INVESTIGATION OF RENAL ISCHEMIA-REPERFUSION INDUCED LIVER INJURY

By

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ABSTRACT

Ischemia-reperfusion (IR) is a major cause of acute kidney injury (AKI). AKI is often associated with dysfunction of remote organs also known as distant organ injury. Previous clinical and animal studies have identified that oxidative stress and inflammation are main pathological components in renal IR injury. However, their roles and regulatory mechanisms in distant organs are not well understood. Liver is a key metabolic organ for redox balance, immune regulation and detoxification. Liver function is often compromised by AKI. The general objective of my research was to investigate the mechanism by which renal ischemia-reperfusion led to oxidative stress and inflammatory responses in the liver. The left kidney of Sprague-Dawley rats was subjected to 45min ischemia followed by 1h or 6h of reperfusion. Renal ischemia-reperfusion impaired kidney and liver function as indicated by increased plasma creatinine and aminotransferase levels. A decrease in glutathione level was observed in the liver and plasma, along with increased hepatic lipid peroxidation and plasma homocysteine levels, as indicators of oxidative stress. Renal ischemia-reperfusion caused a significant decrease in mRNA and protein levels of hepatic enzyme cystathionine γ-lyase, which regulates the transsulfuration pathway. A decrease in gene expression of glutamate-cysteine ligase subunits for glutathione biosynthesis was mediated through inhibition of the transcription factor Nrf2. Renal IR also caused a significant increase in proinflammatory cytokine (MCP-1, TNF-α, IL-1β and IL-6) protein levels in the liver, kidney and plasma. Activation of nuclear transcription factor kappa B (NF-κB) was detected in the liver at 1h and 6h after renal IR. This was accompanied with a significant increase in the mRNA levels of proinflammatory cytokines in the liver. An elevation of myeloperoxidase activity and inflammatory foci were detected in the liver at 6h after renal IR, indicating neutrophil infiltration. In conclusion, results from our research have demonstrated that

renal IR can induce acute liver injury. We have identified that during renal IR, down-regulation of hepatic Nrf2/glutathione production and up-regulation of NF-κB/cytokine expression are responsible for increased oxidative stress and inflammatory response, which, in turn, exert detrimental effects to distant organs.

DEDICATION

I dedicate this thesis to my parents: Lian Shang and Hui Zhu Jiang for their forever love and unlimited support during my entire life. The thesis is also dedicated to friends and extended family that I have met in Winnipeg Manitoba. I would have not been able to achieve this far without everyone's encouragement.

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PERMISSION STATEMENTS

Figure 1.7 Keap1-Nrf2 stress response system, in Kobayashi, E., T. Suzuki and M. Yamamoto (2013), Roles Nrf2 Plays in Myeloid Cells and Related Disorders, Oxidative Medicine and Cellular Longevity 2013: 7, is released under the Creative Commons Attribution License in Oxidative Medicine and Cell Longevity by Hindawi Publishing Co., which permits unrestricted use, distribution, and reproduction of the material in any medium.

Figure 1.9 Nrf2 activation and NF-κB inhibition though Keap1, in Stefanson, A. L. and M. Bakovic (2014), Dietary regulation of Keap1/Nrf2/ARE pathway: focus on plant-derived compounds and trace minerals, Nutrients 6(9): 3777-3801, is released under the Creative Commons Attribution License in Nutrients by Multidisciplinary Digital Publishing Institute, which permits unrestricted use, distribution, and reproduction of the material in any medium.

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Manuscript II in Chapter IV is under preparation for submission, which requests a hold for release before its publication.

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8-oxodG	8-hydroxydeoxyguanosine
8-oxoG	8-hydroxyguanosine
ACE	Angiotensin-converting enzyme
ADQI	Acute dialysis quality initiative
AKI	Acute kidney injury
AKIN	Acute kidney injury network
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AQP	Aquaporin
ARE	Antioxidant response element
ARF	Acute renal failure
AST	Aspartate aminotransferase
ATN	Acute tubular necrosis
ATP	Adenosine triphosphates
BAFF	B-cell activating factor
BNx	Bilateral nephrectomy
CAD	Coronary artery disease
CBP	CREB binding protein
CBS	Cystathionine-β-synthase
CCARM	Canadian Centre for Agri-Food Research in Health and Medicine

cDNA	Complementary deoxyribonucleic acid
CRP	C-reactive protein
CSE	Cystathionine-γ-lyase
CVD	Cardiovascular disease
CXCL	Chemokine (C-X-C motif) ligand
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetra-acetic acid
ENA	Epithelial neutrophil-activating protein
ENaC	Epithelial sodium channel
Gcl	Glutamate-cysteine ligase
GFR	Glomerular filtration rate
GGT	γ-glutamyltranspeptidase
GLM	General linear models
GN	Glomerulonephritis
GPx	Glutathione peroxidase
GS	Glutathione synthase
GSH	Glutathione
GSSG	Oxidized glutathione
Hcy	Homocysteine
HDAC3	Histone deacetylase 3
HDL	High-density lipoprotein
HO	Heme oxygenase

ICAM	Intercellular adhesion molecule
ICU	Intensive care unit
IFN	Interferon
IKK	IκB-kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Interferon invasive protein
IR	Ischemia-reperfusion
JAK/STAT	Janus kinase/signal transducer and activator of transcription
KDIGO	Kidney Disease Improving Global Outcomes
Keap1	Kelch-like ECH-associated protein 1
KIM-1	Kidney injury molecule-1
Klf9	Kruppel-like factor 9
LDL	Low-density lipoprotein
L-FBP	Liver-type fatty acid binding protein
LOOH	Lipid hydroperoxide
LPS	Lipopolysaccharides
MCP	Monocyte chemoattractant protein
MDA	Malondialdehyde
MPO	Myeloperoxidase
NADP	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology

	Information
NF-κB	Nuclear factor kappa B
NGAL	Neutrophil gelatinase-associated lipocalin
NIK	NF-κB inducing kinase
NK	Natural killer
NO	Nitrogen oxide
Nqo1	NAD(P)H: quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid 2-related factor 2
NSAIDs	Nonsteroidal anti-inflammatory drugs
NSERC	Natural Sciences and Engineering Research Council of Canada
PAG	Propargylglycine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLP	Pyridoxal-5'-phosphate
PUFAs	Polyunsaturated fatty acids
qRT-PCR	Quantitative real-time PCR
RIFLE	The risk of renal failure, injury to the kidney, failure of kidney function, loss of kidney function, and end-stage renal failure
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

RRT	Renal replacement therapy
SCr	Serum creatinine
SD	Sprague Dawley
SEM	Standard error of the mean
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TGF-β	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UO	Urine output
VLDL-TG	Very-low-density lipoprotein triglyceride

Chapter I. Introduction

1.1 Physiological role of kidney

The kidneys are important organs that can extract waste from blood, balance fluid and electrolyte, form urine, control pH and aid in other metabolic functions of the body. The basic functions of the kidney include: 1) regulation of extracellular fluid volume and osmolarity, 2) regulation of ion concentrations and pH, 3) excretion of wastes and toxins and 4) production of hormones such as erythropoietin and renin to stimulate red blood cell synthesis, and help control salt and water balance, respectively.

The kidneys are bean shaped organs that reside against the back muscles in the upper abdominal cavity and sit on either side of the spine. There are three major regions in the kidney, including renal cortex, medulla and pelvis. The outer, granulated layer is the renal cortex. The inner radially striated layer is the renal medulla, which contains cone shaped tissue mass called renal pyramids, separated by renal columns. The ureters are continuous with the renal pelvis and at the very center of the kidney (Mader, 2003).

Three main processes enable the kidney to filter blood, which include: 1) glomerular filtration, where fluid such as water and nitrogenous waste in the blood is filtered across the capillaries of the glomerulus, and nonfilterable components such as cells and serum albumins will exit via an arteriole. The force of hydrostatic pressure is the driving force that pushes filtrate out of the capillaries and into the slits in the nephron. 2) tubular reabsorption, where molecules and ions is removed from the tubular fluid and reabsorbed into the circulatory system. The mechanisms of this process include: passive diffusion, passing through plasma membranes of the kidney epithelial cells by concentration gradients; active transport by using ATPase pumps such as Na^+/K^+ ATPase pumps with carrier proteins; and co-transport to reabsorb water. 3) tubular secretion, where substances such as hydrogen ions, creatinine, drugs and urea are removed from

the blood through the peritubular capillary network into the collecting duct. Urine is the end product of these processes, which is a collection of substances that has not been reabsorbed during glomerular filtration or tubular reabsorption. Urea is one of the main components in the urine, which is a highly soluble molecule composed of ammonia and carbon dioxide, and provides a way for nitrogen removal from the body (Mader, 2003).

1.2 Acute kidney injury (AKI)

Acute kidney injury (AKI) is also known as acute renal failure (ARF), which is a sudden episode of kidney function deterioration that happens within a few hours or a few days. AKI causes waste accumulation in the blood and disrupts the balance of the body fluid. Thus, AKI can induce a broad range of complications from less severe forms of injury such as subtle biochemical and structural changes, to more advanced injury that requires renal replacement therapy (RRT) (Lewington and Kanagasundaram, 2011). It can also affect other organs such as the brain, heart, liver, intestine and lungs. AKI is common in patients who are in the hospital, in intensive care units, and especially in older adults.

1.2.1 Classification

To standardize AKI classification and definition, the Acute Dialysis Quality Initiative (ADQI) group published the risk of renal failure, injury to the kidney, failure of kidney function, loss of kidney function, and end-stage renal failure (RIFLE) criteria in 2004. It classified AKI into three categories (risk, injury, and failure) according to the status of serum creatinine (SCr) and urine output (UO). In 2007, a modified version of the RIFLE classification was published by the AKI Network (AKIN) working group, and is also known as the AKIN classification (Mehta et al., 2007, Lin and Chen, 2012). In AKIN stage-1, a smaller change within 48 h in SCr of over 0.3 mg/dL ($\geq$ 26.2 μmol/L) was suggested as the threshold for AKI. Patients receiving RRT were re-

classified as AKIN stage-3. Furthermore, the loss and end-stage kidney disease categories were eliminated in the AKIN classification. Both RIFLE and AKIN classifications have been proven to be useful for diagnosing and classifying the severity of AKI in critical patients (Lin and Chen, 2012). More recently, the Kidney Disease Improving Global Outcomes (KDIGO) group published a clinical practice guideline for acute kidney injury, which combined the RIFLE criteria and the AKIN definition. The criteria of RIFLE, AKIN and KDIGO are listed in Table 1.1 (Khwaja, 2012, Singbartl and Kellum, 2012). Standardized AKI classification serves as a useful tool in estimating the incidence of AKI, improving the specificity of diagnosis, determining the appropriate participants and helping clinicians to conduct evidence-based clinical practice.

Table 1.1 Classification schemes for acute kidney injury (AKI) defined by RIFLE, AKIN and KDIGO*

	Stage	SCr[1] or GFR[2] Criteria	Urine Output (UO) Criteria
RIFLE[3]	**Risk**	↑SCr ≥1.5×Baseline or ↓GFR ≥25%	UO <0.5 ml/kg/h ×6 h
	Injury	↑SCr ≥2.0×Baseline or ↓GFR ≥50%	UO <0.5 ml/kg/h ×12 h
	Failure	↑SCr ≥3.0×Baseline or ↑SCr ≥4 mg/dl (353.6 μmol/l) or ↓GFR ≥75%	UO <0.3 ml/kg/h ×24 h or anuria ×12 h
	Loss	Complete loss of renal function for >4 weeks	
	ESKD[4]	Complete loss of renal function for >3 months	
AKIN[5]	**Stage 1**	Same as RIFLE Risk plus ↑SCr ≥0.3 mg/dl (26.4 μmol/l)	Same as RIFLE Risk
	Stage 2	↑SCr ≥2.0×Baseline	Same as RIFLE Injury
	Stage 3	Same as RIFLE Failure plus initiation of renal replacement therapy (RRT)	Same as RIFLE Loss
KDIGO[6]	**Stage 1**	↑SCr ≥1.5×Baseline within 7 days or ↑SCr ≥0.3 mg/dl (26.4 μmol/l) within 48 h	Same as RIFLE Risk
	Stage 2	↑SCr ≥2.0×Baseline	Same as RIFLE Injury
	Stage 3	↑SCr ≥3.0×Baseline or ↑SCr ≥4 mg/dl (353.6 μmol/l) plus initiation of RRT	Same as RIFLE Loss

* Table adapted from Khwaja (2012), Lin and Chen (2012), and Singbartl and Kellum (2012). [1]SCr: Serum creatinine; [2] GFR: Glomerular filtration rate; [3] RIFLE: Risk of renal failure, injury to the kidney, failure of kidney function, loss of kidney function, and end-stage renal failure; [4] ESKD: End-stage kidney disease; [5]AKIN: Acute Kidney Injury Network; [6] KDIGO: the Kidney Disease Improving Global Outcomes.

1.2.2 Pathophysiology and diagnosis of AKI

1.2.2.1 Pathophysiology of AKI

Ischemia, hypoxia and nephrotoxicity are the primary causes for AKI. Particularly, AKI features a rapid decline in GFR, which is usually associated with a decrease in renal blood flow. Furthermore, inflammation represents an important component of AKI, leading to an exacerbation of the injury (Basile et al., 2012).

Clinically, AKI can be conveniently grouped into three primary etiologies: prerenal, renal (intrinsic), and postrenal (Basile et al., 2012, Singbartl and Kellum, 2012). Prerenal azotemia is characterized by a decrease in GFR due to a decrease in renal perfusion pressure without damage to the renal parenchyma, which can be caused by 1) hypovolemia resulting from hemorrhage, vomiting, diarrhea, poor nutrition intake, burns, excessive sweating, renal losses; 2) impaired cardiac output resulting from congestive heart failure or decreased cardiac output states; 3) decreased vascular resistance resulting from sepsis, vasodilator medications, autonomic neuropathy, or anaphylaxis; and 4) renal vasoconstriction from hypercalcemia or vasoconstrictive medications (Badr and Ichikawa, 1988). Intrinsic renal etiologies of AKI can be induced by 1) tubular damage caused by ischemic or nephrotoxic acute tubular necrosis (ATN); 2) glomerular damage caused by acute glomerulonephritis (GN); 3) interstitial damage result from acute interstitial nephritis due to an allergic reaction to infection; and 4) vascular damage from injury to intrarenal vessels which decreases renal perfusion and diminishes GFR. Postrenal causes of AKI are characterized by acute obstruction of urinary flow. The urinary tract obstruction increases intratubular pressure and thus decreases GFR. Acute urinary tract obstruction can also lead to impaired renal blood flow and inflammatory processes which also decrease GFR (Basile et al., 2012). The etiology of AKI is summarized in Figure 1.1.

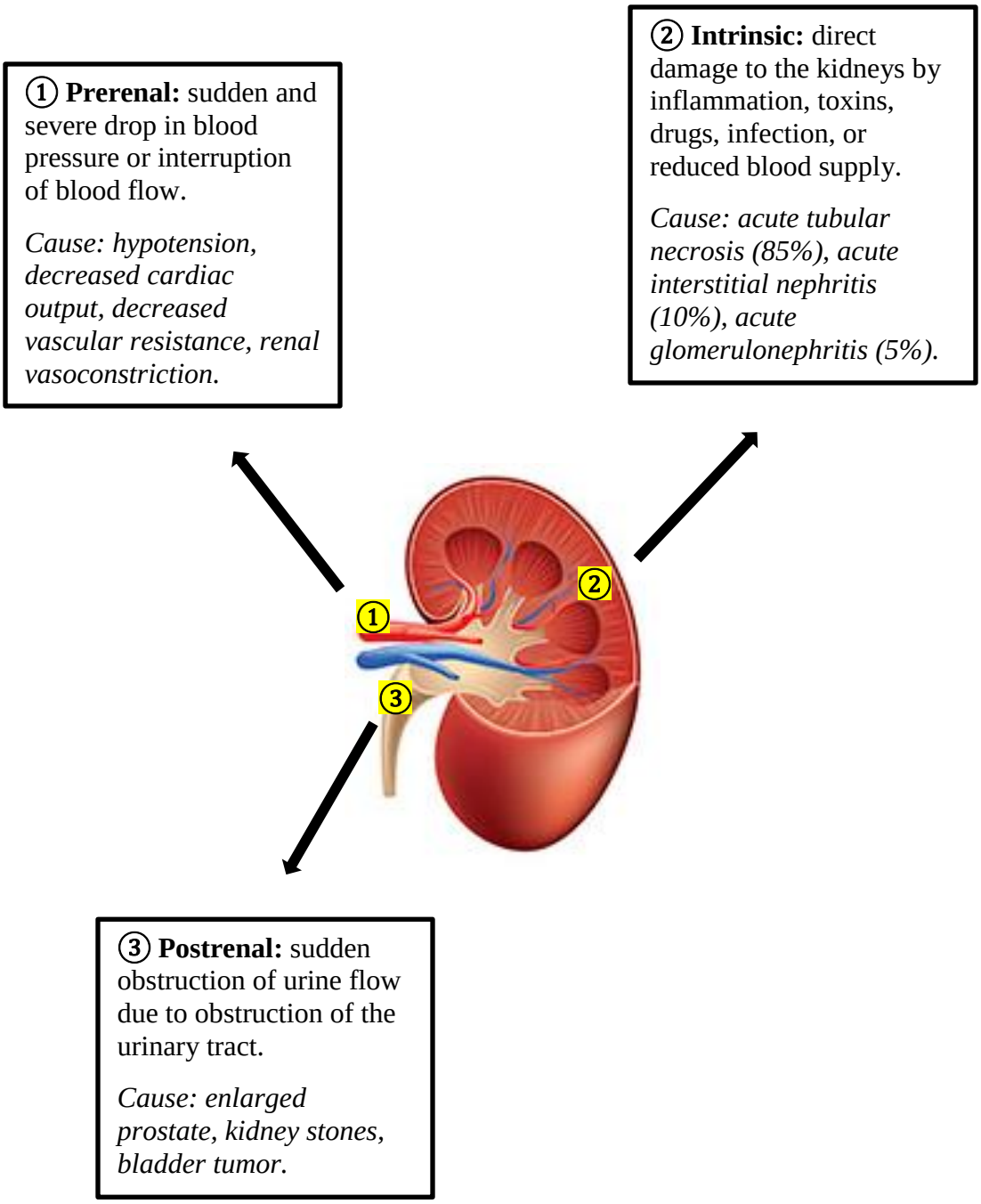

Figure 1.1 The etiology of acute kidney injury (AKI).

The mechanisms involved in the etiology of AKI include: 1) endothelial injury from vascular perturbations with the loss of ability to regulate vascular tone and elevated endothelial injury markers; 2) abolishment of renal autoregulation with elevated intracellular calcium levels from tubular damage, causing increased sensitivity to vasoconstrictors and renal nerve stimulation which increases tubuloglomerular feedback; 3) production of inflammatory mediators and oxygen radicals, such as tumor necrosis factor (TNF)-α, interleukin (IL)-18, intercellular adhesion molecule (ICAM)-1 and reactive oxygen species (ROS), triggered by inflammatory cytokines, as well as immune cell attachment and migration; and 4) direct effect of nephrotoxins (Lattanzio and Kopyt, 2009). The mechanism of AKI is illustrated in Figure 1.2.

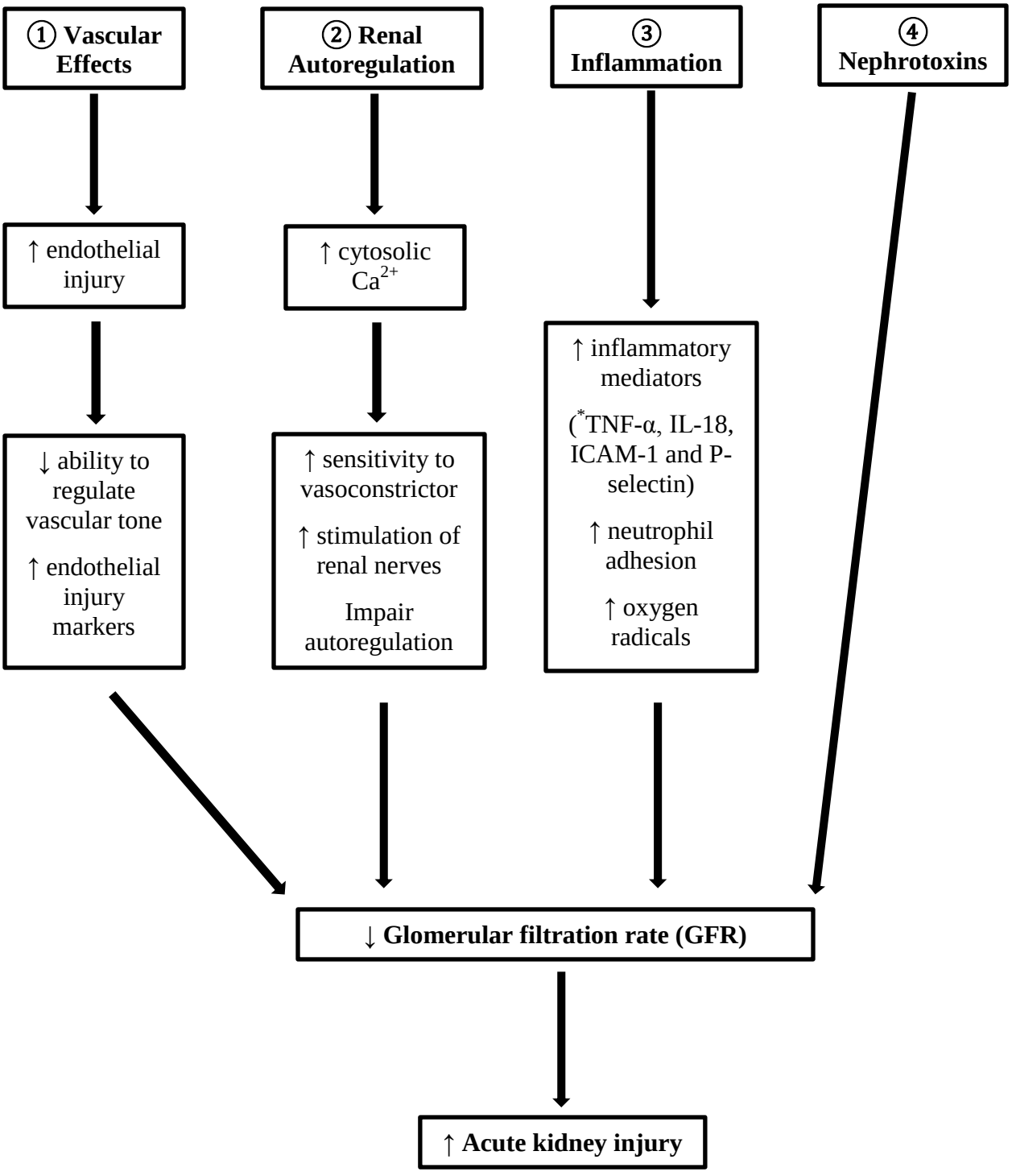

Figure 1.2 The mechanisms of acute kidney injury (AKI)

(*TNF-α: tumor necrosis factor-α, IL-18: interleukin-18, ICAM-1: intercellular adhesion molecule-1)

1.2.2.2 Diagnosis of AKI

The diagnosis of AKI is traditionally based on functional parameters such as an increase in serum creatinine level as a surrogate for impaired GFR; physical examination such as assessment of intravascular volume status and skin manifestations as indicators of systemic illness; review of medical history for possible nephrotoxic insults, such as exposure to contrast materials, medications, or hypotension; complete blood count, urinalysis, serial measurement of blood urea nitrogen, and electrolyte levels including fractional excretion of sodium (FE_{Na}); and renal ultrasonography to identify obstruction (Bellomo et al., 2004, Rahman et al., 2012). The diagnosis tests are listed in Table 1.2.

Table 1.2 Diagnosis tests for acute kidney injury (AKI)

Test	Function
Urine output measurement	Measurement of the amount of urine excretion to determine the cause of AKI.
Urinalysis	To determine abnormalities suggested by kidney failure.
Blood tests	Measure the levels of creatinine, urea nitrogen phosphorus, potassium, glomerular filtration rate (GFR) and protein level to determine kidney function.
Imaging tests	Imaging tests such as ultrasound and computerized tomography to see abnormality of the kidneys
Kidney biopsy	To remove a small sample of kidney tissue for lab microscopy.

Creatinine is currently the most widely used marker of renal function. However, estimation of GFR with creatinine is often inaccurate due to poor predictive accuracy for early stage AKI and changing determinants such as nutrition, medication and serum volume (Bellomo et al., 2004). Furthermore, significant renal impairment could occur with only a subtle change of serum creatinine depends on the reserved and/or increased creatinine level in the kidney. Therefore,

there is great interest in identifying early biomarkers that are elevated during ischemia and within hours of the AKI event. Neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), IL-18, cystatin C and liver-type fatty acid binding protein (L-FBP) are among the biomarkers that have shown promising preliminary results. Further validation of these new biomarkers needs to be conducted in order to improve the accuracy and efficiency for AKI diagnosis (Bellomo et al., 2004, McDougal, 2005, Parikh et al., 2005, Portilla et al., 2008, Thurman and Parikh, 2008).

1.2.3 Prevalence and current treatment of AKI

1.2.3.1 Prevalence of AKI

AKI is a complication that occurs frequently in hospitalized patients, with a reported incidence of 2% to 5%, and accounts for approximately 2 million deaths every year worldwide. The incidence of AKI in hospitalized patients also shows an increase of 11% per year (Xue et al., 2006). In addition, AKI has a poor prognosis with a mortality rate of 50%-60%, depending on associated organ dysfunctions and comorbidity. The survivors face marked increases in morbidity and prolonged hospitalization, and around 5-20% of the patients remain dialysis dependent at hospital discharge (Hoste and Schurgers, 2008).

Many studies have analyzed the incidence and mortality of AKI at an intensive care unit (ICU). In a multinational, multicenter trial, the overall hospital mortality was 60.3% and dialysis dependence at hospital discharge was 13.8% for survivors, during the study period from September 2000 to December 2001 (Uchino et al., 2005). There has been an increase in the incidence of AKI treated with RRT over time (Hoste and Schurgers, 2008). Between 1996 and 2003, the incidence of non-dialysis depending AKI increased from 0.32% to 0.52% annually, whereas the dialysis-depending AKI increased 10 per million people per year (Hsu et al. 2007).

