17D) demonstrated that the motor outcome is improved in LCN2 antibody injection mice after tMCAO, suggesting that LCN2 reduction decreases the brain damage in stroke-reperfusion injury.
Figure 16. Cerebral infarction after tMCAO mice with LCN2 antibody treatment.

Representative images of TTC-stained brain slices from control IgG (left) and LCN2 antibody injection mice (right) after one hour of middle cerebral artery occlusion and 23 hours of reperfusion. Viable tissue is stained in red color, while the infarcted area in white color.
Figure 17. Stroke injury after tMCAO is reduced in LCN2 antibody injection mice.

Total infarct volume (A) (n=5), brain swelling volume (B) (n=5), neurological deficit scores (C) (n=7), and corner tests (D) (n=7) were determined after one hour of tMCAO and 23 hours of reperfusion from control IgG and LCN2 antibody injection mice. **P < 0.01 and ***P < 0.001 compared with IgG injection mice (two-tailed, unpaired t test).
4.3.2. Effect of anti-LCN2 antibody on blood brain barrier leakage after stroke-reperfusion injury

Blood brain barrier (BBB) permeability increases after ischemic stroke reperfusion injury (Jin et al., 2014). The reduction of edema after LCN2 antibody treatment (Figure 17) suggests that the BBB permeability could also be reduced after tMCAO. To determine whether the BBB breakdown is reduced with LCN2 antibody treatment, 2% Evans blue was injected immediately after reperfusion. The mice injected with the same amount of IgG was used as control. The BBB leakage was evaluated by Evans Blue extravasation in cerebral hemispheres (Figure 18A and 18B). The amount of extravasated Evans Blue was reduced by 59% in LCN2 antibody injection mice (5.779 ± 1.104 µg of Evans Blue per g of brain proteins) as compared to the control mice (13.791 ± 1.079 µg/g) (P< 0.01; Figure 18B). The expression of BBB tight junction protein (Claudin-5) was reduced in ipsilateral hemisphere of both LCN2 antibody injection mice and IgG injection mice at 23 hours after tMCAO (P< 0.05; Figure 18C). However, the reduction of Claudin-5 was significantly improved in the LCN2 antibody injection mice compared to the control mice (Figure 18C). These results indicated that reduction of LCN2 by the LCN2 antibody treatment attenuated the BBB leakage after stroke-reperfusion injury.
Figure 18. **Blood brain barrier leakage is reduced in LCN2 antibody injection mice after tMCAO.** (A) Representative images of blood brain barrier leakage from mice with IgG or LCN2 antibody injection after tMCAO (n=5). Blood brain barrier leakage area was shown in blue color. (B) Quantification of brain Evans Blue extravasation from the IgG or LCN2 antibody injection mice after tMCAO (n=5). **P < 0.01 compared to the IgG injection mice (two-tailed, unpaired t test).** (C) Claudin-5 immunoreactivity normalized to β-Actin expression (claudin-5/Actin) indicated that the claudin-5 was significantly reduced in the ipsilateral hemispheres of IgG injection mice (n=4) compared to the LCN2 antibody injection mice (*P < 0.05, one-tailed, unpaired t test).
4.3.3. Effect of anti-LCN2 antibody on neutrophil infiltration after stroke-reperfusion injury

The infiltration of immune cells (e.g. neutrophils) exacerbates brain damage in stroke-reperfusion injury (Iadecola and Anrather, 2011). We used the myeloperoxidase (MPO) level in cerebral hemispheres to determine immune cell infiltration in IgG or LCN2 antibody injection mice after tMCAO. The expression of MPO was detected in the ipsilateral hemisphere of both IgG and LCN2 antibody injection mice (Figure 19A). However, the level of MPO was reduced significantly in the LCN2 antibody injection mice as compared to the IgG antibody injection mice ($P < 0.05$; Figure 19A). In addition, the expression of ICAM-1, which facilitates the attachment of immune cells to vascular endothelial cells, was also significantly reduced in the ipsilateral hemisphere of LCN2 injection mice after tMCAO ($P < 0.05$; Figure 19B). These results suggested that the reduction of LCN2 lessens immune cell infiltration after stroke-reperfusion injury.
Figure 19. Reduction of neutrophil infiltration in LCN2 antibody injection mice after stroke-reperfusion injury. The ipsilateral (I) and contralateral (C) hemispheres of IgG or LCN2 antibody injection mice (n = 5) were collected at 23 hours after 1 hour of tMCAO. (A) The neutrophil infiltration was evaluated by determining the expression of MPO of cerebral homogenates using Western blots. β-Actin was used as a loading control. Representative image shows the expression of MPO heavy chain (~55 kDa) of brain homogenates. There was a significant reduction of MPO in ipsilateral hemispheres of mice with the LCN2 antibody treatment (*P< 0.05, one-tailed, unpaired t test) compared to the control IgG treatment. (B) The expression of ICAM-1 in cerebral hemispheres was analyzed by Western blots. The expression of ICAM-1 was significantly reduced in ipsilateral hemisphere of LCN2 antibody injection mice, compared to the control IgG antibody injection mice (*P< 0.05, one-tailed, unpaired t test).
4.4 Conclusion

Recent studies indicated that LCN2 deficiency impairs cerebral damage in stroke-reperfusion injury (Jin et al., 2014; Wang et al., 2015). My finding indicated that anti-LCN2 antibody can reduce the level of LCN2 in solution (Figure 15). Moreover, anti-LCN2 antibody treatment attenuated cerebral infarct volume, edema volume and neurological outcomes after one hour of tMCAO and 23 hours of reperfusion (Figure 16 and 17), suggesting that anti-LCN2 monoclonal antibody may work as an effective therapeutic agent for stroke-reperfusion injury.

Recent study demonstrates that LCN2 deficiency prevented the reduction of tight junction proteins (claudin 5 and β-catenin) after stroke-reperfusion injury (Jin et al., 2014). I found that the BBB leakage after tMCAO is attenuated with anti-LCN2 antibody treatment (Figure 18). Reduction of BBB tight junction protein, claudin-5, is attenuated with anti-LCN2 antibody treatment 23 hours after ischemic stroke (Figure 18C), suggesting that reduction of LCN2 prevented the BBB degradation in stroke-reperfusion injury.

My previous study shows immune cell (neutrophils and macrophages) infiltration is reduced in ipsilateral hemisphere 23 hours after ischemic stroke in LCN2 deficiency mice (Wang et al., 2015). My recent findings demonstrate that after anti-LCN2 antibody treatment, the induction of MPO and ICAM-1 were reduced in ipsilateral hemisphere after 23 hours of reperfusion (Figure 19). Our result suggests that anti-LCN2 antibody reduced cerebral edema by impairing infiltration of immune cells. My current findings suggest that anti-LCN2 antibody may work as an effective therapeutic agent to treat stroke-reperfusion injury in humans.
5.1 Molecular mechanism of LCN2 in stroke-reperfusion injury

Minutes after ischemic stroke, resident brain cells including astrocytes release ROS, proinflammatory cytokines and chemokines, thus inducing the expression of adhesion molecules on cerebral endothelium and promoting transendothelial migration of circulating neutrophils (Iadecola and Anrather, 2011). Infiltrating neutrophils release ROS as well as cytokines and chemokines that further amplify the brain-inflammatory response. It is likely that LCN2 from reactive astrocytes initiates the neuroinflammatory event, causing release of LCN2 from cerebral endothelium and circulating neutrophils (Figure 20). A recent study also confirmed the induction of LCN2 in reactive astrocytes as well as cerebral endothelium after tMCAO (Zamanian et al., 2012).
Figure 20. A proposed model for the sequence of LCN2 induction and its effect on neurons. (1) LCN2 is induced and released from astrocytes in response to stroke-reperfusion injury. (2) The released LCN2 may cause the induction and release of LCN2 from cerebral endothelial cells and neutrophils, and stimulate the infiltration of circulating neutrophils. (3) LCN2 released from astrocytes and neutrophils may cause additional neuronal cell death, and (4) recruit circulating macrophages.
5.2 Discussion