The incidence is also greater in men compared with women (Hsu et al., 2007). Patients on RRT or those who already have chronic kidney disease have a higher incidence in developing end-stage kidney disease. In Canada, 22% of surviving patients developed end-stage kidney disease. The annual mortality rate was 7.3 per million population with the highest rates among males over 65 years of age (Bagshaw et al. 2005). The 1 month, 3 months, and 1 year fatality rates were 51%, 60%, and 64%, respectively (Bagshaw et al., 2005). Similarly, studies in Sweden reported that 8.3% of patients on continuous RRT and 16.5% of patients on intermittent RRT developed end-stage kidney disease (Bell et al., 2007). In addition, patients who have chronic kidney dysfunction (53%) are more prone to the development of end-stage kidney disease when compared to patients with normal baseline kidney function (13%) (Prescott et al., 2007). Emerging data of the high incidence and mortality rate of AKI should encourage researchers and medical providers to find feasible prevention approaches, early diagnosis and management strategies for its complications.

1.2.3.2 Treatment of AKI

Treatment of AKI is mostly support-based, including adjustment of medications, avoidance of nephrotoxins, nutritional support, correction of volume status including hyperkalemia, hypovolemia and acidosis, management of blood pressure and fluid, and dialysis to remove toxins (Bellomo et al., 2004). However, because the kidneys are very vulnerable to the toxic effects of different chemicals, to date, no therapeutic agents have shown efficacy in treating the condition (Rigatto et al., 2013).

For patients with AKI, prescription and non-prescription medications should be carefully examined to avoid potentially nephrotoxic components. Some nephrotoxic agents include aminoglycosides, amphotericin, nonsteroidal anti-inflammatory drugs (NSAIDs), angiotensin-

converting enzyme (ACE) inhibitors, cisplatin, ifosfamide, and herbal remedies (Choudhury and Ahmed, 2006). If endogenous nephrotoxicity is diagnosed at an early point, it can often be reversed with urinary alkalinisation to prevent kidney failure and minimize the need for dialysis (Rahman et al., 2012). Nutritional support is recommended for balancing the protein and energy level to alleviate negative protein-energy wasting effects in AKI patients. Due to the loss of homeostatic function of the kidneys, patients with AKI are prone to have complications such as hyperglycemia, hypertriglyceridemia and fluid retention. It has been suggested that AKI patients on RRT should receive at least 1.5 g/kg/day of protein, and less than 30 kcal non-protein calories (Fiaccadori and Cremaschi, 2009). Maintaining adequate renal perfusion is the key to management. An increase in urine output should be attempted by hydrating the patient with saline and a loop diuretic to lower the risk of volume overload as well as hypervolemia and hyperkalemia. Measurement of intravascular volume status or central venous pressures may be helpful for the correction of volume status (Rahman et al., 2012). Correction of electrolyte imbalances such as hyperkalemia, hyperphosphatemia, hypermagnesemia, hyponatremia, hypernatremia, and metabolic acidosis is important (Rahman et al., 2012). Calcium gluconate is often used to stabilize the plasma membrane and reduce the risk of arrhythmias when the patients display symptoms of hyperkalemia. Dietary intake of potassium should be restricted. Supportive therapies such as renal replacement therapy, fluid therapy, acute dialysis therapy, mechanical ventilation, glycemic control, and anemia management should also be considered (Bellomo et al., 2004, Rahman et al., 2012).

1.2.4 Animal models of AKI

1.2.4.1 Implications for humans

Animal models provide a dynamic and integrative system for the understanding of pathophysiology and they are fundamental for the study of human diseases. Animal models of AKI are well-established and allow us to study the response in the context of the whole kidney and the whole organism. Currently, three types of animal models are in use for AKI research, which include: 1) ischemia-reperfusion (IR), induced by clamping the renal artery or the pedicle unilaterally or bilaterally to introduce a period (30 – 60 min) of cold or warm ischemia, followed by reperfusion established for minutes to days before kidney harvesting; 2) toxins and sepsis models, induced by toxins such as endotoxin, bacterial or lipopolysaccharides (LPS) infusion and polymicrobial sepsis due to cecal ligation and puncture; and 3) chemicals and radiocontrast media, such as glycerol, gentamicin, cisplatin, NSAIDs, radiocontrast media and some other chemicals to induce metabolic reactions such as nephrotoxicity, apoptosis, necrosis and oxidative stress, leading to renal failure (Bellomo et al., 2004, Singh et al., 2012). Table 1.3 summarized the list of animal models for the study of acute kidney injury.

There are limitations associated with using animal models of AKI on human situations. For example: hypotension and shock may lead to hypoperfusion of the kidneys and result in ischemic tubular necrosis. However, hypotension in rats does not typically induce renal injury. On the contrary, renal IR in humans leads to subtle and focal histological changes, whereas in rats it may result in extensive necrosis of the proximal tubules (Steen Olsen et al., 1985, Yap and Lee, 2012). Despite all the challenges, animal models are often simple and reproducible. The major mechanisms and outcomes of AKI in the animals are similar and predictive for humans, which include increased injury-related biomarkers, up-regulated inflammatory immune responses,

elevated oxidative stress, etc (Heyman et al., 2009, Shanks et al., 2009). Furthermore, as the animal models allow us to investigate the organism as a whole, they can be used to demonstrate that AKI is not an isolated event and it can lead to multi-organ injury (Yap and Lee, 2012).

1.2.4.2 Implications for non-human subjects

Acute kidney injury also occurs in small and domestic animals and has been documented in cats and dogs due to a sudden and major insult in the kidneys. The major causes of AKI in the animals are nephrotoxins from the diets and environments, such as mycotoxins, ethylene glycol, heavy metals, aminoglycoside antibiotics, hemoglobinuria, melamine-cyanuric acid, lily plant, grapes or raisins, envenomation (e.g., snake, bee, wasp, bull ants) and NSAIDs; ischemia from disseminated intravascular coagulation or severe prolonged hypoperfusion; and infection such as pyelonephritis, leptospirosis and borreliosis (Ross, 2011). The pathophysiology and mechanisms of AKI in the animals are similar to that of humans, which include increased injury-related biomarkers, inflammation, oxidative stress and tissue necrosis. The use of laboratory animal models can also mimic the disease condition of the domestic animals and thus it can help develop effective treatment strategies for veterinary uses.

Table 1.3 List of animal models for the study of acute kidney injury (AKI)

Model	Mechanisms	Tubular injury	Medullary injury	Inflammation
Ischemia-reperfusion (IR)	Acute tubular necrosis with flattened epithelia and tubular dilation and cast formation. 45 min ischemia followed by 24 h reperfusion is more commonly used for the induction of AKI (Lieberthal and Levine, 1996, Singh et al., 2012)	+[1]	+	+
Toxins and sepsis				
Endotoxin	Nephrotoxicity			+
Bacterial infusion	Intravenous or intraperitoneal infusion of lipopolysaccharide (LPS).			+
Sepsis	Cecal ligation and puncture induced polymicrobial sepsis (Yang et al., 2009).			+
Chemicals and radiocontrast media				
Glycerol	Tubular nephrotoxicity caused by myoglobin, and resembles clinical rhabdomyolysis (Karam et al., 1995).	+		+
Gentamicin	Polycationic aminoglycoside gentamicin binds to acidic phospholipids, causing the production of metabolites. It also alters mitochondrial respiration (Mingeot-Leclercq and Tulkens, 1999, Wargo and Edwards, 2014).	±[2]		
Cisplatin	Direct tubular toxicity in the form of apoptosis and necrosis, reactive oxygen species (ROS) production, calcium overload, phospholipase activation,	+		+

	depletion of reduced glutathione, inhibition of mitochondrial respiratory chain function, and ATP depletion (Miller et al., 2010).			
NSAIDs[3]	Cause renal failure, induce oxidative stress (e.g. acetaminophen, and diclofenac sodium) (Singh et al., 2012).			
Other chemicals	Induce nephrotoxicity, oxidative stress, tubular epithelial injury (e.g. Uranium, Mercuric chloride ($HgCl_2$), Potassium dichromate, Ferric nitrilotriacetate, bipyridyls, etc.) (Singh et al., 2012).	+		+
Radiocontrast	Deleterious reduction of renal arteriolar blood flow and glomerular filtration, direct renal tubular toxicity, generation of free radicals, inflammatory mediators, alteration of anti-oxidant defense systems and development of apoptosis (Brown and Thompson, 2010).	+	+	+

[1]'+' Indicates the presence of a given feature; [2]'±' indicates only the partial presence of that feature; and the absence of any sign indicates the lack of such a feature; [3]NSAIDs, Non-steroidal anti-inflammatory drugs.

1.2.5 Kidney ischemia-reperfusion injury: causes and outcomes

Ischemia-reperfusion (IR) injury is caused by a sudden impairment of the blood flow to an area or organ, followed by a restoration of perfusion and re-oxygenation (Hesketh et al., 2014, Malek and Nematbakhsh, 2015). Renal IR injury may result in AKI and remote organ impairment in patients, leading to many clinical situations such as RRT, renal transplantation and sepsis. Renal IR injury often leads to high mortality and morbidity, and currently there is no specific treatment available (Hesketh et al., 2014).

IR injury is a pathological condition that is usually associated with both innate and adaptive immune responses, along with an oxidative stress response to hypoxia and reperfusion. Renal IR injury triggers a cascade of inflammatory responses including activation of chemokines and proinflammatory cytokines such as IL-6 and TNF-α, activation of Janus kinase/signal transducer, activation of the transcription (JAK/STAT) pathway, activation of complement systems and release of biologically active complements such as C4a, C3a, and C5a, increased adhesion molecule expression, and leukocyte infiltration (Patel et al., 2005, Yang et al., 2008, Malek and Nematbakhsh, 2015). The damaged tissue from IR injury produces excessive amounts of free radicals including ROS and reactive nitrogen species (RNS) through partial reduction of oxygen, lipid membrane peroxidation and suppressed mitochondrial oxidative phosphorylation. These products further induce oxidative stress, increase intracellular calcium level (calcium overload), impair ATP synthesis, diminish activity of cellular energy-dependent processes and cause lipid peroxidation, and eventually contributes to apoptosis and cell death (Bonventre, 1993, Lloberas et al., 2002). In the meantime, IR injury also decreases the production of endogenous antioxidant enzymes including catalase, superoxide dismutase (SOD), and glutathione peroxidase (Chatauret et al., 2014, Malek and Nematbakhsh, 2015). Hyperhomocysteinemia, reduced hydrogen sulfide

generation, increased oxidative stress, and elevated inflammation of the kidney have been previously reported by our lab using a rat IR model (Prathapasinghe et al., 2007, Xu et al., 2009, Wu et al., 2010, Wang et al., 2013, Wang et al., 2014)

1.3 Distant organ injury

Pathophysiological factors associated with AKI are also implicated in the failure of other organs. AKI is often part of a multiple organ failure syndrome, known as distant organ injury (Grams and Rabb, 2012, Lane et al., 2013, Druml, 2014, Dépret et al., 2017). Today, awareness and attention to multiple organ failure has been increased among clinicians. Animal models of AKI are shown to cause injury of multiple organs including brain, heart, intestine, lung, bone marrow and liver (Grams and Rabb, 2012, Druml, 2014, Ologunde et al., 2014). Among these organs, pulmonary and cardiovascular dysfunctions have been studied the most. Different pathways are associated with distant organ manifestations of AKI, which include induction of remote oxidative stress, systemic inflammation and apoptotic pathway activation (Kelly, 2003, Hassoun et al., 2007, Ologunde et al., 2014). However, mechanisms of AKI-induced distant organ injury are not well understood. The interaction between AKI and distant organ injury is summarized in Figure 1.3.

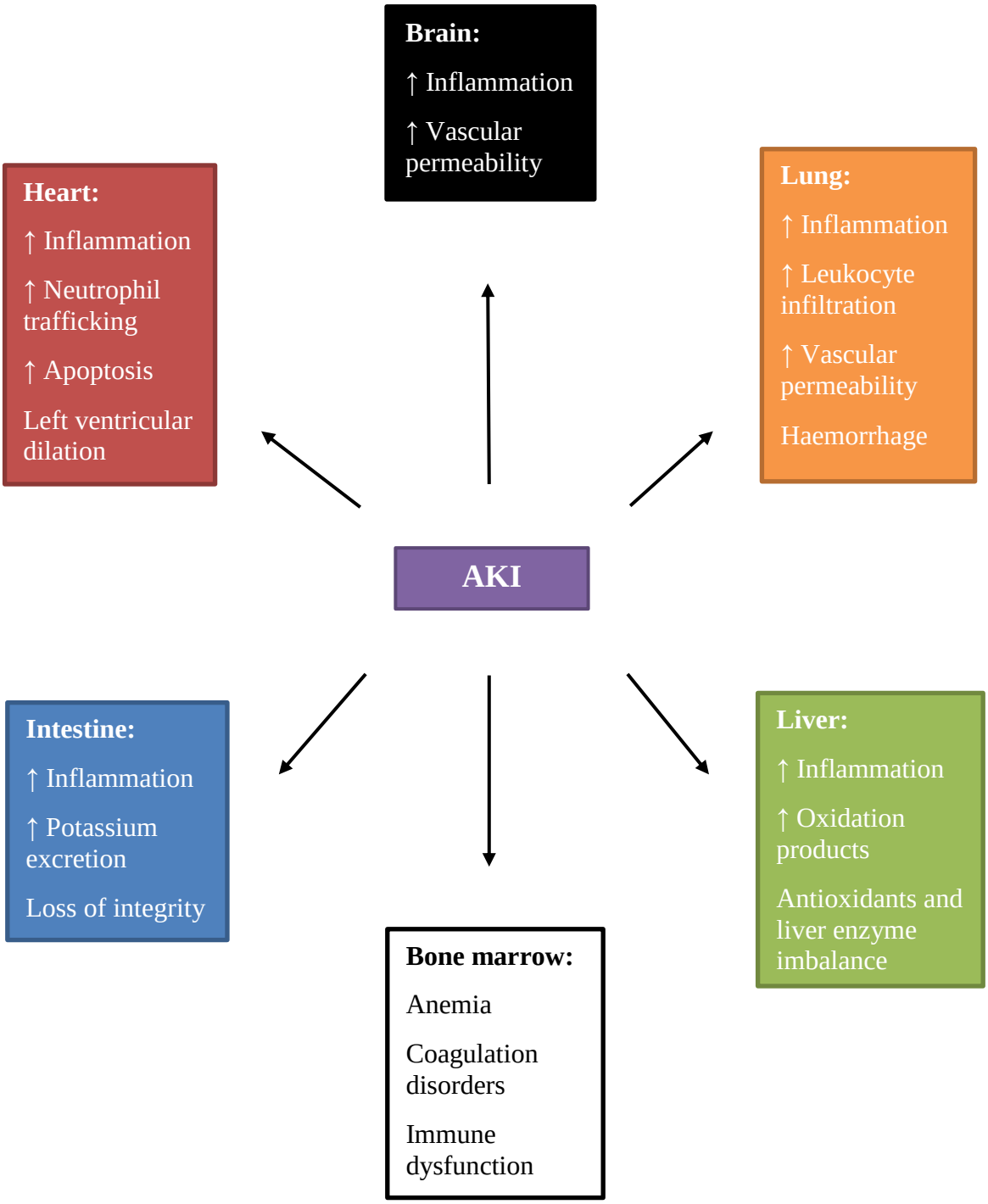

Figure 1.3 The impact of acute kidney injury (AKI) on distant organs

1.3.1 AKI and lung injury

The remote effects of AKI on pulmonary functions are characterized by increased vascular permeability, increased leukocyte infiltration, increased concentration of cytokines such as TNF-α and IL-6, down-regulation of the sodium-potassium pump and water channels, alveolar edema formation and tissue migration (Rabb et al., 2003, Yap and Lee, 2012, Druml, 2014). Histological changes such as enhanced pulmonary endothelial and epithelial cell apoptosis can also be found in the lungs after renal IR (Hassoun et al., 2009).

Animal models of sham surgery, unilateral IR, bilateral IR, and bilateral nephrectomy (BNx) have been used to study the distant effects of AKI on the lungs. In a rat model of bilateral renal IR injury, increased lung vascular permeability along with interstitial edema and alveolar hemorrhage were observed at 24 and 48 h post ischemia (Hoke et al., 2007). Another study in rats subjected to unilateral IR, bilateral IR and BNx surgery demonstrated that renal IR down-regulated pulmonary epithelial sodium channel (ENaC), Na, K-ATPase and aquaporin-5 (AQP-5), and increased pulmonary vascular permeability mediated in part by macrophage activation (Rabb et al., 2003). Pulmonary inflammation and disruption in salt and water balance are the direct results from AKI. The up-regulation of cytokines and circulating leukocytes are indicators of the pathogenesis of lung injury (Ologunde et al., 2014). Cytokines including IL-6, ICAM-1, IL-1β, IL-12, and G-CSF are shown to mediate lung injury in AKI through the activation of chemokines, adhesion molecules and neutrophils (Deng et al., 2004, Hoke et al., 2007, Ologunde et al., 2014). Both IR and BNx resulted in increased IL-6 and IL-1β with pulmonary vascular congestion and neutrophil infiltration (Kim et al., 2006, Hoke et al., 2007). Administration of IL-10, an anti-inflammatory cytokine, showed a protective effect by reducing inflammatory markers, such as proinflammatory cytokine productions, bronchoalveolar lavage fluid total protein,

pulmonary myeloperoxidase (MPO) activity, and chemokine macrophage inflammatory protein (Hoke et al., 2007). It also showed a positive effect for improving lung histology (Hoke et al., 2007). The kidneys play an important role in the elimination of systemic cytokines through proximal tubule regulation, and in the case of AKI, impairment of cytokine regulation may lead to increased levels of TNF-α and IL-6, which in turn, result in the reduction of pulmonary expression of AQP-1 and α-EnaC (Ma and Liu, 2013).

1.3.2 AKI and cardiovascular disease (CVD)

AKI and cardiovascular disease is often characterized as cardiorenal syndrome, meaning that the acute or chronic pathophysiological disorder of one organ may induce acute or chronic dysfunction in the other organ (Ronco et al., 2008). Cardiorenal syndromes type 1 characterizes that the acute dysfunction of the heart induces abrupt worsening of the renal function, whereas cardiorenal syndrome type 3 describes that the acute dysfunction of kidney worsens the heart function (Grams and Rabb, 2012, Doi and Rabb, 2016). For patients with congestive heart failure, around 20-30% will reach stage 1 AKI with increased mortality, and for those who have AKI, cardiac failure is a common cause of death (Damman et al., 2009, Grams and Rabb, 2012). Epidemiologic studies have also demonstrated that the risk of progressive chronic kidney disease (CKD) is increased after AKI, and thus CKD further contributes to cardiovascular diseases and mortality (Ishani et al., 2011, Bucaloiu et al., 2012, Doi and Rabb, 2016).

Several mechanisms are related to cardiac dysfunction after AKI, including increased preload secondary to AKI-induced salt and water retention, fluid overload contributing to pulmonary edema, myocardial damage due to neutrophil trafficking, arrhythmias due to hyperkalemia, myocyte apoptosis in response to oxidative stress, decreased myocardial contractility due to decreased influx of calcium, endothelial dysfunction, increased mitochondrial fragmentation and

elevated level of inflammatory cytokines (IL-1, IL-6, and TNF-α) from increased production and impaired clearance (Kelly, 2003, Ronco et al., 2008, Yap and Lee, 2012, Shiao et al., 2015, Dépret et al., 2017).

In a mouse model of AKI, increased mRNA level of immunoreactive TNF-α, IL-1 and ICAM-1 were observed along with increased MPO activity in the heart. Treatment with anti-TNF-α antibody attenuated cardiomyocytes apoptosis (Kelly, 2003). In addition, significantly increased left ventricular diastolic and systolic diameter and decreased fractional shortening were observed by echocardiography (Kelly, 2003). A bilateral renal IR injury in transgenic sickle mice showed marked cardiac vascular congestion and increased serum amyloid P-component, which is equivalent to c-reactive protein (CRP) in humans (Nath et al., 2005, Grams and Rabb, 2012). Heme oxygenase (HO)-$1^{-/-}$ knockout mice showed an increased level of IL-6 mRNA expression in the heart and lungs after AKI, and the level of renal dysfunction was reduced after applying IL-6 neutralizing antibody (Tracz et al., 2007). In contrast, mild renal IR injury has been found to have protective effects against myocardial ischemia. A 15 minute brief renal ischemia under hypothermic conditions decreased infarction size at the relative myocardial area subjected to coronary artery occlusion (Gho et al., 1996). These remote ischemic preconditioning effects on myocardial ischemic protection may be due to a systemic anti-inflammatory and anti-apoptotic response (Yap and Lee, 2012).

1.3.3 AKI and liver injury

1.3.3.1 The liver

The liver is the largest gland in the body, which lies in the upper right section of the abdominal cavity and under the diaphragm. The main role of the liver is to help maintain metabolic homeostasis, which includes: 1) removing and detoxifying poisonous substances; 2)

storing iron and fat-soluble vitamins A, D, E and K; 3) making plasma proteins from amino acids and produces urea after breaking down amino acids; 4) storing glucose as glycogen and break down of glycogen to maintain glucose concentration after or before meals, respectively; 5) regulating blood lipids levels (cholesterol, triglycerides, low-density lipoprotein (LDL), high-density lipoprotein (HDL), etc.); and 6) removing bilirubin, derived from the breakdown of hemoglobin and excreting it in the liver produced bile (Mader, 2003). Liver plays an important role in the detoxification of xenobiotics and the production of antioxidants against oxidative stress (Klaassen and Reisman, 2010). Impairment of liver function can be acute or chronic, involving a progressive destruction and regeneration of the liver parenchyma, and it can lead to fibrosis and cirrhosis. An increased ratio of the liver enzymes aspartate aminotransferase (AST) to alanine aminotransferase (ALT) has been used as an indicator of liver injury (Giannini et al., 2005).

1.3.3.2 Distant effects of AKI on the liver

The distant effects of AKI on the liver include altered level of liver enzymes, increased leukocyte influx, increased vascular permeability which leads to neutrophil and T-lymphocyte infiltration (Grams and Rabb, 2012, Lane et al., 2013, Ologunde et al., 2014), elevated inflammatory cytokine concentrations (such as TNF-α, IL-6 and IL-17A), increased apoptosis and markers of oxidative stress (Golab et al., 2009, Park et al., 2011, Lane et al., 2013), decreased levels of total glutathione (GSH) and increased malondialdehyde (MDA), an index of lipid peroxidation (Golab et al., 2009, Kadkhodaee et al., 2009). Pre-treatment with GSH (pentobarbital sodium) showed improved liver histology and a reduced level of MDA (Golab et al., 2009). AKI may also affect liver function by alterations of lipid metabolism (Lane et al., 2013). As both kidney and liver play critical roles in the detoxification of metabolites and

exogenous drugs, the remote effect of AKI can be further complicated in the setting of multiple organ failure, and thus contributes to higher mortality (Ologunde et al., 2014).

The effect of liver disease on kidney damage (hepatorenal syndrome) is well studied, however, knowledge of AKI on hepatic dysfunction is lacking. Few animal studies have investigated the effect of AKI on hepatic responses. A significant increase of serum and hepatic TNF-α level was observed after 6 and 24 h of renal IR (45 min of ischemia) in rats subjected to IR injury and BNx. The expression of liver ALT, as an indicator of hepatic injury was significantly increased 6 h after AKI. Hepatocyte apoptosis increased 24 h after nephrectomy. Early leucocyte infiltration and congestion, increased oxidative stress (MDA), and decreased GSH levels were also found (Golab et al., 2009). Shorter reperfusion time (45 min of ischemia followed by 60 min reperfusion) also caused a significant reduction of liver GSH and a significant increase of TNF-α and IL-10 concentrations (Kadkhodaee et al., 2009). Treatment with antioxidant and scavenger melatonin reduced hepatic inflammation and decreased the levels of oxidants and MDA (Fadillioglu et al., 2008). Similar results were observed in mice studies. IR injury elevated hepatic IL-6, chemokine (C-X-C motif) ligand (CXCL)-1, IL-1β, and TNF-α expression within 6 h of ischemic AKI (Andrés-Hernando et al., 2011). A minimum of 30 min ischemia and 1 h of reperfusion is enough to produce remote effects on the liver from renal IR injury (Serteser et al., 2002). Hepatic TNF-α and MPO activity as an indicator of neutrophil recruitment, are found to be significantly increased. Decreased levels of antioxidant enzymes and GSH, and increased levels of thiobarbituric acid reactive substances (TBARS) and protein carbonyls were indicators of oxidative stress upon 60 min of ischemia and 1 h of reperfusion injury (Serteser et al., 2002). IR injury and BNx in mice also increased ALT and bilirubin levels, elevated peri-portal hepatocyte necrosis, vacuolization, neutrophil infiltration, and up-regulated

the mRNA expressions of proinflammatory TNF-α, IL-6, and IL-17A (Park et al., 2011). Another study reported that increased accumulations of neutrophils and cytotoxic T cells were observed in both kidney and liver at 1 to 3 hours after renal ischemia (Miyazawa et al., 2002). A study in dogs with bilateral renal IR reported an impairment of hepatic oxidative drug metabolism along with elevated serum TNF-α and IL-6 levels (Gurley et al., 1997). In a recent swine renal IR study, the authors observed significant, short-term (24h reperfusion) increment of hepatic enzymes, as noted by increased AST: ALT ratio, an indicator of acute liver damage. However, no changes on histopathology, edema, apoptosis, or immune cell infiltration were observed in the short and long term (Gardner et al., 2016).

The studies listed above have examined the outcomes of AKI induced liver injury, however, the mechanisms of these distant effects are still unknown. It is essential to understand the regulations and mechanisms of AKI induced oxidative stress and inflammatory responses in the liver, in order to find therapeutic targets to ameliorate local and systemic injuries.

1.4 Oxidative stress

Oxidative stress is commonly defined as an imbalance between the production of free radicles including reactive oxygen species (ROS) as well as reactive nitrogen species (RNS), and the ability of the body to detoxify or to repair their harmful effects through antioxidant defense mechanisms. At low or moderate levels, ROS and RNS exert beneficial effects on cellular responses and immune function. On the contrary, at high concentrations, these free radicals induce oxidative stress, a deleterious process that can damage cell components, including nucleic acids, proteins, carbohydrates and lipids. Endogenous and exogenous antioxidants act as free radical scavengers by preventing and repairing damage caused by ROS and RNS, and therefore enhance the immune defense and lower the risk of degenerative diseases and cancer (Fang et al.,

2002, Halliwell, 2007, Pham-Huy et al., 2008, Lobo et al., 2010, Ray et al., 2012). However, when free radicals overwhelm the cellular antioxidant defense system through either an increase in the level of reactive species or a decrease in the cellular antioxidant capacity, oxidative stress occurs. The redox imbalance is demonstrated in Figure 1.4.

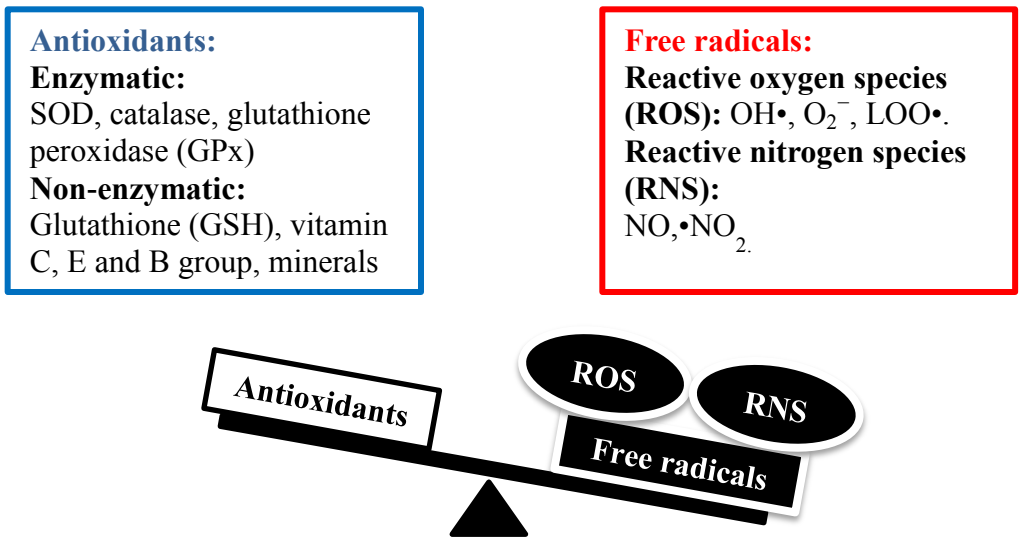

Figure 1.4 The redox imbalance

Oxidative stress occurs when there is an increase in the level of free radicals and/ or a decrease in cellular antioxidants. The antioxidants are categorized into enzymatic and non-enzymatic antioxidants. Some of the major enzymatic antioxidants include superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). Some of the non-enzymatic antioxidants such as glutathione (GSH) can be produced in the body, whereas other micronutrients such as the mineral and vitamin groups have to be supplied in the diet. Free radicals are categorized into reactive oxygen species (ROS) and reactive nitrogen species (RNS). Some highly reactive radicals are hydroxyl radical ($OH^{\bullet}$), superoxide anion (O_2^-), nitrogen oxide (NO), nitrogen dioxide ($^{\bullet}NO_2$) and lipid peroxyl radical ($LOO^{\bullet}$).

1.4.1 Reactive free radicals

Free radicals are molecules that have an unpaired electron in an atomic orbital (Fang et al., 2002, Lobo et al., 2010). Due to the presence of an unpaired electron, these molecules are unstable and highly reactive (Valko et al., 2007). ROS and RNS are collective terms for reactive chemical species that consist of radical and non-radical species formed by the partial reduction of oxygen (Uttara et al., 2009, Ray et al., 2012, El-Hosseiny et al., 2016). Some highly reactive radicals are hydroxyl radical ($OH^{\bullet}$), superoxide anion ($O_2^{\bullet-}$), nitrogen oxide (NO), nitrogen dioxide ($^{\bullet}NO_2$), nitric monoxide ($NO^{\bullet}$) and the lipid peroxyl radical ($LOO^{\bullet}$). Other non-radical species such as hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^{\bullet}O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), dinitrogen trioxide (N_2O_3), lipid hydroperoxide (LOOH) and peroxynitrite ($ONOO^-$) can be easily generated from free radicals (Gilgun-Sherki et al., 2001, Uttara et al., 2009, Ray et al., 2012). The reactive species are mainly generated endogenously as a result of aerobic metabolism such as mitochondrial oxidative phosphorylation, or they may arise from interactions with xenobiotic compounds, cytokines and bacterial invasion (Fang et al., 2002, Pham-Huy et al., 2008, Ray et al., 2012). Formation of ROS and RNS can occur in two ways: enzymatic and non-enzymatic reactions. Enzymatic reactions generate free radicals including those involved in the respiratory chain, phagocytosis, prostaglandin synthesis and the cytochrome P450 system (Halliwell, 1995, Genestra, 2007, Valko et al., 2007, Pham-Huy et al., 2008, Lobo et al., 2010). Non-enzymatic reactions occur during oxidative phosphorylation in the mitochondria as well as from non-enzymatic reactions of oxygen with organic compounds (Genestra, 2007, Pham-Huy et al., 2008). These reactive species that are generated attack important macromolecules leading to oxidative damage and homeostatic disruption (Lobo et al.,

2010). Lipids, nucleic acids, and proteins are the major targets of free radicals (Fang et al., 2002, Valko et al., 2007, Lobo et al., 2010).

1.4.2 Antioxidants: enzymatic and non-enzymatic

To defend against the generation of free radicals, antioxidants are produced to prevent the consumption of oxygen and to inhibit the oxidation of other molecules. Antioxidants are stable molecules that can donate an electron to a free radical and neutralize it, thus reducing oxidative damage (Halliwell, 1995, Lobo et al., 2010). These antioxidants are further categorized into enzymatic and non-enzymatic antioxidants. Some of the enzymatic antioxidants include superoxide dismutase (SOD), catalase, glutathione reductase, glutathione S-transferase and glutathione peroxidase (GPx) (Fang et al., 2002, Uttara et al., 2009). Some of the non-enzymatic antioxidants such as GSH, ubiquinol and uric acid can be produced during normal metabolism in the body, especially in the liver for detoxification, whereas other micronutrients such as the mineral and vitamin groups have to be supplied in the diet (Lobo et al., 2010). Both enzymatic and non-enzymatic antioxidants exist in the intra- and extra- cellular environment to scavenge free radicals.

1.4.2.1 Enzymatic antioxidants

The enzymatic antioxidants include superoxide dismutase (SOD), catalase and the glutathione systems (glutathione reductase, glutathione S-transferase and glutathione peroxidase).

Superoxide dismutase (SOD) catalyses the breakdown of superoxide anion (O_2^-) to oxygen (O_2) and hydrogen peroxide (H_2O_2). Based on the metal co-factors, SOD can be categorized into three families: 1) Cu/ Zn-SOD or SOD1, which binds with both copper and zinc, and are found in the cytoplasm; 2) Mn-SOD or SOD2, which binds with manganese, presents in the

mitochondria; and 3) SOD3, which exists as a copper and zinc-containing tetramer and is exclusively restricted to extracellular spaces (Zelko et al., 2002).

Catalase is a common enzyme that can catalyze the decomposition of hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2). Nearly all living organisms utilize catalase, and in animals, high catalase concentration is found in the liver (Gaetani et al., 1996).

Glutathione systems contain enzymes such as glutathione reductase, glutathione S-transferase and glutathione peroxidase (GPx). GSH reductase reduces GSSG back to GSH and facilitates the recycling of GSH. Glutathione S-transferase has high activity with lipid peroxidase and provides protection against lipid peroxidation (Nauseef, 2014). Glutathione peroxidase contains four selenium (Se) co-factors and it catalyses the breakdown of hydrogen peroxide (H_2O_2) and lipid hydroperoxides (LOOH) (Fang et al., 2002). Different types of GPx exhibit tissue specific functions (Marcus, 1957). These GSH related enzymes are at high levels in the liver for detoxification metabolism and play vital role in maintaining cellular redox status by counteracting oxidants and preventing oxidative damages.

1.4.2.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants can be synthesized endogenously or derived exogenously from dietary sources. The major non-enzymatic antioxidants include GSH, vitamins (vitamin C, E and B), minerals, lipids (omega-3) and phytochemicals.

GSH is one of the most important cellular antioxidants due to its central role and high abundance in regulating redox balance (Lobo et al., 2010, Shelton et al., 2013). It is a thiol-containing substance that can be *de novo* synthesized from glutamate, cysteine and glycine, or absorbed in the small intestine from the diet. The synthesis of GSH will be reviewed in section

1.5.1.2. GSH oxidation forms glutathione radical ($GS^{\bullet}$), a prooxidant radical, which can react with another $GS^{\bullet}$ to yield oxidized glutathione (GSSG). GSH regulates redox dependant cell signaling by modifying the oxidation state of protein cysteine residues, preventing oxidation of SH groups, and reducing disulfide bonds induced by oxidative stress (Lu, 1999). It directly scavenges ROS (e.g. hydroxyl radical, peroxynitrite, lipid peroxyl radical and singlet oxygen), and indirectly detoxifies hydrogen peroxide and lipid peroxides through enzymatic reactions. Furthermore, it can regenerate other antioxidants such as Vitamins C and E, back to their active forms, and can modulate critical cellular processes such as DNA synthesis and immune function (Fang et al., 2002).

Vitamins are able to directly scavenge free radicals and upregulate the activities of antioxidant enzymes (Fang et al., 2002). Among them, vitamin E (γ-tocopherol) and vitamin C (ascorbic acid) are two important antioxidants. Vitamin E inhibits ROS-induced lipid peroxyl generation and protects cells from the peroxidation of polyunsaturated fatty acids (PUFAs) in membrane phospholipids. A dietary deficiency of vitamin E reduces the activities of hepatic catalase, GPx, and glutathione reductase, induces liver lipid peroxidation, as well as causing neurologic and cardiovascular disorders (Chow et al., 1973, Fang et al., 2002). Vitamin C is a water-soluble vitamin that also exhibits protective effects against oxidative damage. Vitamin C is a reducing agent, it can work as an immunomodulator which has antioxidant, anti-atherogenic and anti-carcinogenic properties (Pham-Huy et al., 2008). Vitamin B groups such as vitamin B_6, B_9 (folic acid) and B_{12} are cofactors for methionine synthase (vitamin B_{12}), cystathionine-β-synthase (CBS) (vitamin B_6), cystathionine-γ-lyase (CSE) (vitamin B_6) and as a substrate (5-methyltetrahydrofolate) in homocysteine metabolism. These vitamins are also essential for the methylation of DNA and proteins (Fang et al., 2002, Sanchez-Moreno et al., 2009). Because

homocysteine contributes to oxidative stress, these vitamins help to reduce the risk of hyperhomocysteinemia induced oxidative stress, especially in cardiovascular diseases (Sanchez-Moreno et al., 2009).

Several minerals have been reported to play a role as antioxidants. Magnesium (Mg) is a cofactor for glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which catalyze the production of nicotinamide adenine dinucleotide phosphate reduced (NADPH) from NADP+. Magnesium deficiency reduces glutathione reductase activity and results in radical-induced protein oxidation and marked lesions in skeletal muscle, brain and kidneys (Rock et al., 1995, Fang et al., 2002). Copper (Cu), zinc (Zn) and manganese (Mn) are indispensable metals for Cu, Zn-SOD and Mn-SOD activities. Dietary deficiencies of these minerals result in peroxidative damage, mitochondrial dysfunction, lipid peroxidation and infection (Fang et al., 2002). Selenium (Se) has been identified as an essential cofactor for selenoproteins, which are important for selenium-dependant enzymes such as glutathione peroxidase. A dietary deficiency of Se decreases tissue glutathione peroxidase activity by 90% and results in peroxidative damage and mitochondrial dysfunction (Xia et al., 1985). It also exhibits antioxidant, anti-carcinogenic and immunomodulation functions (Fang et al., 2002, Pham-Huy et al., 2008).

Fish oil contains omega-3 and omega-6 PUFAs, and it has been reported to have beneficial effects towards reducing CVD risk in humans. This effect is partially due to an inhibition of lipogenesis and reduction of fatty acid β-oxidation in the liver (Fang et al., 2002). However, like other PUFAs, fish oil can be peroxidised to form hydroperoxides and result in oxidation. Although no adverse effect has been reported clinically, the oxidized lipids may interfere with the intended biological benefits (Mason and Sherratt, 2017). Therefore, it is necessary to monitor the oxidation level of the fish oil (Cameron-Smith et al., 2015). Furthermore, it is also important

to maintain an appropriate balance of omega-3 and omega-6 in the diet. For humans, a healthy diet should consist of 2-4 times more omega-6 fatty acids than omega-3 fatty acids (Pham-Huy et al., 2008). Omega-3 fatty acids are inhibitors for free radical generation and inflammation. They can upregulate the expressions of antioxidant and lipid catabolism genes in the liver, and at the same time inhibit inducible nitric oxide synthase (iNOS) expression and iNOS synthesis by cytokine-activated macrophages (Ohata et al., 1997, Takahashi et al., 2002). Omega-6 fatty acids can induce inflammation but with appropriate balance it can improve diabetic neuropathy, rheumatoid arthritis and aid in cancer treatment (Pham-Huy et al., 2008).

Dietary phytochemical antioxidants from plant sources are rich in phenolic and polyphenolic compounds, such as flavonoids and catechin, which are capable of scavenging free radicals (Fang et al., 2002). Flavonoids have potent antioxidant activity that can prevent or delay several chronic or degenerative diseases such as cancer, CVD, arthritis, aging, and Alzheimer's disease. The main natural sources of flavonoids include green tea, grapes (red wine), cocoa (chocolate), onion and broccoli (Pham-Huy et al., 2008). Carotenoids, such as β-carotene and lycopene from plant algae and bacterial sources also have antioxidant properties. Beta-carotene can react with peroxyl radicals to form a stabilized carbon-centered radical within its conjugated alkyl structure, thereby inhibiting the chain propagation effect of ROS (Fang et al., 2002). Lycopene possesses antioxidant and antiproliferative properties, and was found to be protective towards prostate cancer (Pham-Huy et al., 2008).

1.4.3 Regulation of redox balance

Redox reaction is an oxidation-reduction reaction, in which one reactant is oxidized and one reactant is reduced simultaneously. Redox balance is disturbed by either an increase in free radicals or a decrease in the activity of antioxidant systems (Valko et al., 2007). The production

of reactive free radicals creates an imbalance in the redox reaction. Antioxidants can add an appropriate number of water molecules to balance the oxygen atoms, add H^+ ions to balance hydrogen atoms, and add enough electrons (e^-) to the more positive side to make the charge equal. This method in organic chemistry is referred as the half equation method (Marcus, 1957). There are three levels of defence mechanisms against free radical induced oxidative stress, which are: 1) preventive mechanisms, to suppress the formation of free radicals such as decomposition of hydroperoxides and hydrogen peroxide; 2) scavenge mechanisms, to suppress the chain initiation or break the chain propagation reactions by scavenging radicals; and 3) repair mechanisms, to remove oxidatively modified proteins and prevent the accumulation of oxidized proteins (Cadenas, 1997, Valko et al., 2007, Lobo et al., 2010).

The production and the scavenging of free radicals is illustrated in Figure 1.5. In brief, ① nitric oxide (NO) is formed from L-arginine by one of the three NO synthase (NOS) isoforms: nNOS, iNOS or eNOS. ② The superoxide anion radical (O_2^-) is generated from O_2 by multiple pathways including NADPH oxidation by NADPH oxidase, xanthine oxidation by xanthine oxidase, autoxidation of monoamines and one-electron reduction of O_2 by cytochrome P-450 or NOS. ③ Further, Superoxide dismutase (SOD) converts O_2^- to hydrogen peroxide (H_2O_2), which is then reduced to H_2O by either glutathione peroxidase (GPx) with GSH as the electron donor or catalase. ④ The oxidized GSSG is reduced back to GSH by glutathione reductase, which uses NADPH as an electron donor. ⑤ Hydrogen peroxide breaks down to reactive hydroxyl radicals ($OH^\bullet$) by the Fenton reaction with transition metals (e.g. Fe^{2+}, Cu^+). When both O_2^- and NO are synthesized close to each other, ⑥ NO can react with O_2^- or H_2O_2 to form peroxynitrite ($ONOO^-$). ⑦ The free radicals $OH^\bullet$ and $ONOO^-$ can abstract an electron from polyunsaturated fatty acid (LH) to form a carbon-centred lipid radical ($L^\bullet$). ⑧ $L^\bullet$ further

interacts with O_2 to generate a lipid peroxyl radical ($LOO^{\bullet}$). ⑨ $LOO^{\bullet}$ abstracts a hydrogen atom from nearby fatty acids to form a lipid hydroperoxide (LOOH), LOOH decomposes to yield a alkoxyl radical ($LO^{\bullet}$), thus propagates lipid peroxidation chain reaction. ⑩ $LOO^{\bullet}$ can also be reduced to LOOH by vitamin E with vitamin C or GSH as reducing agents, and LOOH is then reduced to dioxygen and alcohol (LOH) by GPx with GSH as the electron donor (Fang et al., 2002, Valko et al., 2007). Specific mechanisms of oxidative damage of nucleic acids, lipids and protein are reviewed in the following section 1.4.4.

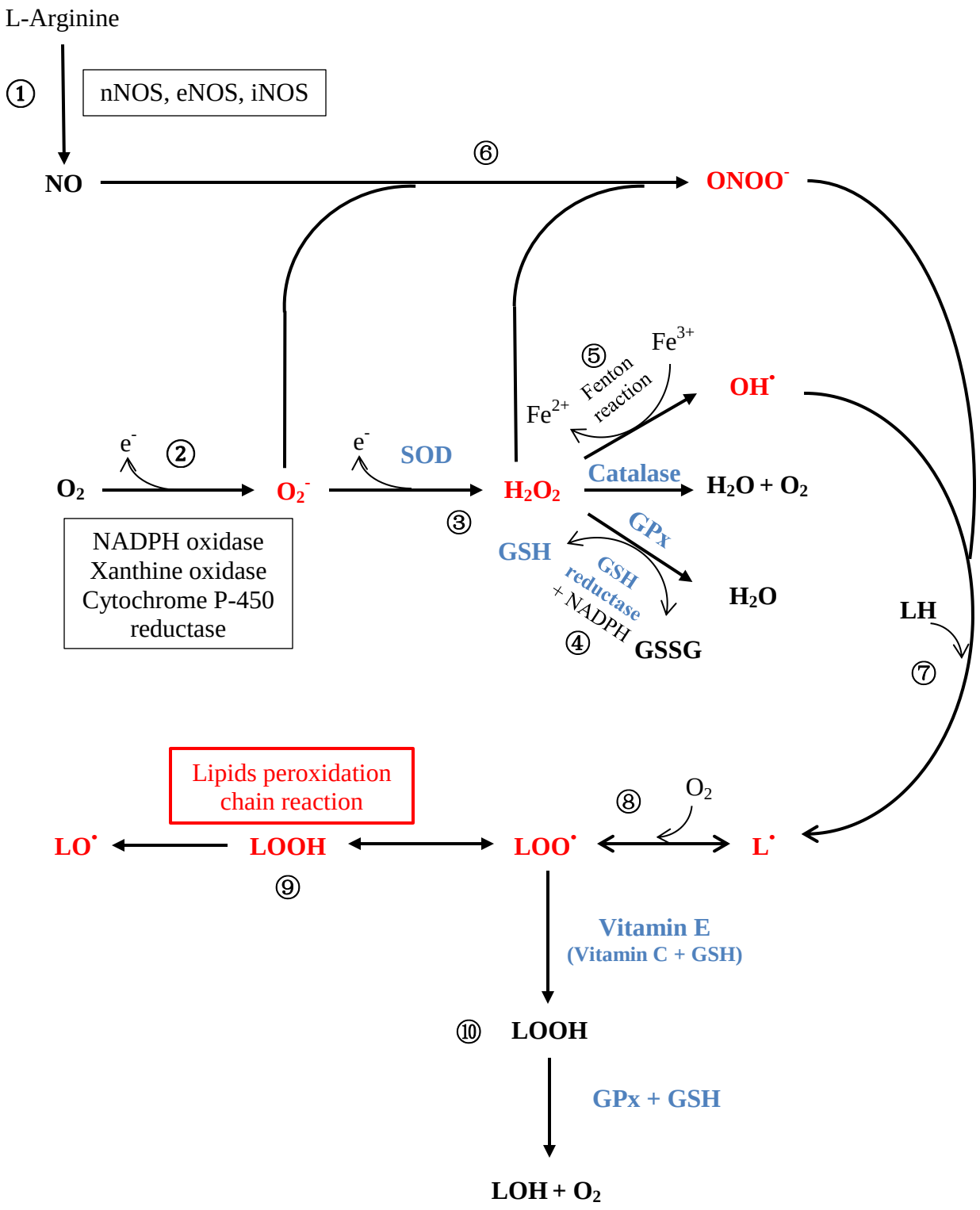

Figure 1.5 The production and the scavenging of free radicals.

1.4.4 Biomarkers of oxidative stress

Oxidative stress can be measured through three approaches: 1) determination of endogenous antioxidant levels by examining the concentrations of antioxidants (e.g. GSH, vitamin C, vitamin E, carotenoids and minerals), antioxidant enzyme activities (e.g. SOD, catalase, GPx, GSH reductase), and/or measurement of GSH to GSSG ratio; 2) direct detection of free radicals by electron spin resonance technology; and 3) measurement of oxidized products, which include the three major targets of the free radicals: nucleic acids, lipids and protein (Fang et al., 2002). The mechanisms of oxidative damage and biomarkers of these molecules are reviewed in the following sections.

1.4.4.1 Nucleic acids

Many studies have provided evidence that DNA and RNA are susceptible to oxidative damage. DNA oxidation occurs most readily at guanine residues due to a high oxidation potential of its base compared to cytosine, thymine or adenine. It has been reported that DNA, in particular mitochondrial DNA, is a major target in the case of aging and cancer (Valko et al., 2004, Genestra, 2007). Oxidative damage to DNA may promote microsatellite instability, inhibit methylation and accelerate telomere shortening (Lobo et al., 2010). Oxidative DNA damage also produces a series of modifications in the DNA structure including base and sugar lesions, strand breaks, DNA-protein cross-links and base-free sites (Pham-Huy et al., 2008). Oxidative nucleotides such as 8-hydroxyguanine, 8-hydroxydeoxyguanosine (8-oxodG) and 8-hydroxyguanosine (8-oxoG) are increased during oxidative damage to DNA and RNA under UV radiation or free radical exposure. It has been suggested that 8-oxodG can be used as a biological marker for oxidative stress and it is frequently associated with carcinogenesis and disease (Hattori et al., 1996, Valavanidis et al., 2013).

1.4.4.2 Lipids

Lipid peroxidation refers to the oxidative degradation of lipids. It features a free radical chain mechanism, by which a free carbon-centred lipid radical ($L^{\bullet}$) reacts rapidly with molecular oxygen, creating a peroxyl-fatty acid radical ($LOO^{\bullet}$). This radical extracts a hydrogen atom from a nearby unsaturated fatty acid producing a lipid hydroperoxide (LOOH) and a new fatty acid radical. The LOOH decomposes to form a lipid alkoxyl radical ($LO^{\bullet}$). The continuous production of lipid peroxyl and alkoxyl radicals propagates the lipid peroxidation chain reaction (Fang et al., 2002). Similar to the other reactions, lipid peroxidation consists of three major steps: initiation, propagation, and termination. The radical reaction terminates when two radicals react and produce a non-radical species, only when the concentration of these radical species is high enough to have a probability of collision. Human or animal bodies can utilize antioxidants to speed up the termination process by neutralizing free radicals and, therefore, protecting the cell membrane. Polyunsaturated fatty acids (PUFA), which are located in the cell membranes, are mostly affected by a radical chain reaction, because they contain multiple double bonds and have methylene bridges ($-CH_2-$) that possess highly reactive hydrogen atoms. During lipid peroxidation, compounds such as malondialdehyde (MDA), and isoprostanes are formed. These compounds can be used as markers in a lipid peroxidation assay and have been verified as present in many diseases such as neurogenerative diseases, ischemic reperfusion injury, and diabetes (Fang et al., 2002, Lobo et al., 2010). In addition, oxidized LDL has been found to be a predictive biomarker for coronary artery disease (CAD) (Meisinger et al., 2005).

1.4.4.3 Proteins

Protein oxidation is defined as the covalent modification of a protein induced either by the direct reactions with free radicals or indirect reactions with secondary by-products of oxidative

stress (Zhang et al., 2013). Proteins oxidation can occur in three ways: 1) oxidative modification of specific amino acid; 2) free radical mediated peptide cleavage; and 3) formation of protein cross-linkages due to a reaction with lipid peroxidation products (Berlett and Stadtman, 1997, Stadtman and Berlett, 1998, Zhang et al., 2013). Peroxyl radicals are one of the free radical species produced from the oxidation of proteins (Lobo et al., 2010). Although all amino acids can be modified by free radicals, cysteine and methionine contain sulfur groups that are the most susceptible to oxidative damage. Oxidative modifications of proteins can change their physical and chemical properties, including conformation, structure, solubility, heat stability, proteolysis susceptibility, signal transduction function and enzyme activities (Lobo et al., 2010, Zhang et al., 2013). For assessing protein oxidation, the production of protein carbonyls (loss of free thiol groups in proteins) and nitrotyrosine (nitration of protein-bound tyrosine residues) are commonly used as stable markers (Fang et al., 2002).

1.4.5 Oxidative stress in renal IR and distant organ injury

Oxidative stress often results in oxidative injuries in both local and distant organs. Ischemia-reperfusion injury can introduce massive amounts of ROS back to the organ during reperfusion, which in turn can lead to tissue injury and causing serious complications in organ transplantation, stroke, and myocardial infarction (Kasparova et al., 2005, Valko et al., 2007). During ischemia, anaerobic glycolysis produces acidosis which leads to H^+ accumulation and a decrease in intracellular pH. Once perfusion is restored, H^+ is transported extracellularly through Na^+/H^+ exchanger in the exchange of Na^+ to normalize the pH. Ischemic damage changes the electrolytes homeostasis by causing dysfunction of the Na^+/ K^+ pump that is responsible for Na^+ entry and down-regulating the enzymatic function of Na^+/K^+-ATPase which has Na^+-regulating capacity through its activation/ deactivation, resulting in an increased intracellular Na^+ concentration

(Fekete et al., 2004). The increased intracellular Na^+ in turn activates the Na^+/Ca^{2+} exchanger, resulting in exchange of intracellular Na^+ with extracellular Ca^{2+}, and eventually leading to calcium (Ca^{2+}) overload (Frank et al., 2012). Calcium overload further induces mitochondrial dysfunction and hypercontracture eventually leading to cell death (Garcia-Ruiz and Fernandez-Checa, 2006). Increased ATP consumption leads to accumulation of purine catabolites hypoxanthine and xanthine, which are later metabolized by xanthine oxidase to produce superoxide radicals, peroxynitrite and hydrogen peroxide upon reperfusion with oxygen (Valko et al., 2007, Malek and Nematbakhsh, 2015). With the restoration of blood flow, reperfusion of the ischemic tissue leads to a cascade of oxidative damage. The following events of lipid peroxidation and ROS production result in tubular cell damage, endothelial cell damage and impaired endothelial function, eventually leading to DNA and protein damage and apoptosis (Bonventre, 1993, Gloire et al., 2006, Malek and Nematbakhsh, 2015). At the same time, down-regulation of the antioxidant enzyme system could also contribute to the pathophysiology of renal IR injury (Wang et al., 2014, Malek and Nematbakhsh, 2015). Hyperhomocysteinemia, a condition of elevated homocysteine (Hcy) level along with reduced activity of cystathionine-β-synthase (CBS), a key enzyme for Hcy metabolism has been previously reported by our lab to show detrimental effects in renal IR injury due to oxidative stress (Prathapasinghe et al., 2007). The severity of oxidative stress is closely associated with inflammation and apoptosis. Renal IR injury down-regulated the transsulfuration pathway thus leading to a decrease in endogenous hydrogen sulfide and GSH production. These reductions increased proinflammatory cytokine expressions in the kidney and tubular cells (Wang et al., 2014). In addition, an alteration of transcriptional factors AP-1, SP-1, NF-κB and Nrf2 has been reported to induce oxidative stress

in renal ischemia reperfusion injury (Sung et al., 2002, Gloire et al., 2006, Wu et al., 2010, Shelton et al., 2013).