Our results identify LCN2 as an acute-phase protein mediating reperfusion injury after ischemic stroke in mice. The elevated level of LCN2 after stroke may exacerbate cerebral infarction and contribute to poor neurological outcomes by enhancing neutrophil infiltration and neuronal apoptosis. We detected the induction of LCN2 in mouse serum within one hour after tMCAO, suggesting the exciting possibility of using LCN2 as an early blood biomarker of stroke. Nearly 95% of stroke patients are unable to benefit from thrombolytic therapy because they are diagnosed beyond a critical 4.5-hour time period following the onset of symptoms (Del Zoppo et al., 2009; Kidwell and Wintermark, 2008; Saver et al., 2013). Development of rapid diagnostic assays using LCN2 may facilitate early diagnosis, reduce the risk of cerebral hemorrhage, and ultimately increase the overall efficacy of thrombolytic therapy for stroke victims (Reynolds et al., 2003; Whiteley et al., 2009).

Expression of LCN2 after stroke was increased not only in sera, but also in brain, where it was localized to infiltrating neutrophils and a subset of reactive astrocytes (Figure 3, 4 and 5). A recent study confirmed the induction of LCN2 in reactive astrocytes as well as cerebral endothelium cells after tMCAO (Zamanian et al., 2012). A few minutes after ischemic stroke, resident brain cells including astrocytes release ROS, proinflammatory cytokines and chemokines, thus inducing the expression of adhesion molecules on the cerebral endothelium cells and promoting transendothelial migration of circulating neutrophils (Iadecola and Anrather, 2011; Jin et al., 2010). Infiltrating neutrophils release excessive amount of ROS as well as cytokines and chemokines that further amplify the brain-inflammatory response by causing extensive activation of brain cells and infiltration of more neutrophils and other leukocytes such as monocytes/macrophages and T-cells (Mayadas et al., 2014). It is unclear which cell types
contribute primarily to plasma LCN2, but it is possible that LCN2 from reactive astrocytes initiate the neuroinflammatory event, causing release of LCN2 from cerebral endothelium and circulating neutrophils. The sequential secretion of LCN2 from astrocytes, endothelia and neutrophils may set up the pathway for infiltrating neutrophils to follow. Infiltrating neutrophils may release additional LCN2 and establish a self-amplifying loop of neuroinflammation.

Several studies have demonstrated that neutrophils accumulate in ischemic brains within 24 hours after the onset of cerebral infarction in both experimental stroke models and clinical studies (Iadecola and Anrather, 2011; Jin et al., 2010; Price et al., 2004). Our finding that the infiltration of MPO-positive immune cells is reduced in LCN2 null mice after tMCAO is consistent with recent reports focused on LCN2 function in other models. For example, immune cell recruitment to the site of injury in LCN2 null mice is diminished in mouse models of spinal cord injury (Rathore et al., 2011) and heterotopic heart transplantation (Aigner et al., 2007). Neutrophils isolated from LCN2 null mice display defective chemotaxis in vitro (Liu et al., 2013). Moreover, recombinant LCN2 stimulates migration of neutrophils in vitro and in vivo (Schroll et al., 2012). These results suggest that LCN2 is a paracrine chemoattractant for immune cells during ischemia-reperfusion injury. Similar pro-neuroinflammatory effects have been observed for S100A8 and S100A9, two members in the S100 family of calcium-binding proteins (Schiopu and Cotoi, 2013). S100A8 and S100A9, also known as myeloid-related proteins (MRP) 8 and 14, are abundantly expressed in neutrophils and stored in the secondary granules where LCN2 was detected (Kjeldsen et al., 2000; Kjeldsen et al., 1993). These two S100 proteins have been characterized as endogenous ligands of toll-like receptor 4 (TLR4) (Vogl et al., 2007) and chemotactic agents and modulators of neutrophil transmigration (Ryckman et al., 2003). The S100A8 and S100A9 are induced in ischemic hemispheres after
tMCAO and deficiency of these proteins the accumulation of inflammatory cells as well as cerebral swelling and infarction in post-ischemic brain (Ziegler et al., 2009).