Limited studies have reported oxidative stress in distant organs upon renal IR injury. As reviewed in section 1.3.3.2, decreased level of antioxidants such as total GSH, increased levels of MDA as an indicator of lipid peroxidation, increased protein carbonyls and increased oxidants were reported in the liver (Serteser et al., 2002, Golab et al., 2009, Kadkhodaee et al., 2009). Treatments with the antioxidants GSH and melatonin have been shown to be effective in decreasing the levels of oxidants and MDA in the liver (Fadillioglu et al., 2008, Golab et al., 2009).

1.5 Regulation of glutathione homeostasis

1.5.1 Role of transsulfuration pathway

1.5.1.1 Transsulfuration pathway

The transsulfuration pathway is a metabolic pathway which is involved in the conversion of homocysteine to cysteine, through cystathionine. It is the only source of *de novo* synthesized cysteine in mammalian cells and it allows the utilization of methionine for GSH synthesis (Mosharov et al., 2000, McBean, 2012, Lu, 2013). Cysteine is an essential precursor for GSH biosynthesis. Hcy can be converted to cysteine through a two-enzyme process. Cystathionine-β-synthase (CBS, EC 4.2.1.22) catalyzes the initial reaction with pyridoxal-5'-phosphate (PLP, activated form of vitamin B_6) by condensing homocysteine with serine to form cystathionine. Cystathionine is subsequently metabolized to cysteine by another enzyme cystathionine-γ-lyase (CSE, EC 4.4.1.1). In the transsulfuration pathway, CBS is the rate-limiting enzyme, while CSE is the most abundant enzyme that is particularly active in hepatocytes (Lu, 2013). CBS and CSE are also responsible for hydrogen sulfide (H_2S), a gasotransmitter generation through

desulfurization reactions (Hourihan et al., 2013, Wang et al., 2014). A schematic illustration of the transsulfuration pathway, GSH biosynthesis and desulfurization reaction is shown in Figure 1.6.

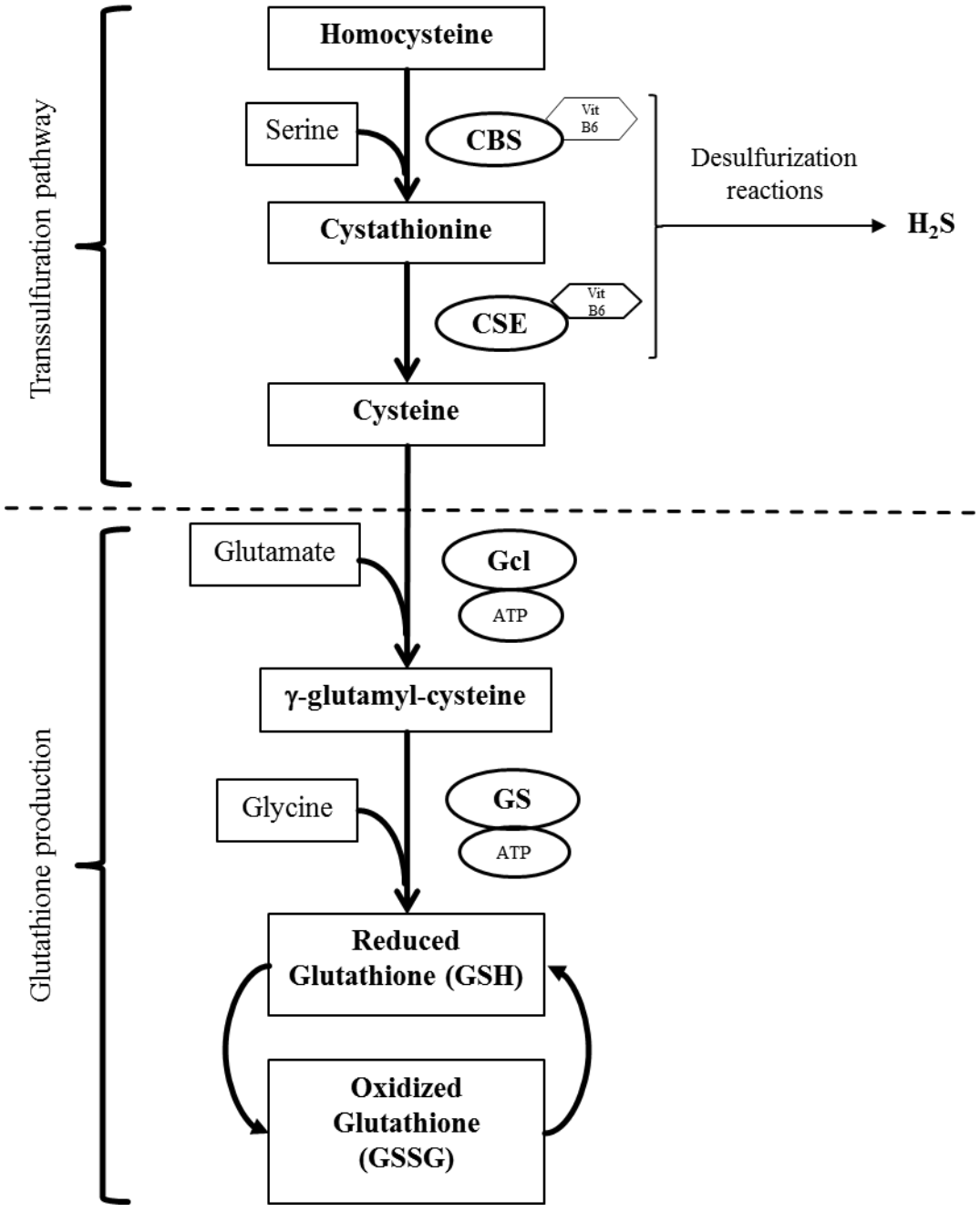

Figure 1.6 Transsulfuration pathway and glutathione production

(CBS: cystathionine β-synthase, CSE: cystathionine γ-lyase, H_2S: hydrogen sulfide, Gcl: glutamine-cysteine ligase, GS: glutathione synthase)

1.5.1.2 Glutathione biosynthesis

Glutathione, γ-L-glutamyl-L-cysteinylglycine, is a thiol-containing tripeptide that defends against both physiologically and pathologically generated oxidative stress (Garcia-Ruiz and Fernandez-Checa, 2006). GSH is also the key and most abundant endogenous non-enzymatic antioxidant produced in the body (DeLeve and Kaplowitz, 1991, Lu, 1999). The liver has high GSH levels due to its efficiency in synthesis (Lu, 1999, Lu, 2013), and about 50% of cysteine in GSH is derived from methionine via transsulfuration (Vitvitsky et al., 2004).

GSH is synthesized by two ATP-requiring enzymes: glutamate-cysteine ligase (Gcl, EC 6.3.2.2) and glutathione synthase (GS, EC 6.3.2.3) (Lu, 2013). As shown in Figure 1.6, Gcl catalyzes the rate-limiting reaction by converting cysteine and glutamate to γ-glutamyl-cysteine, whereas GS catalyzes the reaction of γ-glutamyl-cysteine and glycine to form GSH. Gcl is composed of two subunits: a heavy catalytic subunit Gclc, which has full catalytic capacity and can be inhibited by GSH feedback mechanism, and a light modifier subunit Gclm, which is enzymatically inactive but is important in the regulation of Gclc and GSH (Huang et al., 1993). GS is composed of two identical subunits, and is important in the determination of overall GSH synthetic capacity, free from feedback inhibition by GSH (Lu, 2013). Under normal physiological conditions, more than 90% of the cellular GSH pool is in the reduced form (GSH) and less than 10% is in the oxidized (disulfide) form (GSSG). About 80-85% of the cellular GSH is in the cytosol, 10-15% is in the mitochondria and the rest is in the endoplasmic reticulum. The GSH structure is unique due to the fact that the glutamate and cysteine are bond through γ-carboxyl group, and the only enzyme that can hydrolyze this bond is γ-glutamyltranspeptidase (GGT), which makes GSH resistant to intracellular degradation and can only be metabolized extracellularly by cell surface enzyme GGT (Lu, 1999, Lu, 2013).

1.5.2 Transcriptional regulation of glutathione production: role of Nrf2

1.5.2.1 Regulation of Nrf2

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor involved in cellular responses against oxidative stress (Kim and Vaziri, 2010, Kim et al., 2010, Wakabayashi et al., 2010). Under normal physiological conditions, Nrf2 is kept in the cytoplasm by binding to the repressor protein Kelch-like ECH-associated protein 1 (Keap1), which targets Nrf2 for proteasomal degradation (Kensler et al., 2007). Upon stress stimulation, Nrf2 is dissociated from Keap1 and translocates into the nucleus where it binds to the promoter regions of target genes (Kim and Vaziri, 2010, Kobayashi et al., 2013, Kudoh et al., 2014, Niture et al., 2014). Nrf2 forms heterodimers with small Maf (MafG, MafK and MafF) and Jun (c-Jun, Jun-D, and Jun-B) proteins to bind to antioxidant response element (ARE)), and activates antioxidant and detoxifying gene production (Lu, 2013). The Keap1-Nrf2 signaling pathway is one of the most important defense and survival pathways on fundamental cellular process such as proliferation, apoptosis, angiogenesis and metastasis. It provides protective effects and adaption strategies against various stress conditions including specific diseases such as cancer and drug-induced toxicities, making it a major therapeutic target (Baird and Dinkova-Kostova, 2011, Copple, 2012, Jaramillo and Zhang, 2013, O'Connell and Hayes, 2015). A demonstration of the Keap1-Nrf2 stress response system is shown in Figure 1.7.

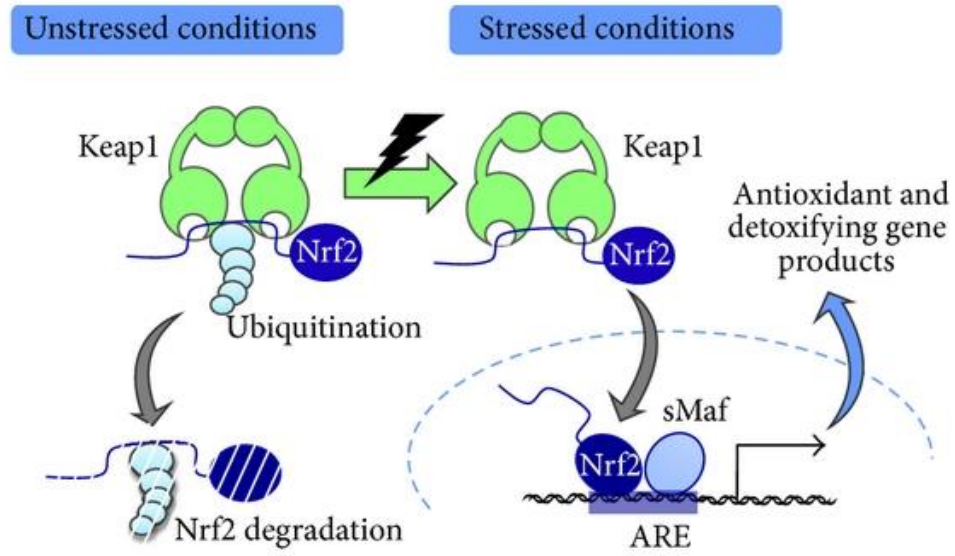

Figure 1.7 Keap1 and Nrf2 stress response system.

Under unstressed conditions, Nrf2 is bond to a repressor protein Kelch-like ECH-associated protein 1 (Keap1), which targets Nrf2 for ubiquitination and degradation. Upon stress stimulation, Nrf2 is dissociated from Keap1 and translocated into the nucleus where it forms heterodimers with small Maf (sMaf) and then binds to the antioxidant response element (ARE), activating antioxidant and detoxifying gene production. (Adapted from Kobayashi et al. (2013), open access and subject to the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction)

1.5.2.2 The function of Nrf2

Activation of Nrf2 induces gene expression of cytoprotective enzymes including those that are responsible for GSH synthesis, such as CSE, Gcl and Gpx, and those that detoxify electrophiles and ROS, such as heme oxygenase-1 (Ho-1), glutathione-S-transferases and NAD(P)H:quinone oxidoreductase 1 (Nqo1) (Lee and Johnson, 2004, Reisman et al., 2009, Kim and Vaziri, 2010, Kim et al., 2010, Wakabayashi et al., 2010). The cytoprotective function of Nrf2 includes: 1) it directly activates antioxidants production; 2) it activates enzymes that can directly inactivate electrophiles and oxidants; 3) it increases GSH synthesis and regeneration; 4) it stimulates NADPH synthesis; 5) it assists in toxin export through multi-drug response transporters; 6) it improves the recognition, repair and removal of damaged nucleotides and proteins; 7) it regulates other transcription factors, growth factors and receptors; and 8) it inhibits cytokine-mediated inflammation (Wakabayashi et al., 2010, Baird and Dinkova-Kostova, 2011, Copple, 2012). Deficiency of Nrf2 leads to increased oxidative stress, renal and hepatic damage and inflammatory responses. Furthermore, Nrf2 knockout mice exhibited lower GSH levels and are more susceptible to liver injury (Chan et al., 2001, Shelton et al., 2013, Kudoh et al., 2014).

1.5.2.3 Nrf2 in renal IR injury

Nrf2 has an important role for the protection of kidney against oxidative damage upon renal IR injury. It has been reported that hyperactivation of Nrf2 in the early phase of renal IR injury prevents the progression of ROS-mediated tubular damage by inducing antioxidant enzymes and NADPH synthesis (Nezu et al., 2017). Up-regulation of Nrf2 during reoxygenation mediates cytoprotective gene expression in IR injury, whereas applying antioxidant N-acetyl-cysteine results in inhibition of Nrf2 activation (Leonard et al., 2006). Nrf2 is down-regulated in longer reperfusion times (45min ischemia and 24h reperfusion) in mice kidney IR (Ryan and Majno,

1977). Nrf2 deficiency increases susceptibility to both ischemic and nephrotoxic AKI and the severity of renal IR injury is exacerbated by the loss of Nrf2 (Liu et al., 2009). Treatment with the antioxidants N-acetyl-cysteine or GSH to Nrf2 knockout mice, or pre-treatment with a small molecular weight Nrf2 inducer to wild-type animals showed an improvement in their renal function (Yoon et al., 2008, Liu et al., 2009, Wu et al., 2011, Shelton et al., 2013). The Nrf2 inducer methyl-2-cyano-3,12-dioxooleano-1,9-dien-28-oate (CDDO-Me, also known as bardoxolone methyl) given after 24h of renal IR, up-regulated Nrf2 activity and altered 2561 transcripts and 240 proteins in the kidneys of Nrf2 knockout mice, making Nrf2 a potential therapeutic target (Noel et al., 2015, Shelton et al., 2015). However, the mechanism for the protective effect of Nrf2 on distant organ injury is not well understood.

1.6 Ischemia-reperfusion and inflammatory response

1.6.1 Inflammatory response

1.6.1.1 Acute and chronic inflammation

Inflammation is a body's response to immune stimulus. It is a reaction of the microcirculation characterized by the movement of fluid and cells from blood into the extravascular compartment (Abbas and Lichtman, 2012). The inflammatory response can be caused by different stimuli including pathogens such as bacteria, virus, parasites and fungus, injuries caused by foreign objects, toxins, chemicals or radiation, or disease conditions such as bronchitis, dermatitis and cystitis (Mader, 2003). The damaged cells release chemicals including proinflammatory cytokines, histamine and prostaglandins, which can cause swelling through fluid leakage from the blood vessels into the tissues.

The inflammatory response can be categorized into two groups: acute and chronic inflammation. Acute inflammation is a short response to injury or infection, which occurs within

seconds to minutes. The duration of acute inflammation is also relatively short, ranging from hours to days. The main processes include: increased blood flow and edema, increased permeability of capillaries, accumulation of fluid and protein, activation of platelets and migration of leukocytes, predominantly neutrophils. Chronic inflammation, on the other hand, is not a part of the body's natural healing process. It is often caused by persistent infections, prolonged exposure to foreign objects, toxic agents or autoimmunity (Abbas and Lichtman, 2012). Chronic inflammation is of a longer duration compared with acute inflammation. It may last from weeks to months. Active inflammation, tissue destruction and repair are proceeded simultaneously during chronic inflammation. The presence of lymphocytes, macrophages, plasma cells, granulation tissue, proliferation of blood vessels and fibrosis are associated with the chronic inflammation (Abbas and Lichtman, 2012).

1.6.1.2 Innate and adaptive immune response

There are two types of immune responses in the immune system: innate and adaptive immune responses. The innate immune response exists before infection and plays an important role as the first line of the defense. It reacts fast (within days) but non-specifically to the foreign antigens. The innate immunity is composed of: 1) barriers such as epithelial layers (skin), defensins and intraepithelial lymphocytes; 2) effector cells including neutrophils, macrophages, natural killer (NK) cells, innate lymphoid cells and platelets; 3) cytokines and chemokines; and 4) effector proteins such as complements, C-reactive protein and mannose-binding lectin. The mechanisms of the innate immune response include phagocytosis by macrophages, dendritic cells and neutrophils, cytotoxicity by NK cells and cell mediated production of cytokines (Ryan and Majno, 1977, Abbas and Lichtman, 2012). In addition, innate immunity activates adaptive immunity in which innate immune cells participate at both priming and effector phases of the

adaptive immunity. It also generates molecules such as cytokines which act as secondary signals for T and B cell activation (Abbas and Lichtman, 2012). The adaptive immune response, on the other hand, is a specific defense system mediated by B and T lymphocytes after exposure to foreign antigens. It lasts for a longer duration (within months or years) and exhibits high specificity and memory in response to recurrent and persistent infections. Types of adaptive immune responses include humoral immunity, where B cells produce antibodies, and cell-mediated immunity, where T cells directly eliminate antigens through cytotoxicity or indirectly through cytokines (Abbas and Lichtman, 2012). Acute kidney injury, as an example, involves in acute inflammation and innate immune response, however if AKI progresses to CKD, the chronic inflammation and adaptive immune responses will act as the main defensive mechanisms.

1.6.2 Regulation of inflammatory response

1.6.2.1 Mediators in inflammation

Many biochemical mediators are released during inflammation. These mediators include cytokines, chemokines, complement, complement derived peptides (C3a, C3b and C5a), kinins, histamine, serotonin, lipid mediators (leukotrienes and prostaglandins), matrix metalloproteases, endothelial-leukocyte adhesion molecules and free radical gas nitric oxide (NO) (Ryan and Majno, 1977). Inflammatory cytokines play important roles in physiological and pathological processes and are reviewed in this section.

Cytokines, including interleukins 1-37 (IL 1-37), tumor necrosis factor-α (TNF-α), and interferon-γ (INF-γ) are produced predominantly by macrophages and lymphocytes (Molls et al., 2006). They are important in mediating the innate immune response. These cytokines are further categorized to proinflammatory and anti-inflammatory cytokines. Major proinflammatory cytokines include IL-1, IL-6 and TNF-α. In acute inflammation, they promote cytotoxicity by

mobilizing and activating leukocytes, enhancing B and T cells proliferation, and are involved in the biologic response to endotoxins. In chronic inflammation, excessive production of inflammatory cytokines contributes to inflammatory diseases such as atherosclerosis and cancer. In addition, these cytokines can activate fibroblasts and osteoblasts, which further cause cartilage and bone resorption (Ryan and Majno, 1977). Major anti-inflammatory cytokines include IL-4, IL-10, and IL-13. Anti-inflammatory cytokines work with specific cytokine inhibitors and soluble cytokine receptors to inhibit the synthesis of proinflammatory cytokines (Opal and DePalo, 2000). A balance between proinflammatory and anti-inflammatory cytokines is necessary to maintain health.

1.6.2.2 Transcriptional regulation of inflammation: Role of NF-κB

Nuclear factor kappa B (NF-κB) is an important transcription factor that plays crucial roles in a number of cellular processes including inflammation, cell proliferation and apoptosis (Gloire et al., 2006). The NF-κB pathway has been well recognized as a proinflammatory signaling pathway, which is responsible for the activation of various cytokines such as IL1 and TNF-α, chemokines and adhesion molecules in inflammation (Lawrence, 2009). NF-κB has also been reported to have important roles in the pathophysiology of renal IR injury. It activates a variety of physiologically important molecules such as IL-1β, IL-6, IL-8, TNF-α, monocyte chemoattractant protein-1 (MCP-1), interferon invasive protein-10 (IP-10), angiotensin II, and inducible nitric oxide synthase (iNOS) (Lawrence 2009). This makes NF-κB an important therapeutic target for renal diseases.

NF-κB is composed of five proteins: p50 and its precursor p105 (NF-κB1), p52 and its precursor p100 (NF-κB2), p65/RelA, c-Rel and RelB (Thurman, 2007). NF-κB is normally in an inactivate state in the cytoplasm, bound by an inhibitory protein called IκB. Upon stimulation,

IκBα is rapidly phosphorylated and ubiquitinated, and degraded by a proteasome, leading to the release of NF-κB. The released NF-κB then translocates into the nucleus and activates the transcription of several target genes. Two NF-κB activation pathways are well studied: the classical and alternative pathways. The classical NF-κB activating pathway is induced by immune mediators such as toll-like receptors (TLRs), proinflammatory cytokines such as TNF-α, IL-1 and antigen receptors ligation, resulting in the activation of IκB-kinase (IKK) complex, composed of IKKγ, IKKα and IKKβ kinases. Phosphorylation of IKK complex activates p65/RelA complex, which regulates the expression of proinflammatory and cell survival genes (Gloire et al., 2006). The alternative NF-κB activating pathway is activated by B-cell activating factor (BAFF), CD40 ligand and lymphotoxin β, but not TNF-α. It results in the activation of RelB/p52 complex through the activation of NF-κB inducing kinase (NIK) and IKKα dependant phosphorylation and processing of p100 into p52. The alternative pathway is related to adaptive immunity which regulates genes required for lymph-organogenesis and B cell activation (Gloire et al., 2006, Lawrence, 2009).

1.6.3 Inflammation in renal IR and distant organ injury

Inflammation plays a major role in the progression of AKI. Studies have reported that a variety of inflammatory reactions are induced by renal IR injury, including the production of proinflammatory cytokines (IL-1, IL-6, TNF-α, and transforming growth factor-β (TGF-β)), chemokines (MCP-1 and epithelial neutrophil-activating protein 78 (ENA-78)), keratinocyte-derived chemokine (IL-8), fibrinogen, P-selectin, adhesion molecules (ICAM-1) and C-reactive protein within the renal parenchyma by tubular epithelial cells and/or leukocytes (Segerer et al., 2000, Kapper et al., 2002, Bonventre and Zuk, 2004, Molls et al., 2006, Thurman, 2007). The release of these inflammatory mediators activates the inflammation pathways and enhances cell

injury. Ischemia reperfusion injury also causes infiltration of neutrophils in the kidney. Myeloperoxidase (MPO) activity is elevated at the early stage after ischemic insult and is most abundantly expressed in neutrophils (Nauseef, 2014). At a later stage, MPO can be produced from macrophages and T cell infiltration, which persists into the recovery phase (Paller, 1989, De et al., 1998, Thurman, 2007). Tissue injury can be ameliorated if neutrophil accumulation is prevented (Paller, 1989, Bonventre and Zuk, 2004). A few mechanisms have been proposed on the activation of the inflammatory response upon renal IR injury, which include: 1) release of cellular factors such as NF-κB which translocate into the nucleus in response to hypoxia or reperfusion (Sung et al., 2002); 2) ATP depletion-induced apoptosis or necrosis, which further induces TNF-α production and leukocyte infiltration; 3) ischemia-induced iNOS production, which exacerbates tubular injury; 4) recognition of altered or injured protein or cell structures; and 5) impaired production of anti-inflammatory factors by injured cells (Thurman, 2007). Inflammation in the kidney tissue is closely related to glomerular and tubulointerstitial lesions, which in turn, greatly affects kidney function (Hiromura et al., 1998). Targeting on different inflammatory pathways may serve as a potential pharmacologic intervention for renal IR injury.

Inflammation is also reported in renal IR-induced distant organ injury. These distant organs include heart, brain, lungs, intestine and liver. Cytokines such as IL-1β, IL-6, IL-12, IL17A, ICAM-1, TNF-α and G-CSF have been reported to be elevated in distant organs (Deng et al., 2004, Park et al., 2011, Park et al., 2012). Increased MPO activity, as an indicator of neutrophil infiltration is found in the heart and lungs (Kelly, 2003, Hoke et al., 2007). Distant organs may respond to systemic inflammation induced by renal IR, however it is possible that the inflammatory signals are generated in a tissue specific manner (Thurman, 2007). For a detailed review of AKI on distant organ injury please refer to section 1.3.

1.7 Oxidative stress and inflammation crosstalk

Crosstalk between oxidative stress and inflammation is an area of increased interest. Oxidative stress is a biochemical dysregulation of the redox status, whereas inflammation is the biological response to oxidative stress that initiates the production of proteins, enzymes and other compounds to restore homeostasis (Stefanson and Bakovic, 2014).

1.7.1 Crosstalk between transcriptional factors Nrf2 and NF-κB

The transcriptional factors Nrf2 and NF-κB regulate oxidative stress and inflammation in a synergistic manner and coordinate the final fate of innate immune cells (Bellezza et al., 2010, Wakabayashi et al., 2010, Cuadrado et al., 2014). The Nrf2 pathway regulates the transcription of antioxidant genes and detoxification genes in order to maintain cellular redox homeostasis and eliminate toxins before damage (Gloire et al., 2006, Stefanson and Bakovic, 2014). The NF-κB pathway regulates cellular immune responses to infection and hypoxia, and coordinates proinflammatory responses by releasing mediators such as cytokines and chemokines (Gloire et al., 2006). Different levels of oxidative stress activate different redox-sensitive transcription factors and coordinate a variety of cellular responses (Bellezza et al., 2010). A mild level of oxidative stress induces Nrf2, which then activates the transcription of antioxidants, whereas an intermediate amount of ROS triggers an inflammatory response through the activation of NF-κB (Stefanson and Bakovic, 2014). An extremely high level of oxidative stress induces perturbation of the mitochondrial permeability transition pore and disruption of electron transfer and activates AP-1, thereby resulting in apoptosis or necrosis (Gloire et al., 2006, Bellezza et al., 2010). The effect of different levels of oxidative stress on cellular responses is shown in Figure 1.8.

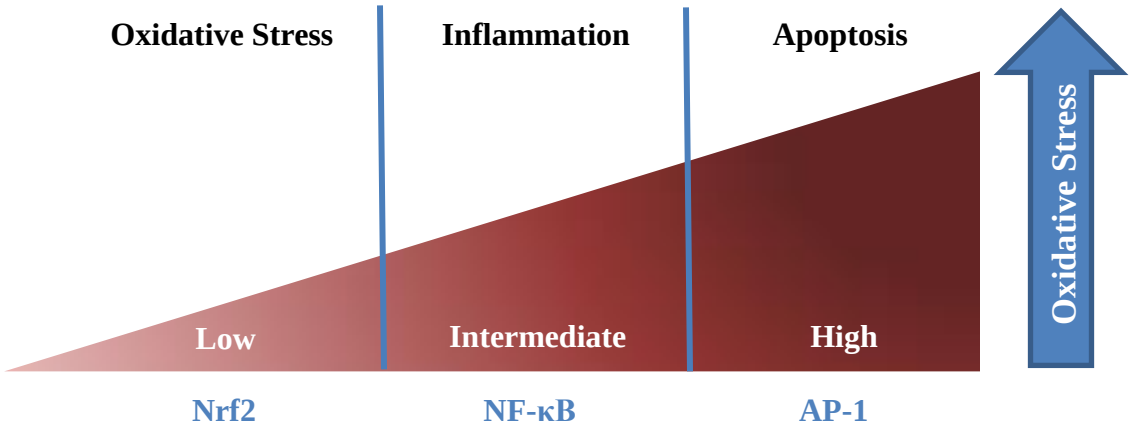

Figure 1.8 Effect of different levels of oxidative stress on cellular responses

A low level of oxidative stress induces Nrf2, which activates the production of antioxidants. An intermediate amount of oxidative stress triggers the activation of NF-κB, leading to proinflammatory cytokine production and inflammation. A high level of oxidative stress activates AP-1, resulting in apoptosis or necrosis.

1.7.2 Activation and inhibition between Nrf2 and NF-κB

The repressor protein Keap1 may act as an coordinating factor between the activation and inhibition of Nrf2 and NF-κB (Stefanson and Bakovic, 2014). Under basal conditions, Nrf2 is bound with Keap1 by an ETGE motif in the cytoplasm (Tong et al., 2006). Similarly, NF-κB is restrained in the cytosol by inhibitor protein IκBα. IKKβ targets IκBα for proteasomal degradation and release of NF-κB for translocation into the nucleus. IKKβ also contains an ETGE motif, which can bind with Keap1 and be targeted for ubiquitination, thus inhibiting NF-κB activation (Lee et al., 2009, Kim et al., 2010). When Nrf2 is activated by oxidative stress, there is an increase in the unbound Keap1 available to capture more intracellular IKKβ, which in turn, inhibits the translocation and activation of NF-κB (Stefanson and Bakovic, 2014). However, beyond a critical intracellular ROS threshold, Nrf2 may amplify oxidative stress via induction of transcription by Kruppel-like factor 9 (Klf9), which can alter the expression of several genes involved in ROS clearance, leading to Klf9-dependant ROS production (Bellezza et al., 2010, Stefanson and Bakovic, 2014, Zucker et al., 2014). Under an intermediate to high oxidative stress situation, Keap1 is unable to revert to a normal protein binding conformation, allowing IKKβ to phosphorylate IκBα, and thus releasing NF-κB for nuclear translocation (Poole et al., 2004, Stefanson and Bakovic, 2014). An activated NF-κB has been known to inhibit Nrf2 activity (Kim and Vaziri, 2010, Kim et al., 2010, Stefanson and Bakovic, 2014). The p65 subunit of NF-κB represses the Nrf2-ARE pathway at a transcriptional level by 1) competitive interaction with the CH1-KIX domain of CREB binding protein (CBP) from Nrf2, which results in Nrf2 inactivation; and 2) p65 facilitates the interaction of a corepressor histone deacetylase 3 (HDAC3) with either CBP or MafK at the ARE, which results in local histone hypoacetylation (Liu et al., 2008). These processes lead to the transition point from oxidative stress to

inflammation. If NF-κB mediated reactions fail to restore homeostasis and oxidative stress is continuously increased to extreme levels, AP-1 mediated apoptosis is triggered (Kim and Vaziri, 2010, Kim et al., 2010, Stefanson and Bakovic, 2014). A schematic illustration of Nrf2 activation and NF-κB inhibition though Keap1 is shown in Figure 1.9.

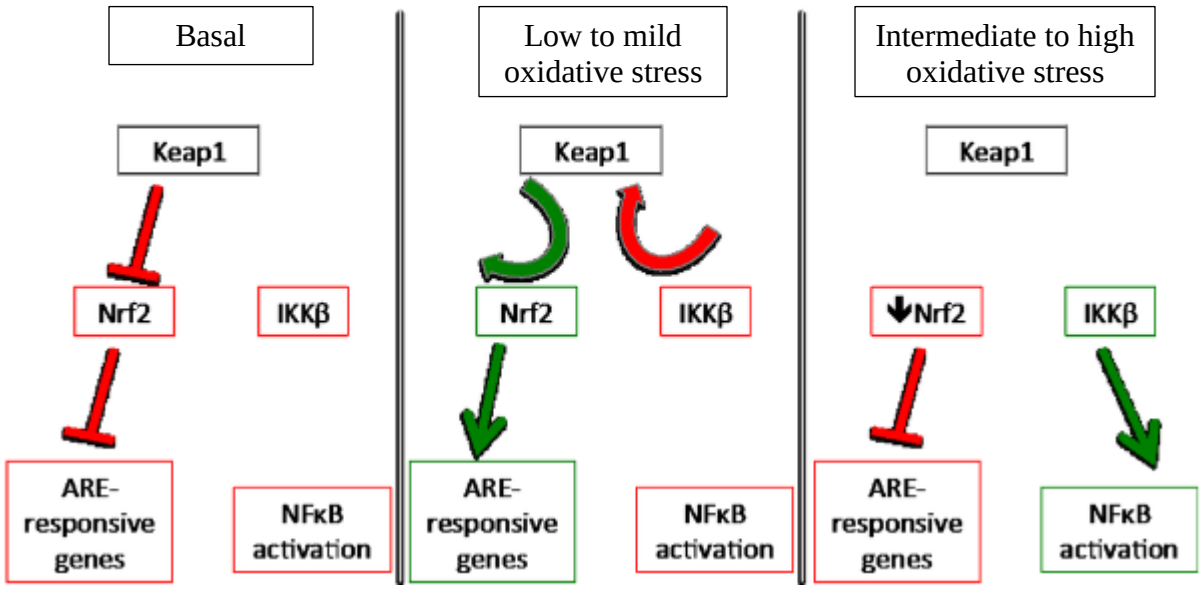

Figure 1.9 Nrf2 activation and NF-κB inhibition though Keap1

Under basal conditions, Nrf2 is inhibited by Keap1. When Nrf2 is activated by oxidative stress, the unbound Keap1 captures intracellular IKKβ, inhibits the translocation and activation of NF-κB. Under an intermediate to high oxidative stress situation, Keap1 is unable to revert to a normal protein binding conformation, allowing IKKβ to phosphorylate IκBα, and thus activates NF-κB. (Adapted from Stefanson and Bakovic (2014), open access and subject to the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction)

The Nrf2 and NF-κB pathway also interact at multiple points to control the transcription of downstream target genes or proteins. Nrf2 activation induces intracellular products that can modulate NF-κB activity, and vice versa. For example, Nrf2 regulates the gene expressions of HO-1 and thioredoxin (Wakabayashi et al., 2010). HO-1 has been shown to modulate NF-κB activation through free iron and bilirubin, prevent phosphorylation of RelA and inhibit IκB degradation (Alam et al., 1999, Jun et al., 2006, Seldon et al., 2007). Thioredoxin regulates NF-κB activation through the reduction of cysteine residues, which can modulate p50 binding to the DNA in the nucleus and blockage of IκB degradation in the cytoplasm (Hirota et al., 1999, Das, 2001). On the other hand, the p65 subunit of NF-κB supresses the Nrf2 pathway at a transcriptional level, and overexpressing p65 that antagonizes the Nrf2 transcriptional activity and further inhibits HO-1 production (Liu et al., 2008). A schematic explanation of crosstalk between Nrf2 and NF-κB activation based on intracellular events is shown in Figure 1.10.

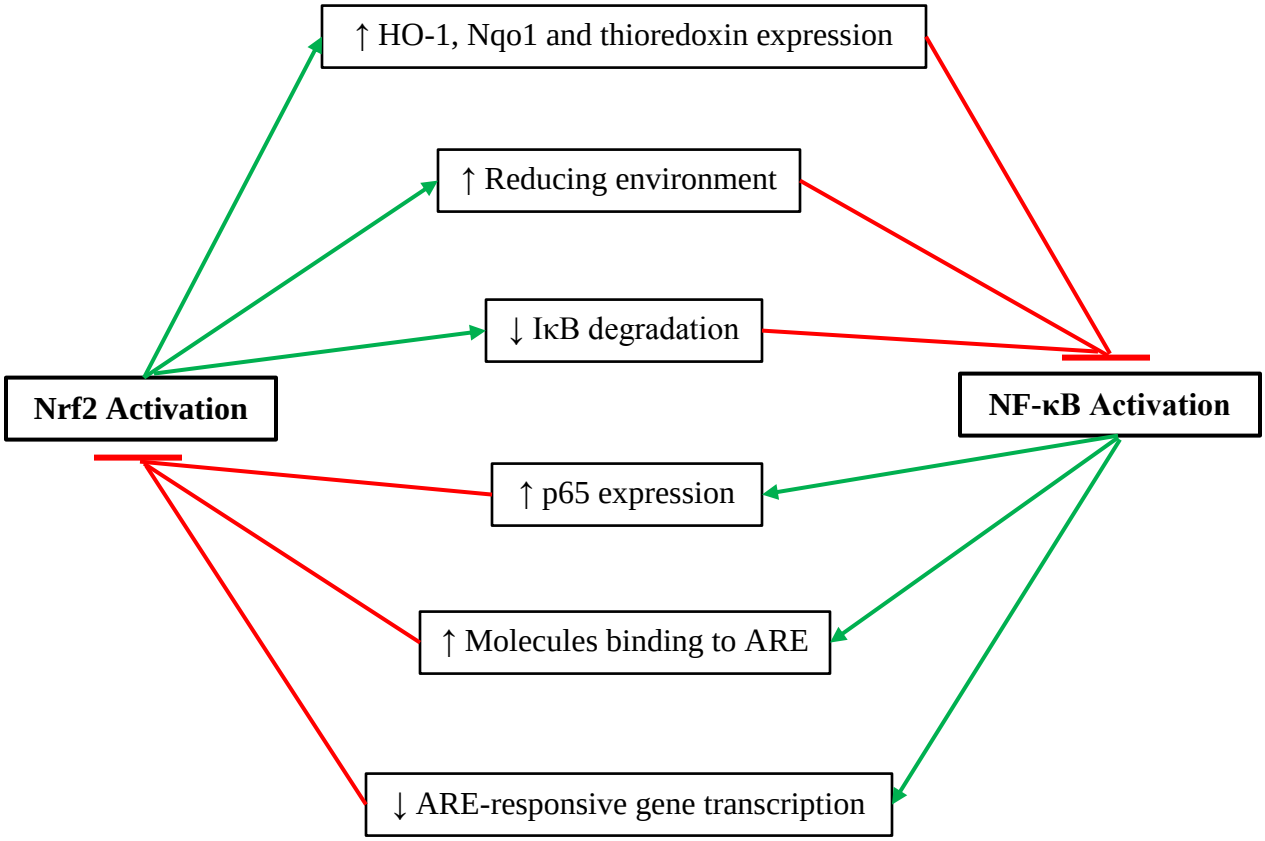

Figure 1.10 Crosstalk between Nrf2 and NF-κB activation based on intracellular events

Green lines: induced events; red lines: inhibitory events; HO-1, Heme oxygenase-1; Nqo1, NAD(P)H: quinone oxidoreductase 1; ARE, antioxidant response element.

Chapter II. Hypothesis and Objectives

2.1 Rationale and hypotheses

Acute kidney injury (AKI) commonly occurs in hospitalized patients, which promotes the development of chronic kidney disease (CKD) and causes end-stage renal disease. AKI also often leads to multi-organ dysfunction known as distant organ injury (Grams and Rabb, 2012, Yap and Lee, 2012). It has been shown in animal models that AKI can cause injury of multiple organs including brain, heart, intestine, lungs and liver (Kelly, 2003, Hassoun et al., 2007, Liu et al., 2008, Grams and Rabb, 2012, Lane et al., 2013, Druml, 2014, Ologunde et al., 2014). However, mechanisms of AKI-induced distant organ injury are not well understood.

Ischemia-reperfusion (IR) is one of the common causes for AKI, which occurs in many clinical situations such as surgical procedures, renal transplantation, vascular disease or as comorbidity in critically ill patients (Thadhani et al., 1996, Mehta et al., 2004, Lameire et al., 2005). Previous findings from our laboratory have reported that renal IR induces oxidative stress and inflammatory responses in the kidney (Sung et al., 2002, Prathapasinghe et al., 2007, Wang et al., 2014). However, the mechanisms for damage to the distant organs are unknown. It is essential to understand renal IR-induced oxidative stress and inflammation in the distant organs, and to further identify therapeutic targets for the amelioration of local and systemic injuries. An in vivo model has been employed in our laboratory to study renal IR. The renal IR will be induced in Sprague Dawley (SD) rats by the clamping of the left renal pedicles for 45 minutes to induce ischemia, followed by different times of reperfusion.

The liver is a major organ for metabolism. It also plays important roles in redox balance, immune regulation and detoxification. Impaired liver function and metabolism has been reported in patients with AKI and renal IR model of animals, which includes induction of systemic inflammation, increased proinflammatory cytokines, increased oxidative stress, and abnormal

liver histology and apoptosis (Mehta, 2002, Golab et al., 2009, Abbas and Lichtman, 2012, Park et al., 2012, Lane et al., 2013, Doi and Rabb, 2016). The mechanism of renal IR-induced liver injury needs to be further investigated.

Oxidative stress occurs when there is an increase in free radicals or a decrease in antioxidants. Glutathione is a major endogenous antioxidant against oxidative stress (DeLeve and Kaplowitz, 1991, Lu, 1999). The homeostasis of GSH is modulated by the transsulfuration pathway and GSH biosynthesis. Transsulfuration enzymes catalyze the *de novo* synthesis of cysteine, which serves as a precursor for the synthesis of GSH. The liver has a high capacity for GSH biosynthesis. Nuclear factor erythroid 2–related factor 2 (Nrf2) is a key transcription factor involved in cellular responses against oxidative stress (Lu, 2013, Kudoh et al., 2014). Activation of Nrf2 induces gene expression of antioxidants and enzymes that are responsible for GSH synthesis (Lee and Johnson, 2004, Kim and Vaziri, 2010, Wakabayashi et al., 2010). Our recent study has shown that IR downregulates the expression of transsulfuration enzymes in the kidney leading to increased oxidative stress and inflammatory responses (Wang et al., 2014). A few studies have reported renal IR-induced oxidative stress in the liver, however, the mechanism by which renal IR impairs hepatic GSH production is not well understood.

An inflammatory response plays a critical role in IR injury (Thurman, 2007). It has been reported that inflammation occurs both locally in the kidney and systemically in the distant organs upon renal IR. The inflammatory response is mediated by several proinflammatory mediators, such as cytokines, chemokines and inflammatory signaling pathways. The remote inflammatory responses of renal IR may be induced by increased influx of cytokines and infiltration of immune cells to the distant organs (Lee et al., 2011). However, the origins of increased cytokines in the liver upon renal IR have not been well identified. Nuclear factor-

kappa B (NF-κB) is one of the key transcription factors that regulates the expression of proinflammatory cytokines and an immune response (Lawrence, 2009). Early stage up-regulation of MCP-1 expression through the activation of NF-κB in the kidney has been previously reported in our laboratory (Sung et al., 2002, Wang et al., 2013). However, the activation of NF-κB and the stimulation of proinflammatory cytokine production in the liver is unknown. Further research is warranted to identify regulatory mechanisms for the hepatic inflammatory response during renal IR.

In our studies, we hypothesized that renal IR induces liver oxidative stress and inflammatory responses through 1) down-regulation of the transsulfuration pathway and inhibition of GSH production through Nrf2 pathway; and 2) activation of NF-κB and increased cytokine expression in the liver.

2.2 Objectives

The general objective of my research was to investigate the mechanisms by which renal IR caused liver injury with increased oxidative stress and inflammatory responses.

Specific objectives of study I - Downregulation of glutathione biosynthesis contributes to oxidative stress and liver dysfunction in acute kidney injury:

(1) To investigate the changes of transsulfuration pathway and glutathione synthesis in the liver upon renal IR.

(2) To investigate the role of transcription factor Nrf2 on antioxidant gene expression in renal IR-induced distant organ injury.

Specific objectives of study II - Kidney ischemia-reperfusion elicits a hepatic inflammatory response and hepatic dysfunction:

(1) To investigate the changes in proinflammatory markers both systemically and locally at different stages in the liver upon renal IR.

(2) To investigate the regulation of proinflammatory cytokines via transcription factor NF-κB in the liver upon renal IR.

Chapter III. Study I

Manuscript I: Downregulation of glutathione biosynthesis contributes to oxidative stress and liver dysfunction in acute kidney injury

Oxidative Medicine and Cellular Longevity, 2016(10):1-13

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3.1 Abstract

Ischemia-reperfusion is a common cause for acute kidney injury and can lead to distant organ dysfunction. However, the mechanisms of distant organ injury are not well understood. Glutathione is a major endogenous antioxidant and its depletion directly correlates to ischemia-reperfusion injury. The liver has a high capacity for producing glutathione and is a key organ in modulating local and systemic redox balance. Liver function is often compromised by acute kidney injury. In the present study, we investigated the mechanism by which kidney ischemia-reperfusion led to glutathione depletion and oxidative stress. The left kidney of Sprague-Dawley rats was subjected to 45min ischemia followed by 6h reperfusion. Renal ischemia-reperfusion impaired kidney and liver function. This was accompanied by a decrease in glutathione levels in the liver and plasma, increased hepatic lipid peroxidation and plasma homocysteine levels. Renal ischemia-reperfusion caused a significant decrease in mRNA and protein levels of hepatic glutamate-cysteine ligase (catalytic and modifier subunits) which regulates the rate-limiting reaction in glutathione biosynthesis. A decrease in gene expression of glutamate-cysteine ligase subunits was mediated through inhibition of a transcription factor Nrf2. Renal ischemia-reperfusion also inhibited hepatic expression of cystathionine γ-lyase, an enzyme responsible for producing cysteine (an essential precursor for glutathione synthesis) through the transsulfuration pathway. These results suggest that inhibition of glutamate-cysteine ligase expression and the transsulfuration pathway lead to reduced hepatic glutathione biosynthesis and elevation of plasma homocysteine levels, which, in turn, may contribute to oxidative stress and distant organ injury during renal ischemia-reperfusion.

3.2 Introduction

Ischemia-reperfusion (IR) is one of the common causes for acute kidney injury (AKI) (Thadhani et al., 1996, Mehta et al., 2004, Lameire et al., 2005). AKI often leads to multi-organ dysfunction known as distant organ injury (Grams and Rabb, 2012, Yap and Lee, 2012). Renal IR occurs in many clinical situations such as surgical procedures, renal transplantation or as comorbidity in critically ill patients. However, mechanisms of AKI-induced distant organ injury are not well understood. Clinically, distant organ injury potentiates the already high rate of AKI-associated morbidity and mortality (Levy et al., 1996, Bagshaw et al., 2005). In animal models, AKI is shown to cause injury of multiple organs including brain, heart, intestine, lung and liver (Kelly, 2003, Hassoun et al., 2007, Liu et al., 2008, Grams and Rabb, 2012, Lane et al., 2013, Druml, 2014, Ologunde et al., 2014).

The pathogenesis of IR injury is multifaceted and oxidative stress is considered one of the important mechanisms responsible for local as well as distant organ injury. Impaired antioxidant defense and/or overproduction of reactive oxygen species (ROS) can lead to oxidative stress. Glutathione is a thiol-containing tripeptide that serves as a major endogenous non-enzymatic antioxidant against oxidative stress (DeLeve and Kaplowitz, 1991, Lu, 1999). The liver has high glutathione levels due to its efficient synthesis (DeLeve and Kaplowitz, 1991, Ookhtens and Kaplowitz, 1998, Lu, 1999, Chen et al., 2013, Lu, 2013). Impaired liver function and metabolism have been found in animal models with ischemic AKI (Serteser et al., 2002, Golab et al., 2009, Kadkhodaee et al., 2009, Wang et al., 2010). It has been shown that renal IR causes a depletion of hepatic glutathione while administration of this antioxidant effectively attenuates oxidative stress and improves liver function (Golab et al., 2009). However, the mechanism by which ischemic AKI elicits hepatic glutathione depletion is not well understood. Glutathione is

synthesized by two enzymes, namely, glutamate-cysteine ligase (EC 6.3.2.2) and glutathione synthase (EC 6.3.2.3) (Figure 3.11) (Lu, 2013). Glutamate-cysteine ligase is composed of a catalytic subunit (Gclc) and a modifier subunit (Gclm). This enzyme catalyzes the rate-limiting reaction by converting cysteine and glutamate to γ-glutamyl-cysteine. The second enzyme, glutathione synthase catalyzes the reaction of γ-glutamyl-cysteine and glycine to form glutathione. Nuclear factor erythroid 2–related factor 2 (Nrf2) is a key transcription factor involved in cellular responses against oxidative stress. It regulates the expression of many antioxidant proteins and enzymes (Kim and Vaziri, 2010, Lu, 2013, Kudoh et al., 2014). Activation of Nrf2 induces gene expression of cytoprotective enzymes including those that are responsible for glutathione synthesis (Lee and Johnson, 2004, Kim and Vaziri, 2010, Kim et al., 2010, Wakabayashi et al., 2010). Under normal conditions, Nrf2 is retained in the cytoplasm through binding to a repressor protein named Kelch-like ECH-associated protein 1 (Keap1). Upon stimulation, Nrf2 is dissociated from Keap1 and translocated into the nucleus where it binds to the promoter regions of the target genes (Kim and Vaziri, 2010, Kudoh et al., 2014). Nuclear translocation of Nrf2 can be stimulated by hydrogen sulfide through S-sulfhydration of Keap1 (Hourihan et al., 2013, Yang et al., 2013). It has been shown that deficiency of Nrf2 leads to increased oxidative stress, renal and hepatic damages and inflammatory responses (Kim and Vaziri, 2010, Kim et al., 2010, Kudoh et al., 2014). Although the protective effect of Nrf2 through upregulation of antioxidant enzymes against IR or toxin induced organ injury has been implicated (Chan et al., 2001, Enomoto et al., 2001, Kudoh et al., 2014), its role in distant organ injury is not clear.

The availability of cysteine is another important determinant in modulating glutathione homeostasis as this sulfur-containing amino acid is an essential precursor for glutathione

biosynthesis. Aside from dietary intake or endogenous protein degradation, the transsulfuration pathway is the only source of de novo synthesized cysteine in mammalian cells (Figure 3.11) (Mosharov et al., 2000, McBean, 2012, Lu, 2013). In the transsulfuration pathway, cystathionine β-synthase (CBS, EC 4.2.1.22) catalyzes the initial reaction in which homocysteine is condensed with serine to form cystathionine. Cystathionine is subsequently metabolized to cysteine by another enzyme, cystathionine γ-lyase (CSE, EC 4.4.1.1). By generating cysteine, the transsulfuration pathway provides the rate limiting amino acid for cellular glutathione biosynthesis (Stipanuk and Beck, 1982, Stipanuk, 1986, Finkelstein, 2000, Mosharov et al., 2000, Brosnan and Brosnan, 2006). This may be particularly important in the liver as 50% of the cellular glutathione pool in hepatocytes is derived from the transsulfuration pathway (Lu, 1999). The transsulfuration pathway enzymes are widely present in mammalian tissues such as liver, kidney, intestine, heart, aorta, brain, lungs and intestine, with liver having the highest enzyme activities of CBS and CSE (Stipanuk and Beck, 1982, Stipanuk, 1986, Lu, 1999, Finkelstein, 2000, Mosharov et al., 2000, Brosnan and Brosnan, 2006, Rosado et al., 2007). Our recent study has shown that IR downregulates the expression of transsulfuration enzymes in the kidney leading to increased oxidative stress and inflammatory response (Wang et al., 2014). Although liver oxidative stress has been reported in ischemic AKI, the mechanism by which renal IR impairs hepatic glutathione production is not well understood. As glutathione is the major endogenous antioxidant and the liver is the key organ for its generation, its depletion could lead to local and systemic oxidative stress. In the present study, we investigated the mechanism by which renal IR caused downregulation of hepatic glutathione biosynthesis and oxidative stress.