Stroke damage was reduced in LCN2 null mice in the tMCAO model, but not in the pMCAO model (Figure 7, 8 and 11). Several studies demonstrated that neutrophil-derived proteolytic enzymes, oxygen radicals, and cytokines increase cerebral edema and neuronal apoptosis after the onset of ischemia-reperfusion (Iadecola and Anrather, 2011; Jin et al., 2010). Co-culturing primary neurons with isolated neutrophils, but not lymphocytes, causes massive neuronal cell death (Dinkel et al., 2004). Thus, it is likely that diminished neutrophil infiltration led to neuroprotection in LCN2 null mice after tMCAO. We found that recombinant LCN2 can directly induce significant neuronal cell death at a concentration of 10 µg/ml (Figure 9), which is close to the range we measured in serum after stroke (5.5 µg/ml) (Figure 5). Higher LCN2 levels may be achieved locally through degranulation by infiltrating neutrophils or accumulation of serum LCN2 through leaky blood-brain barrier. The apoptotic effect of LCN2 appears to be dependent on cell type-specific expression of its putative BOCT receptors (Devireddy et al., 2005; Miharada et al., 2008; Miharada et al., 2005). Our results demonstrate the mutually exclusive expression patterns of LCN2 and BOCT in neutrophils and neurons under non-ischemic conditions. LCN2 is abundantly expressed and packaged in the granules of neutrophils (Kjeldsen et al., 2000; Kjeldsen et al., 1993), whereas the expression of BOCT is low in neutrophils (Miharada et al., 2008). Therefore, neutrophils have been reported to be insensitive to LCN2 mediated apoptosis (Miharada et al., 2008; Miharada et al., 2005). LCN2 protein is nearly absent in the brains of normal control mice, while BOCT is ubiquitously expressed in the neurons of brain regions (striatum and cortex) supplied by middle cerebral arteries (Allen Brain Atlas) (Lein et al., 2007). LCN2 levels increased in mouse brains after tMCAO, possibly
following release by neutrophils and reactive astrocytes, and might exert its apoptotic effect through the BOCT receptors expressed in neurons (MacManus et al., 2004).

The plasma concentration of LCN2 is increased in patients during 1-3 days after ischemic stroke (Anwaar et al., 1998; Elneihoum et al., 1996; Falke et al., 2000). During a four-year follow-up, the stroke patients with higher levels of LCN2 had higher cardiovascular mortality (Falke et al., 2000). These results together with the findings in this report strongly suggest that LCN2 is a detrimental factor induced following stroke.

Immunotherapy has been proven effective in the treatment of stroke-reperfusion injury (Yu et al., 2013). Numerous monoclonal antibodies have been used to block special signaling pathway in murine brain to reduce cerebral damage of stroke-reperfusion injury (Liesz et al., 2011a; Macrez et al., 2011; Wiessner et al., 2003). The result of this study showed that anti-LCN2 antibody reduced the level of mouse LCN2 (Figure 15). Reduced LCN2 also attenuated cerebral infarct volume, edema volume and outcomes after 23 hours of reperfusion (Figure 11), suggesting that anti-LCN2 antibodies could be important therapeutic candidates that may modulate post-ischemic inflammation in patients with ischemic stroke.