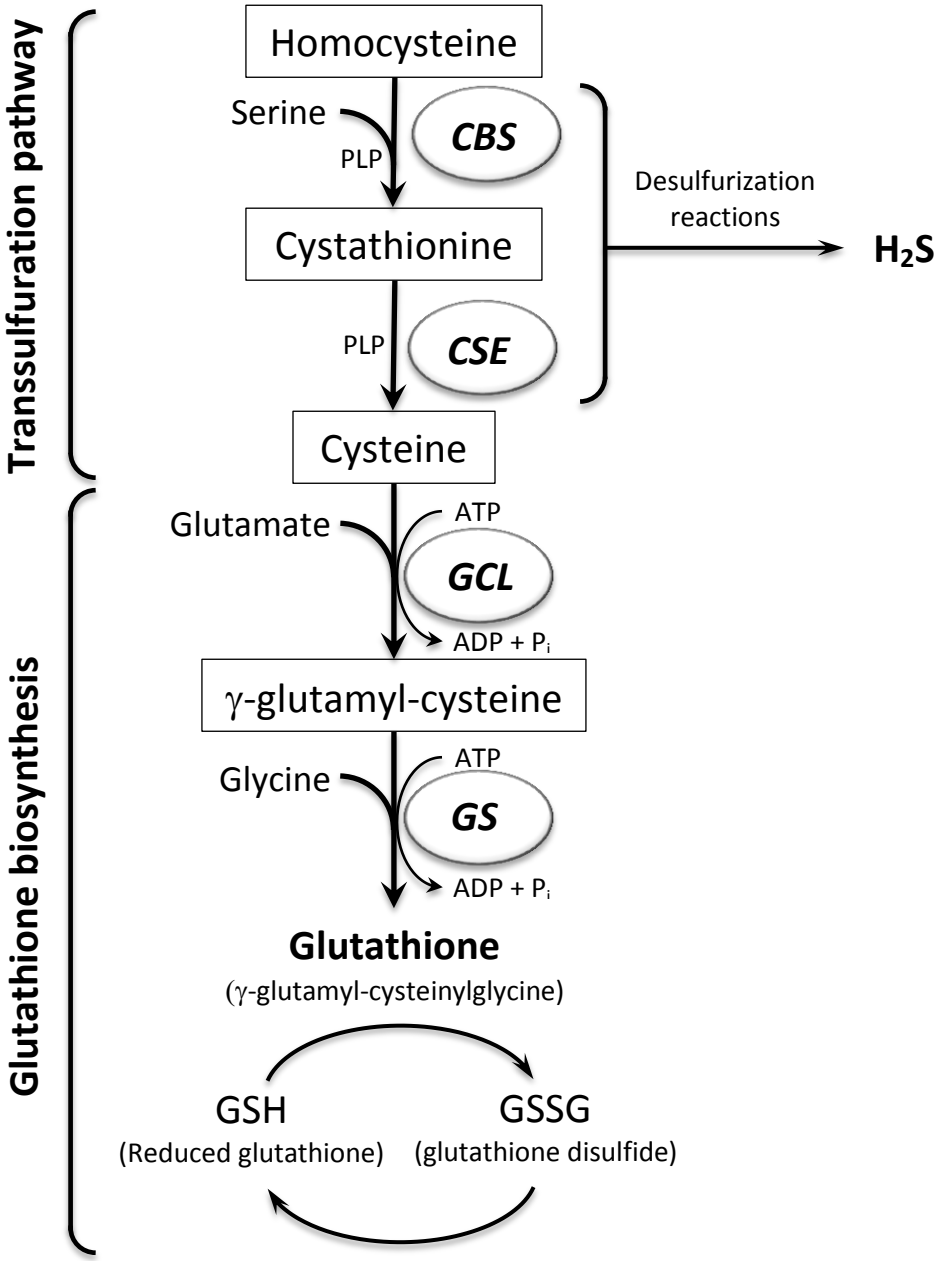


Figure 3.11 Transsulfuration pathway and glutathione biosynthetic pathway

The transsulfuration pathway metabolizes homocysteine to cysteine. In this pathway, cystathionine β-synthase (CBS) catalyzes the initial reaction in which homocysteine is condensed with serine to form cystathionine. Cystathionine is subsequently metabolized to cysteine by the second enzyme, cystathionine γ-lyase (CSE). Both CBS and CSE are pyridoxal-5'-phosphate (PLP)-dependent enzymes. The tri-peptide glutathione is synthesized from glutamate, cysteine and glycine. Glutamate-cysteine ligase (GCL) catalyzes the rate-limiting reaction by converting cysteine and glutamate to γ-glutamyl-cysteine. The second enzyme, glutathione synthase (GS) catalyzes the reaction of γ-glutamyl-cysteine and glycine to form glutathione. The equilibrium between reduced (GSH) and oxidized (GSSG) glutathione reflects the redox potential of a given tissue, with lower GSH: GSSG ratios being indicative of oxidative stress. Alternatively, CBS and CSE also mediate the desulfurization reactions which lead to hydrogen sulfide (H_2S) synthesis.

3.3 Materials and methods

Sprague-Dawley male rats (250-300g, 7-8 weeks old) were fed a commercial diet (Prolab® RMH 3000, 5P00) containing 0.40% of cysteine and 0.58% of methionine (LabDiet, St. Louis, MO) prior to surgery. Rats were anesthetised by 3% isoflurane/oxygen gas. Renal ischemia was induced by clamping the left kidney pedicle for 45 min as described in our previous studies (Prathapasinghe et al., 2007, Wu et al., 2010, Wang et al., 2013, Yang et al., 2013). At the end of ischemia, the rats were subjected to 6h of reperfusion by removal of the clamp and right nephrectomy. During the surgery, the rats were kept on a heating pad and 1-2% isoflurane/oxygen gas was maintained via inhalation. As a control (sham-operated), rats were subjected to the same surgical procedure without inducing ischemia and were sacrificed at the corresponding time point. A blood sample was collected from the portal vein and centrifuged at 3000 *g* for 20 min for plasma preparation. Plasma creatinine, alanine aminotransferase, aspartate aminotransferase and homocysteine levels were measured using the Cobas C111 Analyzer (Roche, Laval, QC). All procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and approved by the University of Manitoba Protocol Management and Review Committee.

3.3.1 Biochemical analyses

Reduced (GSH) and oxidized (GSSG) glutathione in the plasma and liver were measured by a spectrophotometric detection method (Anderson, 1985, Sarna et al., 2010, Hwang et al., 2013, Wang et al., 2014). A ratio of GSH and GSSG was determined as an indicator of redox potential. The degree of lipid peroxidation in the liver tissue was determined by measuring malondialdehyde (MDA) levels with thiobarbituric acid reactive substances (TBARS) assay (Ohkawa et al., 1979, Sung et al., 2002). Cysteine content in the liver was measured by ion

exchange chromatographic method according to the Official Methods of Analysis (982.30, AOAC, 1984). Briefly, approximately 100 mg of each sample was subjected to performic acid oxidation for 20 h at 4 °C and digested in 4 mL of 6 N HCl for 16 h at 110 °C, followed by neutralization with 2 mL of 25% (wt/vol) NaOH and cooling to room temperature. The mixture was then equalized to a 50-mL volume with sodium citrate buffer (pH 2.2) and analyzed using an amino acids analyzer (Sykam, Eresing, Germany). The obtained cysteine content was first equalized with the total oxidized amino acids content and compared between Sham and IR groups. Hydrogen sulfide (H_2S) production was measured based on a spectrophotometric detection method of Stipanuk and Beck (Stipanuk and Beck, 1982) as described in our previous studies (Xu et al., 2009, Hwang et al., 2013, Wang et al., 2014).

3.3.2 Transfection of HepG2 cells with Nrf2 siRNA

HepG2 cells (American Type Culture Collection, MA, a cell line derived from human hepatoblastoma) were transfected with Nrf2 siRNA oligonucleotides (Life Technologies, Carlsbad, CA) or RNAi negative control consisting of scrambled oligonucleotides using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as transfection reagent. After 6h incubation, the medium was replaced with HyClone™ Dulbecco's Modified Eagle Medium (containing L-cystine 2HCl 62.57mg/L and L-methionine 30.00mg/L) supplemented with 10% fetal bovine serum and incubated for another 48h. The mRNA of Nrf2 and glutamate-cysteine ligase subunits (*Gclc*, *Gclm*) was determined. HepG2 cells are from an established human hepatoblastoma cell line, which share many features of hepatocytes and are widely used in physiological and pharmacological studies (Wilkening et al., 2003, Sarna et al., 2016). HepG2 cells are susceptible to free radicals and many studies have used the HepG2 cell line to investigate mitochondrial

toxicity and oxidative stress response (Setzer et al., 2008, Angeles et al., 2010, Savania et al., 2015, Sarna et al., 2016).

3.3.3 Real-time polymerase chain reaction (PCR) analysis

Total RNAs were isolated from the liver tissue with Trizol reagent (Invitrogen, Carlsbad, CA). The mRNA of glutamate-cysteine ligase catalytic subunit (Gclc) and modifier subunit (Gclm), glutathione synthase, enzymes in the transsulfuration pathway (CBS, CSE) and Nrf2 was determined by real-time PCR analysis using the iQ5 real-time PCR detection system (Bio-Rad) and normalized with β-actin (Wu et al., 2010, Hwang et al., 2013, Wang et al., 2014). The primers (Invitrogen) used for rat mRNA measurement were: Gclc (124 bp), 5'-GCCCAATTGTTATGGCTTTG-3' (forward) and 5'- AGTCCTCTCTCCTCCCGTGT-3' (reverse) (GenBankTM accession number NM_012815); Gclm (114 bp), 5'-CGAGGAGCTTCGAGACTGTAT -3' (forward) and 5'- ACTGCATGGGACATGGTACA -3' (reverse) (GenBankTM accession number NM_017305); glutathione synthase (182 bp), 5'-ACAACGAGCGAGTTGGGAT-3' and 5'- TGAGGGGAAGAGCGTGAATG-3' (reverse) (GenBankTM accession number NM_012962); rat CBS (148 bp), 5'-TCGTGATGCCAGAGAAGATG-3' (forward) and 5'-TTGGGGATTTCGTTCTTCAG-3' (reverse) (GenBankTM accession number NM_012522); CSE (150 bp), 5'-GTATGGAGGCACCAACAGGT-3' (forward) and 5'-GTTGGGTTT GTGGGTGTTTC-3' (reverse) (GenBankTM accession number NM_017074); and ß-actin (198 bp), 5'-ACAACCTTCTTGCAGCTCCTC-3' (forward) and 5'- GACCCATACCCACCA TCACA-3' (reverse) (GenBankTM accession number NM_031144). Primers (Invitrogen) used for human mRNA measurement were as follows: Nrf2 (106 bp), 5'- AGTGGATCTGCCAACTACTC-3' (forward) and 5'- CATCTACAAACGGGAATGTCTG -3' (reverse) (GenBankTM accession

number NM_006164); Gclc (105 bp), 5'- TACAGTTGAGGCCAACATGC-3' (forward) and 5'-CTTGTTAAGGTACTGGGAAATG AAG-3' (reverse) (GenBankTM accession number NM_001197115); Gclm (102 bp), 5'- GTTCAGTCCTTGGAGTTGCACA-3' (forward) and 5'-CCCAGTAAGGCTGTAAATGCTC-3' (reverse) (GenBankTM accession number NM_002061); and ß-actin (95 bp), 5'- AGATCAAGATCATTGCTCCTCCT -3' (forward) and 5'-GATCCACATCTGCTGGAAGG -3' (reverse) (GenBankTM accession number NM_001101).

3.3.4 Western immunoblotting analysis

The protein levels of hepatic glutamate-cysteine ligase catalytic (Gclc) and modifier (Gclm) subunits, glutathione synthase and enzymes in the transsulfuration pathway (CBS, CSE) were determined by Western immunoblotting analysis. Total proteins (10µg) were extracted using lysis buffer (20mM Tris, 150mM NaCl, 1mM EDTA, 1mM EGTA, 2.5mM Na pyrophosphate, 1mM β-glycerophosphate, 1mM Na orthocanadate and 10% Triton X-100) and were separated by electrophoresis in 10% SDS polyacrylamide gels. Proteins in the gel were transferred to a nitrocellulose membrane. The membrane was probed with rabbit anti-Gclc monoclonal (1:2000, Abcam Inc., Toronto, Canada), rabbit anti-Gclm monoclonal (1:2000, Abcam Inc., Toronto, Canada), rabbit anti-glutathione synthase monoclonal (1: 4,000; Abcam Inc., Toronto, Canada), mouse anti-CBS monoclonal (1:4000, Abnova Corporation, Taipei, Taiwan) or rabbit anti-CSE monoclonal antibodies (1:4000, GeneTex, Irvine, CA) for total liver proteins. Nuclear proteins (90µg) were extracted using buffers containing HEPES, EDTA, EGTA, KCl and NaCl, and were used to determine Nrf2 protein with anti-rabbit Nrf2 monoclonal antibodies (1:500, Abcam Inc., Toronto, Canada). HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (Cell Signaling Technology, Danvers, MA) were used as the secondary antibodies (1:2000). The corresponding protein bands were visualized using enhanced chemiluminescence reagents and analyzed with a

gel documentation system (Bio-Rad Gel Doc1000). To confirm the equal loading of proteins for each sample, the same membranes were probed with mouse anti-ß-actin monoclonal antibodies (1:5000, Cell Signaling Technology, Danvers, MA) or rabbit anti-Histone H3 monoclonal antibodies (1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA).

3.3.5 Statistical analysis

Results were analyzed using a two-tailed Student's t-test. *P* values less than 0.05 were considered statistically significant.

3.4 Results

3.4.1 Renal ischemia-reperfusion impaired kidney and liver function

Renal IR (45 min ischemia followed by 6h reperfusion) resulted in a significant elevation of plasma creatinine levels (Figure 3.12A), indicating that kidney function was impaired. Renal IR also caused a marked increase in plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Figure 3.12B, 3.12C), suggesting that IR not only impaired renal function but also caused liver injury. Renal IR significantly decreased the reduced glutathione (GSH) levels (Figure 3.13A) while did not change oxidized glutathione (GSSG) levels (Figure 3.13B), leading to a low ratio of GSH to GSSG in the plasma (Figure 3.13C). There was a significant elevation of plasma homocysteine level in rats subjected to renal IR (Figure 3.13D).

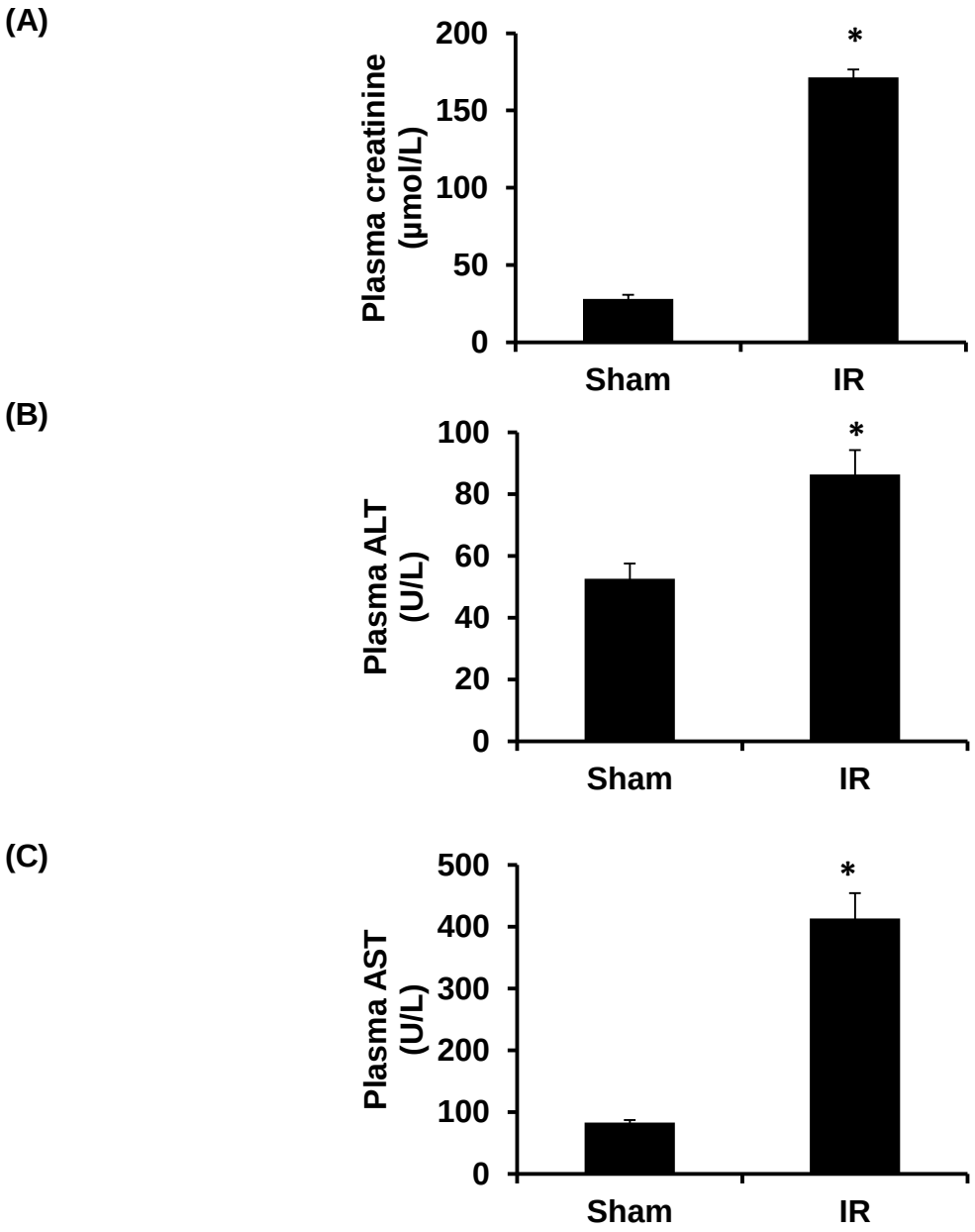


Figure 3.12 Effect of kidney ischemia-reperfusion on kidney and liver function

The left kidney of rats was subjected to 45 min ischemia followed by 6 h reperfusion (IR). As a control, rats were subjected to a sham-operation without inducing ischemia (Sham). Plasma creatinine (A), alanine aminotransferase (ALT) (B) and aspartate aminotransferase (AST) (C) were measured. Results are expressed as mean ± SE (n = 4 for each group). *$p < 0.05$ when compared with the value obtained from the sham-operated group.

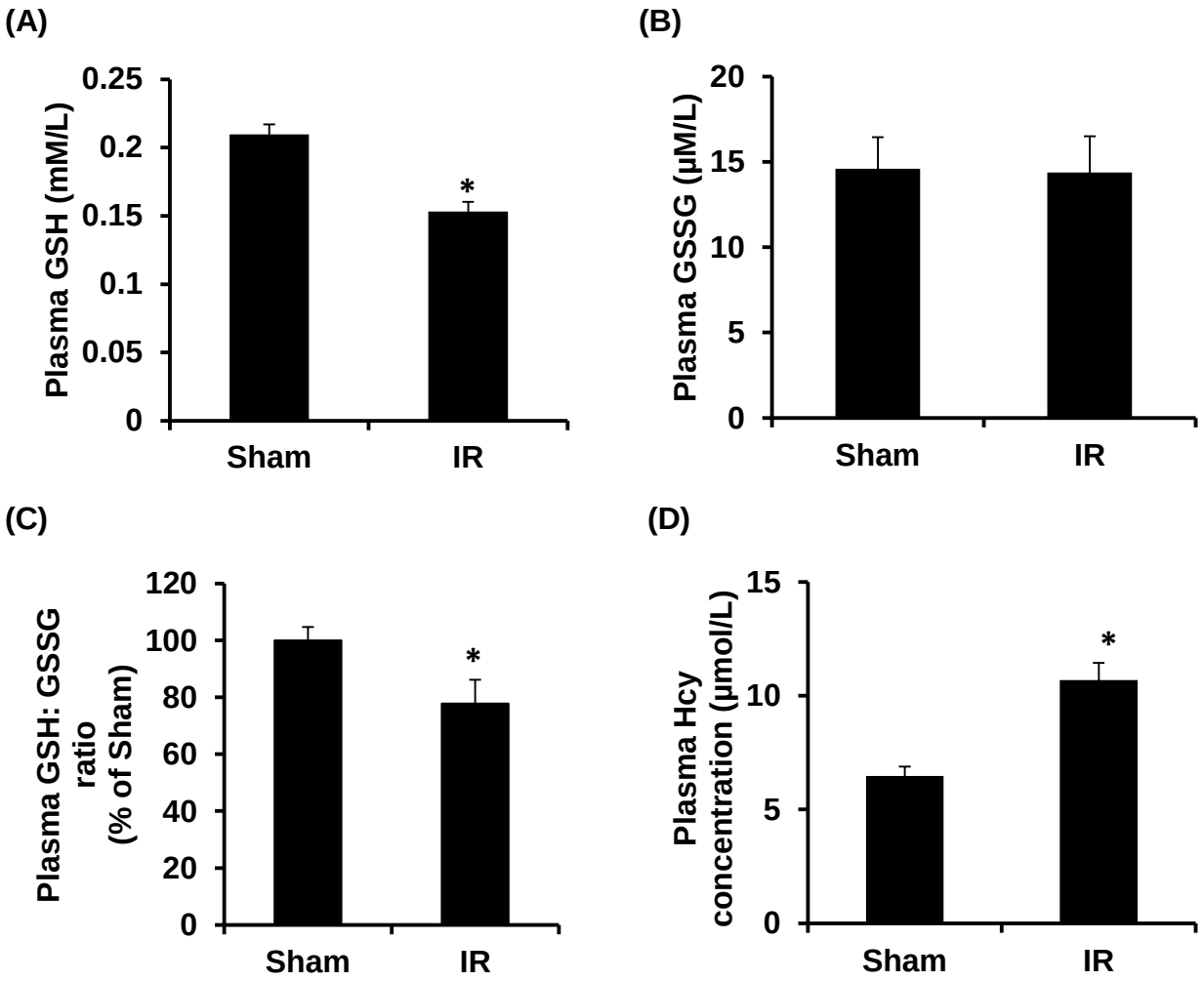


Figure 3.13 Effect of kidney ischemia-reperfusion on glutathione and homocysteine levels in the plasma

Plasma reduced glutathione (GSH) (A), oxidized glutathione (GSSG) (B), a ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) (C) and plasma homocysteine (Hcy) (D) were measured in rats subjected to renal ischemia-reperfusion injury (IR) or sham-operation. Results are expressed as mean ± SE (n = 4 for each group). *$p < 0.05$ when compared with the value obtained from the sham-operated group.

3.4.2 Renal ischemia-reperfusion reduced glutathione synthesis and induced oxidative stress in the liver

Renal IR resulted in a significant decrease in GSH levels and a low ratio of GSH to GSSG in the liver (Figure 3.14A, 3.14B, 3.14C). The level of MDA, a biomarker for lipid peroxidation, was significantly increased in the liver of rats subjected to renal IR, indicating oxidative stress (Figure 3.14D). To investigate whether low level of glutathione in the liver upon renal IR was due to a decrease in glutathione biosynthesis, we examined the expression of the enzymes that were responsible for its synthesis. Renal IR resulted in a significant decrease in the expression of glutamate-cysteine ligase subunits (Gclc and Gclm) mRNA and protein in the liver (Figure 3.15A, 3.15B, 3.15C, 3.15D). However, the expression of glutathione synthase in the liver was not significantly altered by renal IR (Figure 3.15E, 3.15F). The protein level of a transcription factor Nrf2 in the nucleus was significantly lower in the liver of rats subjected to renal IR than that in the sham-operated rats (Figure 3.16).

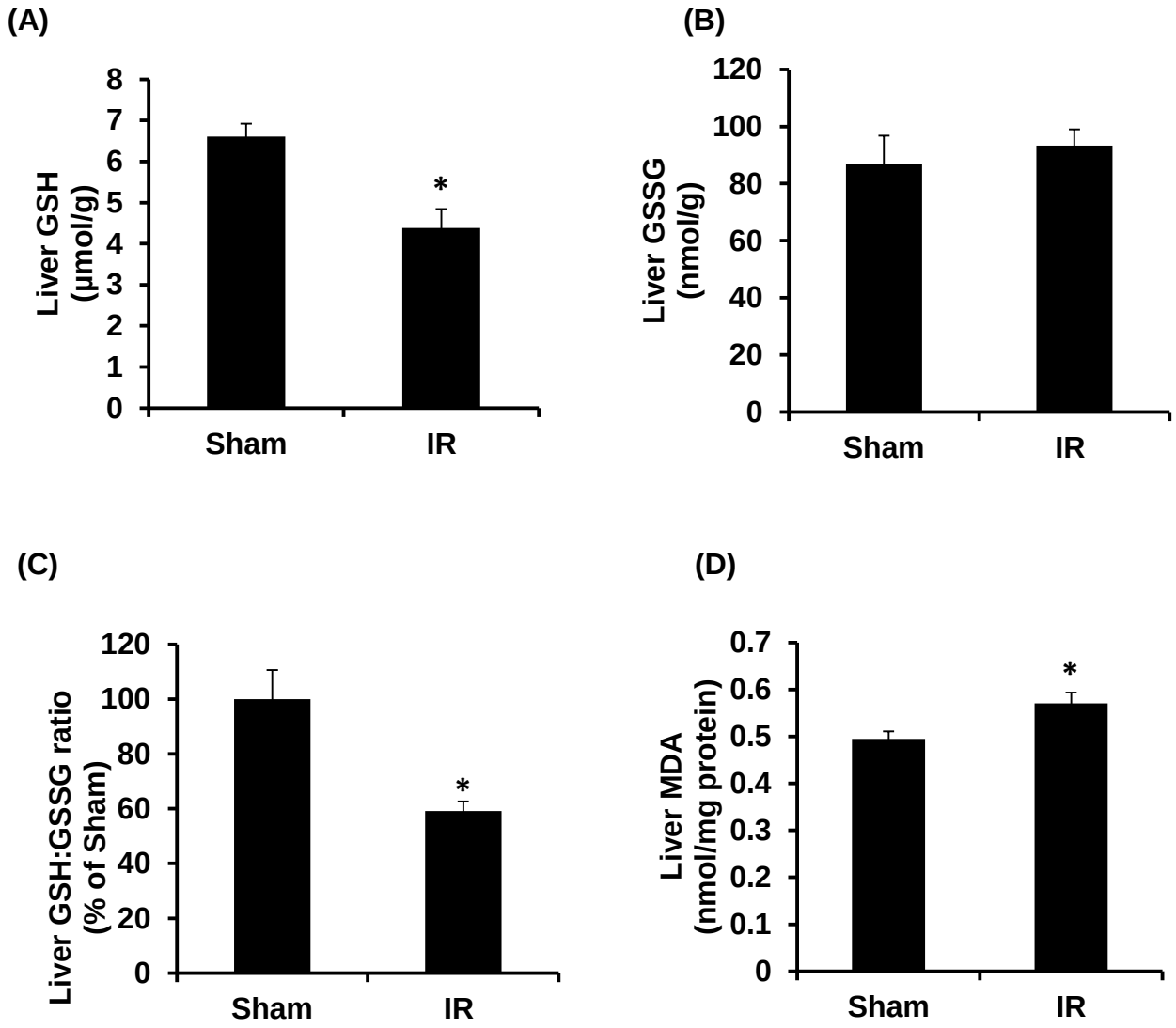
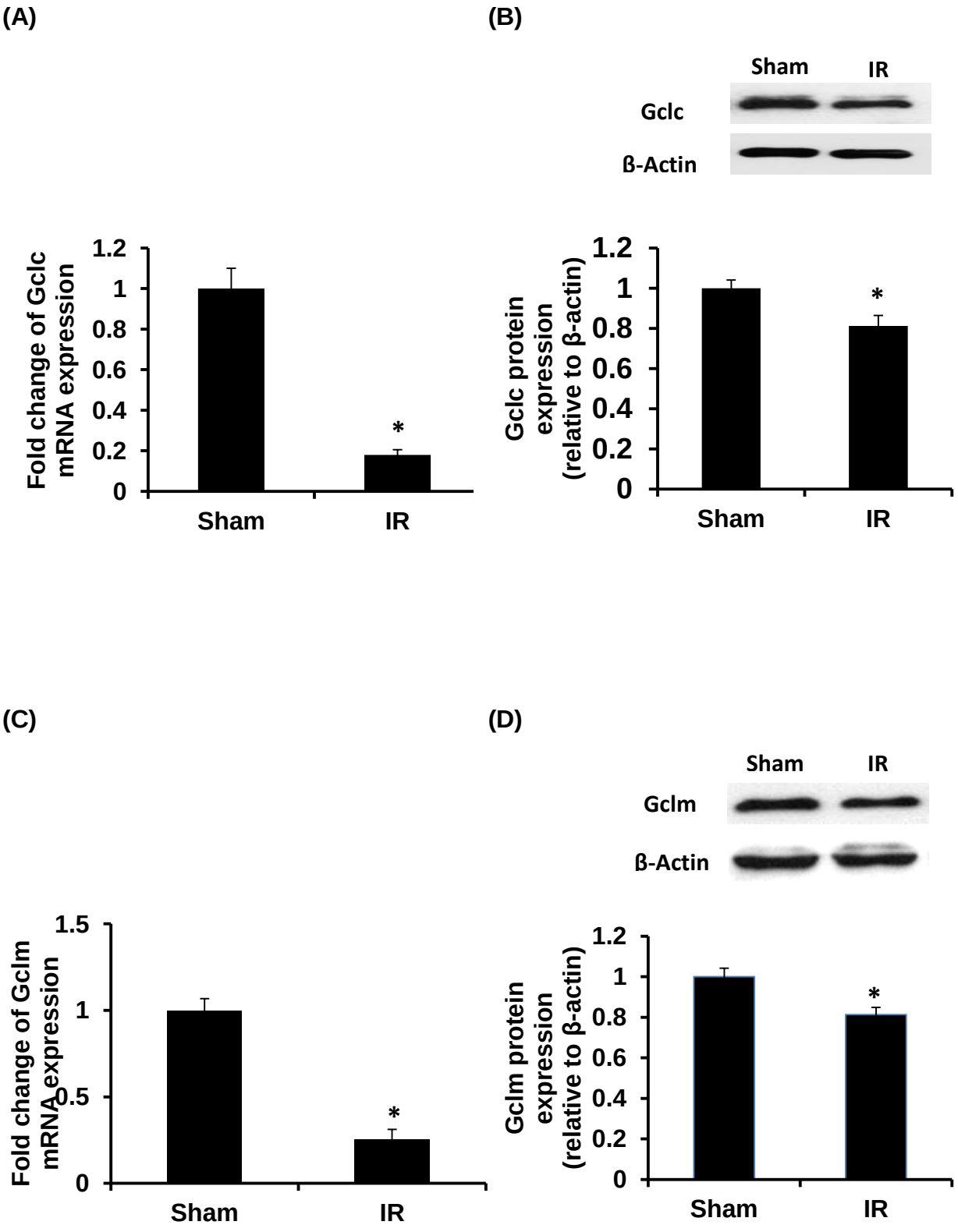


Figure 3.14 Effect of kidney ischemia-reperfusion on glutathione levels and lipid peroxidation in the liver

Liver reduced glutathione (GSH) (A), oxidized glutathione (GSSG) (B), a ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) (C)and liver malondialdehyde (MDA) levels (D) were measured in rats subjected to renal ischemia-reperfusion injury (IR) or sham-operation. Results are expressed as mean ± SE (n = 4 for each group). *$p < 0.05$ when compared with the value obtained from the sham-operated group.


(A)
Fold change of Gclc mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
*
Sham
IR
(B)
Sham
IR
Gclc
β-Actin
Gclc protein expression (relative to β-actin)
1.2
1
0.8
0.6
0.4
0.2
0
*
Sham
IR
(C)
Fold change of Gclm mRNA expression
1.5
1
0.5
0
*
Sham
IR
(D)
Sham
IR
Gclm
β-Actin
Gclm protein expression (relative to β-actin)
1.2
1
0.8
0.6
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0.2
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Sham
IR

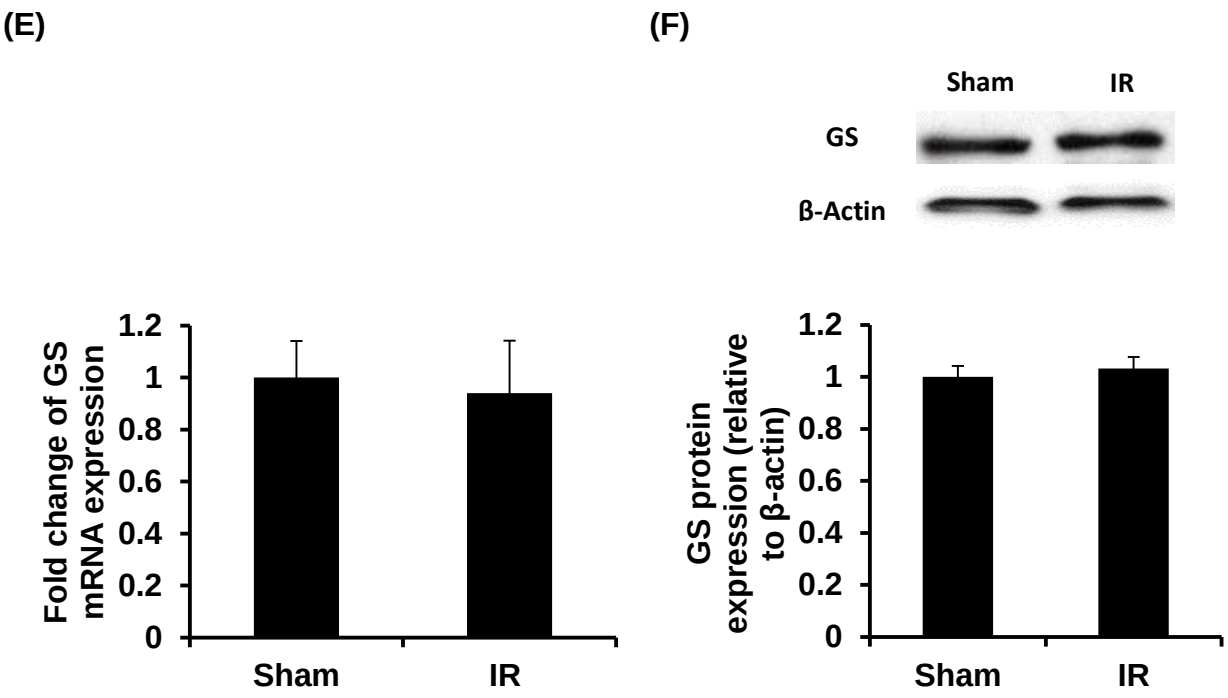

Figure 3.15 Expression of glutathione synthetic enzymes in the liver

Liver glutamate-cysteine ligase catalytic subunit (Gclc) mRNA (A) and protein (B), glutamate-cysteine ligase modifier subunit (Gclm) mRNA (C) and protein (D), and glutathione synthase (GS) mRNA (E) and protein (F) were measured in rats subjected to renal ischemia-reperfusion injury (IR) or sham-operation. Results are expressed as mean ± SE (n = 4 for each group). *p < 0.05 when compared with the value obtained from the sham-operated group.

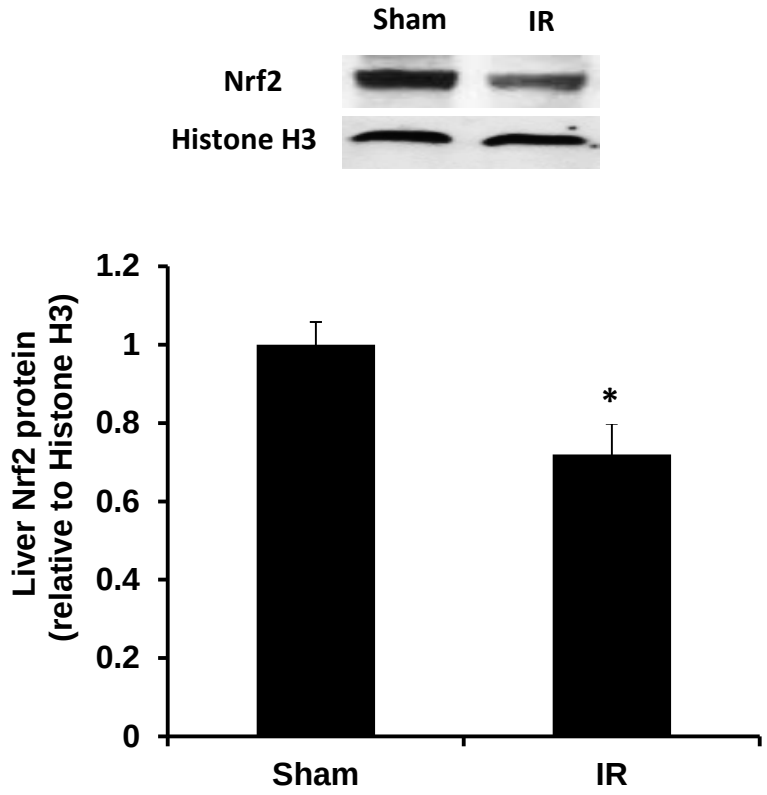

Figure 3.16 Expression of Nrf2 protein in the liver

The Nrf2 protein was determined by Western immunoblotting analysis of the liver nuclear fraction of rats subjected to renal ischemia-reperfusion injury (IR) or sham-operation. Results are expressed as mean ± SE (n = 4 for each group). *$p < 0.05$ when compared with the value obtained from the sham-operated group.

3.4.3 Renal ischemia-reperfusion inhibited the transsulfuration pathway in the liver

The transsulfuration pathway provides cysteine as an essential precursor for glutathione synthesis. To investigate whether renal IR affected this pathway in the liver, the expression of two enzymes (CBS and CSE) was examined. The mRNA and protein levels of CSE were significantly decreased in the liver of rats subjected to renal IR (Figure 3.17A, 3.17B). However, the mRNA and protein levels of CBS were not significantly changed (Figure 3.17C, 3.17D). In accordance with a decreased expression of CSE in the transsulfuration pathway, the level of hepatic cysteine was significantly lower in rats subjected to renal IR than those in the sham-operated group (Figure 3.18A). The production of hydrogen sulfide was significantly reduced in the liver of rats subjected to renal IR (Figure 3.18B).

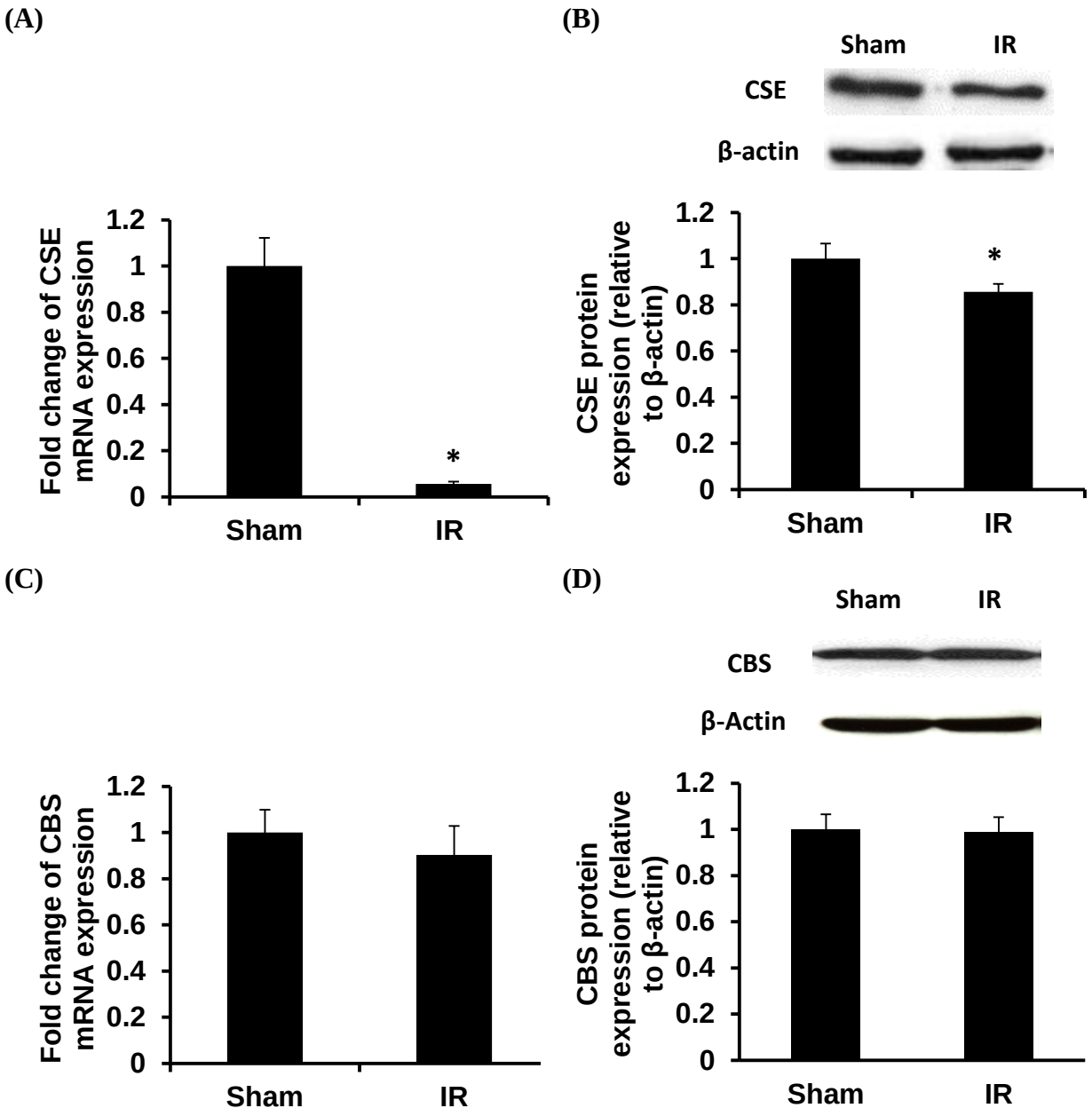

Figure 3.17 Expression of transsulfuration pathway enzymes in the liver

Cystathionine γ-lyase (CSE) mRNA (A) and protein (B), and cystathionine β-synthase (CBS) mRNA (C) and protein (D) were determined in the liver samples of rats subjected to renal ischemia-reperfusion injury (IR) or sham-operation. Results are expressed as mean ± SE (n = 4 for each group). *$p < 0.05$ when compared with the value obtained from the sham-operated group.

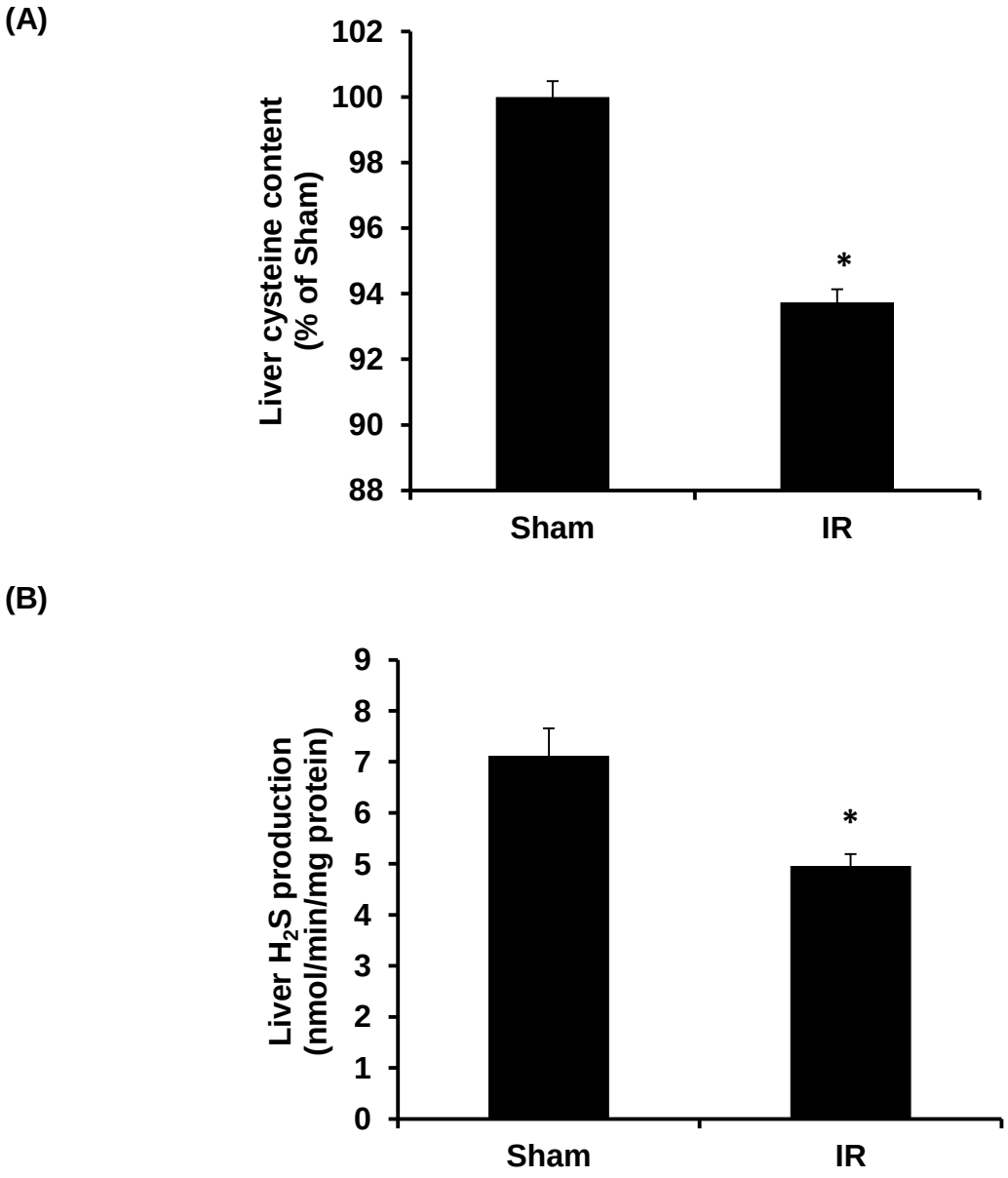

Figure 3.18 Hepatic cysteine content and hydrogen sulfide production

The cysteine content (A) and hydrogen sulfide (H_2S) production were measured in the liver of rats subjected to renal ischemia-reperfusion injury (IR) or sham-operation. Results are expressed as mean ± SE (n = 4 for each group). *$p < 0.05$ when compared with the value obtained from the sham-operated group.

3.4.4 Regulation of glutathione synthesis in HepG2 cells

To further investigate whether downregulation of hepatic CSE expression contributed to decreased glutathione synthesis, experiments were conducted in HepG2 cells. Treatment of cells with a CSE inhibitor (DL-propargylglycine, PAG) (Xu et al., 2009, Wang et al., 2014) significantly reduced intracellular glutathione level (Figure 3.9A). Furthermore, we verified whether an inhibition of Nrf2 expression could contribute to a decrease in Gcl expression as observed in vivo. Our results showed that transfection of HepG2 cells with Nrf2 siRNA not only inhibited Nrf2 expression (Figure 3.19B), but also significantly reduced mRNA expression of glutamate cysteine ligase subunits (Gclc and Gclm) (Figure 3.19C, 3.19D). These results suggested that the transsulfuration pathway and Nrf2 played an important role in regulating glutathione synthesis in hepatocytes.

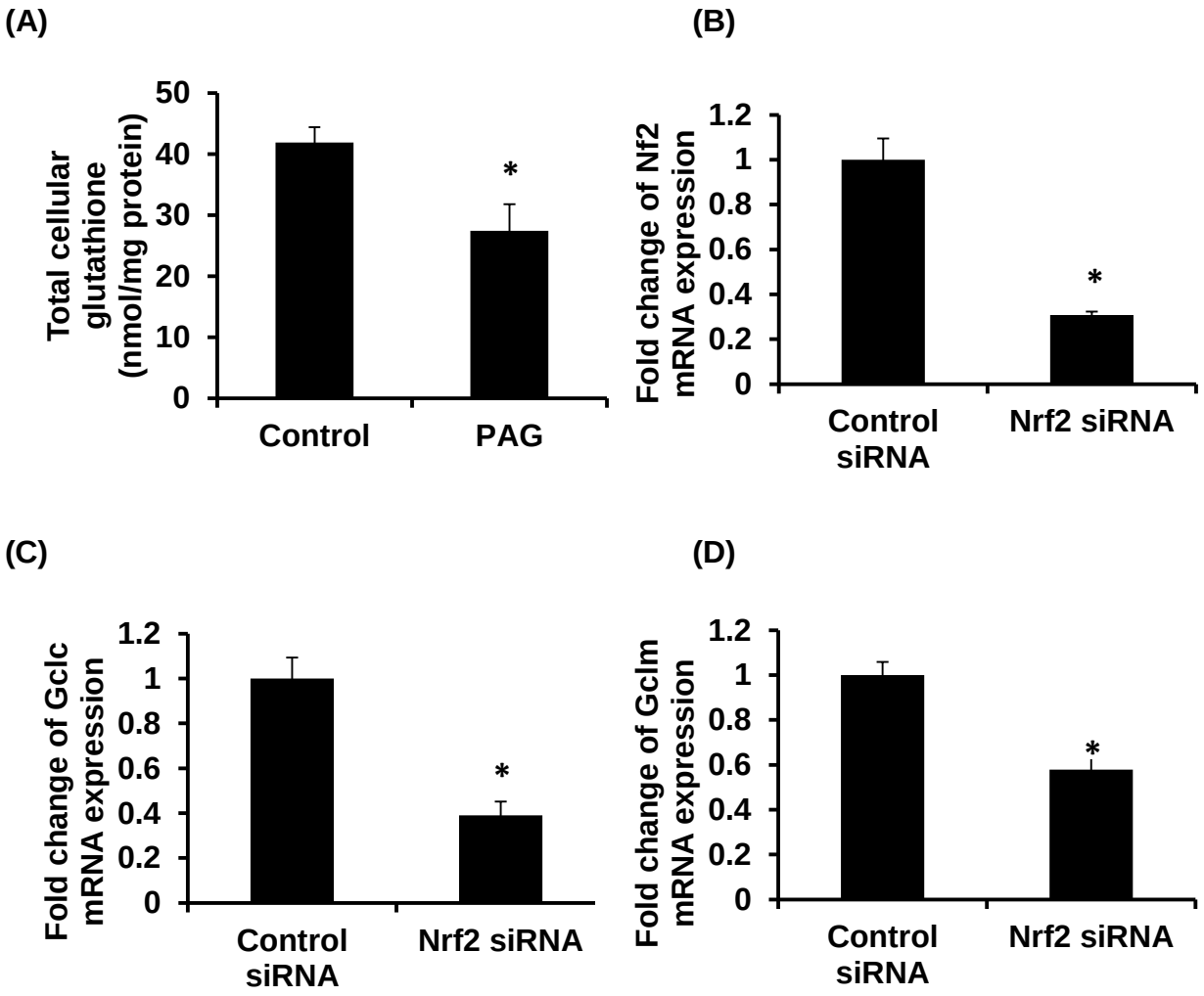

Figure 3.19 Glutathione measurement and siRNA transfection in HepG2

Cells were incubated in the absence (control) or presence of DL-propargylglycine (PAG) for 16h and total glutathione was measured (A). Cells were transfected with Nrf2 siRNA or scrambled siRNA (control). The mRNA of Nrf2 (B) and glutamate-cysteine ligase subunits Gclc (C) and Gclm (D) were determined. Results are expressed as mean ± SE (n = 4 for each group). *$p < 0.05$ when compared with the value obtained from control cells.

3.5 Discussion

In the present study, renal IR caused local and distant organ injury which was accompanied by a marked decrease in plasma and hepatic glutathione levels. Depletion of glutathione, a major endogenous non-enzymatic antioxidant might compromise the ability of the body to cope with oxidative stress locally and systemically. Our study, for the first time, has identified that decreased expression of glutamate-cysteine ligase, a key enzyme for glutathione biosynthesis as well as reduced CSE-mediated cysteine production through the transsulfuration pathway may be responsible for hepatic glutathione depletion upon renal IR. This, in turn, dampens the antioxidant defense mechanism and contributes to renal IR-induced oxidative stress.

Glutathione serves as a major endogenous antioxidant against oxidative stress. Under physiological conditions, more than 90% of the cellular glutathione pool is in the reduced form (GSH) and less than 10% is in the oxidized (disulfide) form (GSSG). In the present study, the ratio of reduced (GSH) to oxidized (GSSG) glutathione in the liver was markedly decreased in rats subjected to renal IR. The equilibrium between reduced (GSH) and oxidized (GSSG) glutathione reflects the redox potential of a given tissue. Lower GSH to GSSG ratio observed in the liver and plasma indicated that oxidative stress occurred in the distant organs as well as systemically upon renal IR. This was accompanied by increased hepatic lipid peroxidation. The liver plays an important role in regulating glutathione homeostasis due to its high capacity for glutathione synthesis. However, renal IR caused a significant reduction in hepatic glutathione levels and impaired liver function, which was in line with findings by other investigators (Golab et al., 2009, Kadkhodaee et al., 2009). Further investigation revealed that renal IR caused a significant decrease in the expression of hepatic glutamate-cysteine ligase, a key enzyme

responsible for glutathione synthesis. Nrf2 is a major transcription factor that induces the expression of antioxidant enzymes including those responsible for glutathione synthesis. Activation of Nrf2 has been implicated as a potential therapeutic target in kidney disease (Liu et al., 2014, Noel et al., 2015, Shelton et al., 2015). In the present study, renal IR resulted in a significant decrease in Nrf2 protein level in the nucleus. Transfection of hepatocytes with Nrf2 siRNA led to a marked reduction of glutamate-cysteine ligase (Gclc, Gclm) expression. It is plausible that renal IR might impair Nrf2-dependent glutamate-cysteine ligase expression, which, in turn, led to a decrease in hepatic glutathione synthesis upon renal IR. However, the mechanism by which renal IR leads to a decrease in hepatic nuclear Nrf2 protein remains to be investigated.