Moreover, I found that anti-LCN2 antibody reduced BBB leakage after 23 hours of tMCAO (Figure 18). Energy failure in ischemic stroke causes depletion of ATP, reduction of Na\(^+\)-K\(^+\) ATPase activity, imbalance of intracellular ions and release of extracellular glutamate (Kulik et al., 2008; Sandoval and Witt, 2008). Moreover, induction of proteases (i.e., matrix metalloproteinase 9) led to BBB extracellular matrix degradation and disassembly of the tight junction proteins (Khatri et al., 2012; Ludewig et al., 2013; Sandoval and Witt, 2008). All of these contribute to the disruption of the BBB. A recent study indicated that LCN2 deficiency prevented the reduction of tight junction proteins (claudin 5 and β-catenin) in stroke-reperfusion.
injury (Jin et al., 2014). The reduction of BBB tight junction protein, claudin-5, attenuated with anti-LCN2 antibody treatment 23 hours after ischemic stroke (Figure 18), suggesting that the reduction of LCN2 prevents BBB degradation in stroke-reperfusion injury.

In reperfusion phase of stroke-reperfusion injury, neutrophil recruitment and extravasation are involved in disruption of the BBB (Khatri et al., 2012; Sandoval and Witt, 2008). My previous study showed that immune cell (neutrophils and macrophages) infiltration is reduced in ipsilateral hemisphere 23 hours after ischemic stroke in LCN2 deficiency mice. After the anti-LCN2 antibody treatment, MPO and ICAM-1 were reduced in ipsilateral hemisphere after 23 hours of reperfusion (Figure 19). Our results suggest that anti-LCN2 antibody reduced cerebral edema by impairing infiltration of immune cells. These findings suggest that anti-LCN2 antibody may work as an effective therapeutic reagent to treat stroke-reperfusion injury in human.
5.3 Future directions

Several monoclonal antibody treatments are very effective in animal models (Yu et al., 2013). However, due to their side effects, these immunotherapy methods failed in clinical trials. Hence, in the future, reducing the side effects of monoclonal antibody would be a big endeavor. The current technology can promote the quality of monoclonal antibody to reduce the side effects of immunotherapy.

Several important factors need to be considered in stroke immunotherapy. First, the current research focused on mice. However, the pathophysiology of stroke in mouse is different from that in human. Hence, the comparison of stroke between human and animal is important for improving the immunotherapy. In our research, the monoclonal LCN2 antibody is produced in rat. However, injecting rat antibody to human will cause immune responses, which may be one of the major causes of its side effects. Thus, it is essential to humanize the antibody and make it suitable for human.

Second, stroke-reperfusion injury is a dynamic process. LCN2 may work as a deleterious factor within 24 hours after stroke. We injected anti-LCN2 antibody 4 hours after stroke, which is the maximum working time for tPA. It would benefit more patients if late antibody injection also reduces stroke-reperfusion injury. Therefore, it is crucial to test the antibody at later time points and choose proper time points to achieve the maximum results and reduce any potential side effects.

Third, different molecules play different roles in stroke-reperfusion injury. Therefore, a combination treatment of several monoclonal antibodies may produce better treatment outcomes. For example, stroke-reperfusion injury actually contains brain injury from stroke and the further
damage induced by reperfusion. Thus, a combination of antibodies against molecules involved in stroke phase and reperfusion phase could achieve better therapeutic outcomes.

Finally, the recanalization is still a very effective therapeutic method in stroke management. However, delayed thrombolytic therapy with tPA may cause serious complications including hemorrhagic transformation and reperfusion injury. Therefore, a combination therapy with tPA and anti-LCN2 antibody may offer a better approach to stroke management. tPA dissolves clots in blood vessels, while anti-LCN2 antibody may reduce the reperfusion injury caused by tPA treatment. This combination therapy has the high potential of reducing the mortality rate and hemorrhagic transformation, and achieving better neurological outcomes for countless stroke patients.
Bibliography