The availability of cysteine is another important determinant in modulating glutathione homeostasis as this amino acid is a precursor for glutathione biosynthesis (Lu, 1999, Chen et al., 2013, Lu, 2013). The transsulfuration pathway catalyzed by two enzymes, CBS and CSE, is the major source of de novo synthesized cysteine in mammalian cells. Relative to other organs, CBS and CSE are highly expressed in the liver. It has been estimated in murine liver that CSE protein levels may be 60 fold higher than CBS protein (Kabil et al., 2011). The liver's high rate of transsulfuration activity may contribute to its high capacity for glutathione biosynthesis, compared to other organs (Lu, 1999). Our results suggested that lower expression of hepatic CSE upon renal IR could lead to a decrease in *de novo* cysteine production, which, in turn, limited the availability of cysteine and hence decreased glutathione biosynthesis in the liver. Downregulation of CSE expression impaired homocysteine metabolism through the transsulfuration pathway, which, in turn, led to a significant elevation of homocysteine levels in

the plasma, a condition known as hyperhomocysteinemia. Hyperhomocysteinemia is regarded as a risk factor for cardiovascular disease due to its association with vascular endothelial dysfunction and atherosclerosis (Clarke et al., 1991, McCully, 1996, Edirimanne et al., 2007, Prathapasinghe et al., 2007). We have demonstrated that elevation of homocysteine levels in the kidney due to downregulation of the transsulfuration pathway directly linked to IR-induced kidney injury (Prathapasinghe et al., 2007, Prathapasinghe et al., 2008). The transsulfuration pathway serves as the source of *de novo* synthesized cysteine in mammalian cells while cysteine is a precursor for glutathione synthesis. Inhibition of CBS and/or CSE in the transsulfuration pathway by renal IR can affect cysteine biosynthesis and subsequently glutathione generation. It is plausible that elevated homocysteine levels together with reduced *de novo* cysteine synthesis and glutathione generation may act synergistically in the development of AKI and multiple organ dysfunction. It has been reported that administration of N-acetylcysteine is effective in improving renal function in rats with acute kidney failure (DiMari et al., 1997, Conesa et al., 2001, Mehta et al., 2002). Future studies are warranted to examine the effect of N-acetylcysteine/cysteine administration on distant organs and homocysteine metabolism in AKI. Aside from metabolizing homocysteine to cysteine through the transsulfuration pathway, CBS and CSE are also responsible for hydrogen sulfide production through desulfurization reactions. In accordance with the low level of CSE expression, hydrogen sulfide production was significantly reduced in the liver isolated from rats subjected to renal IR. Hydrogen sulfide is a potent gasotransmitter that has multifaceted effects under both physiological and pathophysiological processes including antioxidant, anti-inflammatory effect and protection of myocardial IR injury (Kimura and Kimura, 2004, Whiteman et al., 2005, Elrod et al., 2007, Lee

et al., 2014). Renal IR-induced oxidative stress, hyperhomocysteinemia and low hydrogen sulfide generation may pose adverse effect to the cardiovascular system.

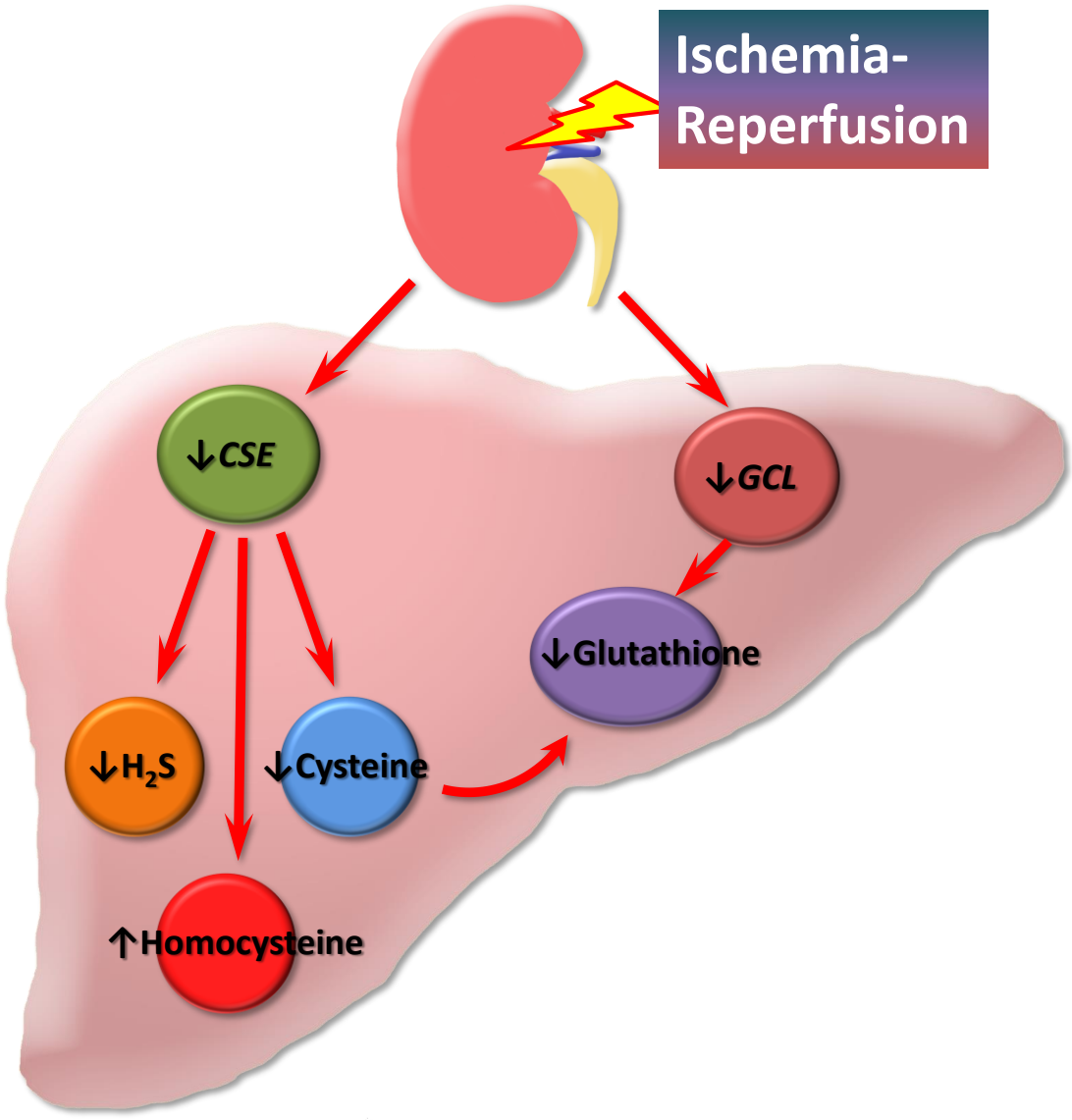

Figure 3.20 Proposed mechanism of kidney ischemia-reperfusion induced reduction of hepatic glutathione biosynthesis

Renal ischemia-reperfusion (IR) inhibits glutathione biosynthesis in the liver through (1) a decrease in the expression of cystathionine γ-lyase (CSE) in the transsulfuration pathway which elevates homocysteine as well as reduces *de novo* cysteine production and hydrogen sulfide (H_2S) generation; and (2) a decrease in the expression of glutamate-cysteine ligase (GCL) which is a key enzyme responsible for glutathione biosynthesis.

In conclusion, renal IR elicits liver injury which is accompanied by reduced hepatic production of glutathione, an important endogenous antioxidant. Several lines of evidence obtained from the present study suggest that the remote effect of renal IR on liver glutathione depletion is caused by (1) inhibition of Nrf2-mediated expression of glutamate-cysteine ligase, a key enzyme that regulates glutathione biosynthesis; and (2) down-regulation of *CSE* expression in the transsulfuration pathway that limits the availability of cysteine, an essential precursor for glutathione biosynthesis as well as elevates homocysteine levels (Figure 3.20). Such an aberrant response may play a critical role in distant organ injury which, in turn, exacerbates kidney injury. Because the liver has such a high rate of the transsulfuration activity and is the major organ for glutathione biosynthesis, future studies are warranted to investigate whether restoration of enzymes that are responsible for glutathione, homocysteine and cysteine homeostasis could alleviate AKI-induced oxidative stress and distant organ injury.

3.6 Acknowledgements

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Chapter IV. Study II

Manuscript II: Kidney ischemia-reperfusion elicits hepatic inflammatory response and dysfunction

Manuscript in preparation

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4.1 Abstract

Ischemia-reperfusion (IR) is a common risk that causes acute kidney injury (AKI). AKI is often associated with dysfunction of remote organs also known as distant organ injury. Inflammatory response plays an important role in IR-induced injury. Although increased proinflammatory cytokines have been detected in the distant organs after renal IR, their sources remain uncertain. The liver function is often compromised in patients with AKI and in animal models. However, the underlying mechanisms are not fully understood. In the present study, we investigated the effect of renal IR on the changes of proinflammatory biomarkers in the liver of Sprague-Dawley rats and the mechanism involved. The left kidney was subjected to ischemia for 45min followed by reperfusion for 1h or 6h with right nephrectomy. Renal IR impaired kidney and liver function as indicated by increased plasma creatinine and transaminase levels. Renal IR caused a significant increase in proinflammatory cytokine (MCP-1, TNF-α, IL-1β and IL-6) protein levels in the liver, kidney and plasma. Activation of a nuclear transcription factor kappa B (NF-κB) was detected in the liver at 1h and 6h after renal IR. This was accompanied with a significant increase in the hepatic mRNA levels of proinflammatory cytokines. The myeloperoxidase activity was elevated and inflammatory foci were detected in the liver at 6h after renal IR, indicating neutrophil infiltration. These results suggest that renal IR can directly activate NF-κB and stimulate early expression of proinflammatory cytokines in the liver, which may contribute to local and distant organ injury.

4.2 Introduction

Acute kidney injury (AKI) is characterized by a decline in kidney function over a short period of time and is associated with high mortality. AKI often leads to multiple organ dysfunctions known as distant organ injury (Miyazawa et al., 2002, Grams and Rabb, 2012, Yap and Lee, 2012, Doi and Rabb, 2016). Kidney ischemia-reperfusion (IR) is one of the most common causes for AKI (Thadhani et al., 1996, Mehta et al., 2004, Lameire et al., 2005). It occurs in clinical situations such as kidney transplantation, cardiac surgery, sepsis and in critically ill patients. Despite the advancement in kidney transplantation, the mortality in patients with AKI complicated by multi-organ dysfunction remains high worldwide (estimated to be 50%). In experimental animals, renal IR is shown to cause distant organ injury in the heart, lung, intestine and liver (Kelly, 2003, Hassoun et al., 2007, Liu et al., 2008, Grams and Rabb, 2012, Lane et al., 2013, Druml, 2014, Ologunde et al., 2014, Shang et al., 2016). Clinical studies have shown that AKI patients with multiple organ dysfunctions have a worse prognosis than those that have AKI alone (Bagshaw et al., 2005, Park et al., 2011). Although the remote effects of AKI have been noted for a long time, the precise mechanisms responsible for pathological changes in the distant organs are not well understood.

Liver function is often impaired in patients with AKI and in animal models with renal IR or nephrectomy (Chertow et al., 1995, Hassoun et al., 2007, Grams and Rabb, 2012). It appears that AKI patients complicated with liver dysfunction have poorer clinical outcomes (Liano et al., 1996, Mehta, 2002, Bagshaw et al., 2005, Park et al., 2011). Oxidative stress, systemic inflammatory response and increased leukocyte trafficking have been implicated in AKI associated distant organ injury. In experimental animals, AKI-induced liver injury manifests with

increased oxidative stress, hepatocyte vacuolization and necrosis, elevated cytokine production and increased leukocyte infiltration (Golab et al., 2009, Park et al., 2011, Doi and Rabb, 2016, Shang et al., 2016). Liver plays a central role in metabolism, redox balance, immune regulation and detoxification. Our recent study shows that renal IR causes liver injury with reduced hepatic production of glutathione, a major endogenous antioxidant. We have identified that renal IR directly inhibits hepatic glutathione production through downregulation of the transsulfuration and Nrf-2 mediated glutathione biosynthesis pathway, leading to oxidative stress in rats (Shang et al., 2016). Renal IR-induced redox imbalance in the liver may contribute to local and systemic oxidative stress (Golab et al., 2009, Shang et al., 2016).

Inflammatory response plays a critical role in IR injury (Thurman, 2007). Upon renal IR, proinflammatory cytokines such as MCP-1, TNF-α, IL-1β and IL-6 generated in the kidney are considered as major contributors to local and systemic inflammation (Sung et al., 2002, Akcay et al., 2009, Lee et al., 2011, Ologunde et al., 2014, Wang et al., 2014). It has been suggested that the remote effect of renal IR is initiated by an increased influx of cytokines and infiltration of immune cells to the distant organs (Lee et al., 2011). Studies have shown increased levels of cytokines in the liver of experimental animals including in mice at 5h after renal IR (Park et al., 2011), in rats at 6h and 24h after renal IR (Golab et al., 2009) and in pigs at 48h after renal IR (Gardner et al., 2016). However, the origin(s) of increased cytokines in the liver after renal IR have not been well identified. Nuclear factor-kappa B (NF-κB) is one of the key transcription factors that regulates the expression of proinflammatory cytokines and immune response (Sung et al., 2002, Lawrence, 2009). Our previous study showed that IR stimulated chemokine MCP-1 expression through the activation of NF-κB in the kidney (Sung et al., 2002, Wang et al., 2013).

It is not known whether renal IR can acutely trigger NF-κB activation and stimulate cytokine expression in the liver. More research is required to identify the molecular mechanisms behind distant organ inflammatory response during renal IR. In the present study, we investigated the early impact of renal IR on liver function and inflammatory response in a rat model.

4.3 Materials and methods

4.3.1 Animal model

Renal IR was induced in rats as described in our previous studies (Prathapasinghe et al., 2007, Wu et al., 2010, Wang et al., 2013). In brief, Sprague-Dawley rats (250-300g, male) were anesthetised through inhalation of 3% isoflurane/oxygen gas prior to surgery. Renal ischemia was induced by clamping the left kidney pedicle for 45 min. At the end of ischemia, the clamp was removed to allow reperfusion in the left kidney with right nephrectomy. Rats were sacrificed at 1h or 6h after reperfusion. As a control (sham-operated), rats were subjected to the same surgical procedure without inducing ischemia and were sacrificed at the corresponding time points. Blood was collected from the portal vein and centrifuged at 3000 *g* for 20 min for plasma preparation. Plasma creatinine, alanine aminotransferase and aspartate aminotransferase were measured by using the Cobas C111 Analyzer (Roche, Laval, QC). Cytokines and neutrophil gelatinase-associated lipocalin (NGAL) in the plasma, kidney and liver were measured by using the MesoScale Discovery electrochemiluminescence platform (Rockville, MD). Liver myeloperoxidase (MPO) activity was measured by using a commercial kit (Abcam Inc., Toronto, Canada). All procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and approved by the University of Manitoba Protocol Management and Review Committee.

4.3.2 Real-time polymerase chain reaction (PCR) analysis

Total RNAs were isolated from the kidney and liver with Trizol reagent (Invitrogen, Carlsbad, CA). The mRNA of MCP-1, TNF-α and IL-6 was determined by a real-time PCR analysis using the iQ5 real-time PCR detection system (Bio-Rad) and normalized with β-actin (Wu et al., 2010, Hwang et al., 2013, Wang et al., 2014). The primers (Invitrogen) used for rat mRNA measurement were: MCP-1 (119 bp), 5'- CAGAAACCAGCCAACTCTCA-3' (forward) and 5'- AGACAGCACGTGGATGCTAC-3' (reverse) (GeneBank accession number NM_031530), TNFα (215 bp), 5'- CCCAGACCCTCACACTCAGAT-3' (forward) and 5'- TTGTCCCTTGAAGAGAACCTG-3' (reverse) (GenBank, accession number NM_012675), IL-6 (161 bp), 5'- CCGGAGAGGAGACTTCACAG-3' (forward) and 5' – ACAGTGCATCATCGCTGTTC-3' (reverse) (GenBank accession number NM_012589) and ß-actin (198 bp), 5'- ACAACCTTCTTGCAGCTCCTC-3' (forward) and 5'- GACCCATACCCACCA TCACA-3' (reverse) (GenBank accession number NM_031144).

4.3.3 Electrophoretic mobility shift assay (EMSA)

The binding activity of NF-κB with DNA was measured by electrophoretic mobility shift assay (EMSA) (Thermo Fisher Scientific, Waltham, MA). In brief, nuclear proteins were prepared from the liver after renal IR or sham-operation as described in our previous studies (Wu et al., 2010). Nuclear proteins (2μg) were incubated with biotin-labeled oligonucleotides containing a consensus sequence specific for the NF-κB/ DNA binding site (5'-AGTTGAGGGGACTTTCCCAGGC-3') (Promega, Madison, WI).

4.3.4 Histological examination

A portion of the kidney and the liver was immersion fixed in 10% neutral-buffered formalin followed by embedding in paraffin. The paraffin-embedded cross sections (5μm) were prepared

and stained with hematoxylin and eosin (H&E) to examine histological changes in the kidney and the liver as described in our previous studies (Wang et al., 2013). Images were captured by using Olympus BX43 microscope with an Olympus QColor3 digital camera. The slides were analyzed at ×100 ×200 and ×400 magnification using Image-Pro Plus software (version 7.0, Media Cybernetics, Bethesda, MD, USA).

4.3.5 Statistical analysis

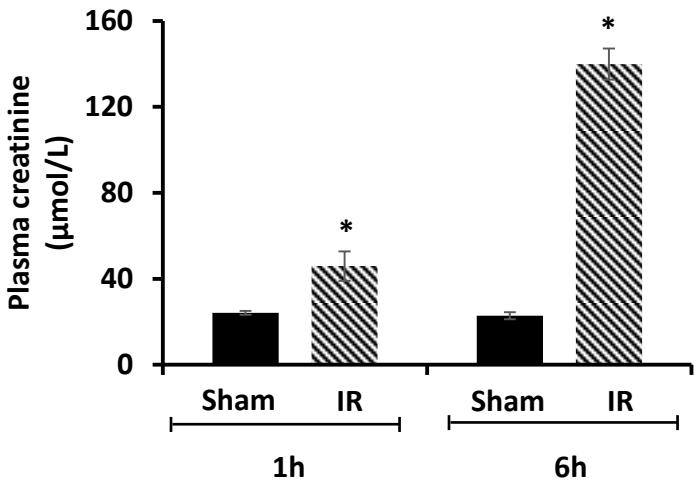
Results were analyzed using a two-tailed Student's t-test. *P* values less than 0.05 were considered statistically significant.

4.4 Results

4.4.1 Renal ischemia-reperfusion caused kidney and liver injury

Renal ischemia for 45 min followed by reperfusion for 1h or 6h caused a significant elevation of plasma creatinine levels (Figure 4.21A), indicating that the kidney was damaged by IR. There was a significant increase in plasma AST levels in rats at 6h after renal IR (Figure 4.21B), suggesting that renal IR caused liver injury. These results indicated that renal IR not only caused kidney injury but also impaired distant organ function of the liver.

(A)



(B)

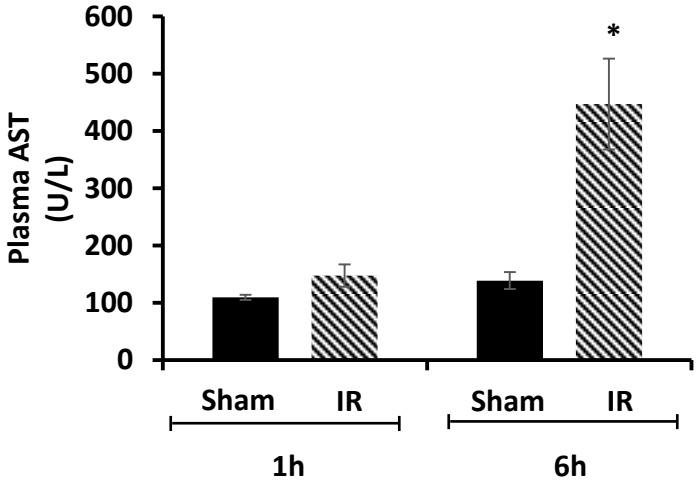


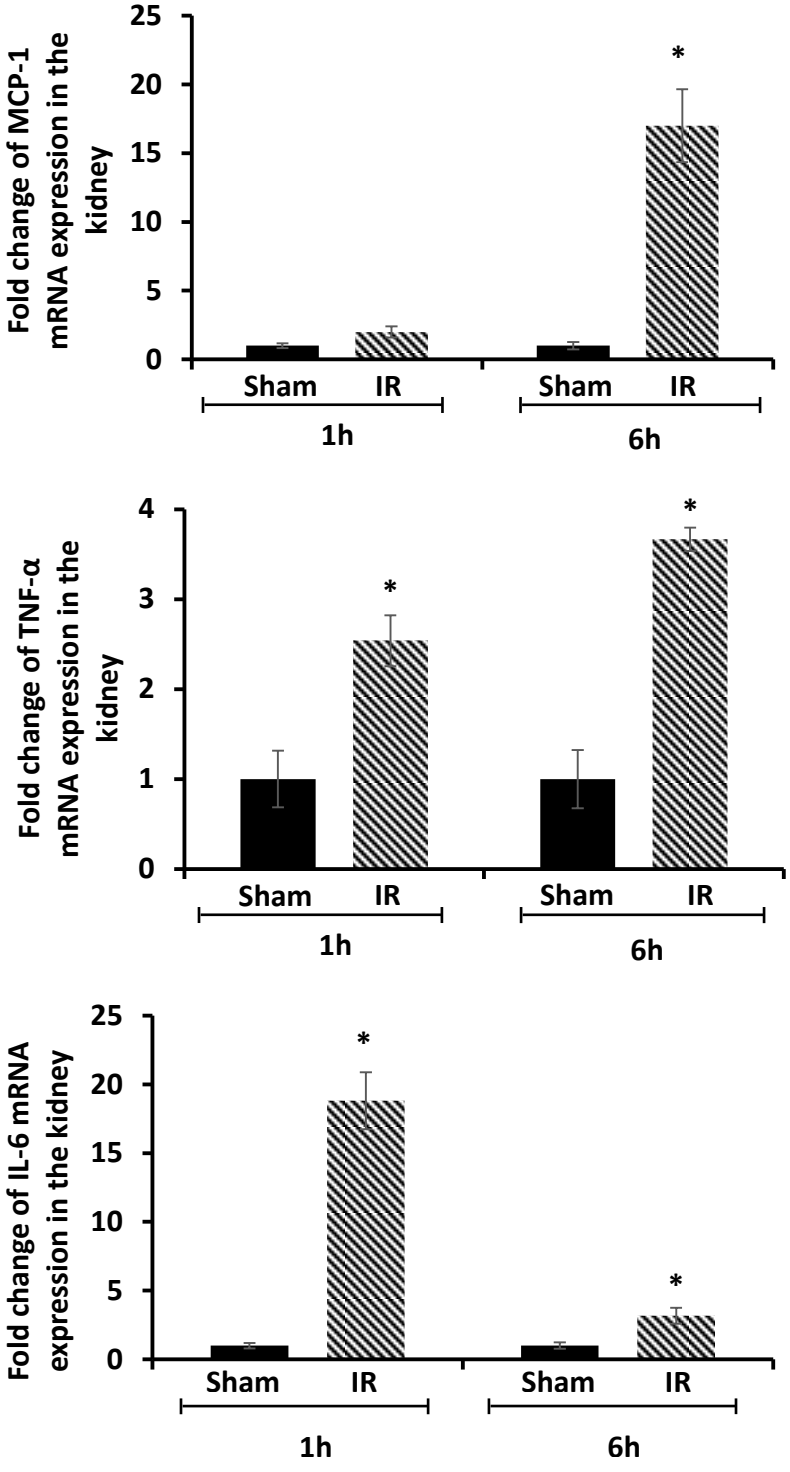
Figure 4.21 Effect of kidney ischemia-reperfusion on kidney and liver function

The left kidney of rats was subjected to 45 min ischemia followed by 1 h and 6h of reperfusion (IR). As a control, rats were subjected to a sham-operation without inducing ischemia (Sham). Plasma creatinine (A) and aspartate aminotransferase (AST) (B) were measured. Results are expressed as mean ± SE (n = 5 for each group). *p < 0.05 when compared with the value obtained from the sham-operated group.

4.4.2 Renal ischemia-reperfusion increased inflammatory cytokine expression in the kidney and liver

To examine whether renal IR-induced inflammatory response in local and distant organs, we first examined changes in mRNA expression of proinflammatory cytokines in the kidney and in the liver. Compared to the sham-operated rats, there was a significant elevation of TNFα and IL6 mRNA in the kidney (Figure 4.22A) as well as in the liver (Figure 4.22B) at 1h and 6h after renal IR. Interestingly, a significant elevation of MCP-1 mRNA in the kidney was observed at 6h after renal IR (Figure 4.22A), while it was more significant at 1h after renal IR in the liver (Figure 4.22B). These results indicated that the expression of MCP-1 may be time-dependant in different tissues. The protein levels of these proinflammatory cytokines were measured in the kidney, liver and plasma at 6h after renal IR. Renal IR resulted in a significant increase in TNF-α, IL-6 and MCP-1 protein levels in the kidney as well as in the liver (Figure 4.23A, 4.23B). In accordance, there was a significant elevation of these proinflammatory cytokines in the plasma (Figure 4.23C).

(A)


Fold change of MCP-1 mRNA expression in the kidney
25
20
15
10
5
0
*
Sham
IR
Sham
IR
1h
6h
Fold change of TNF-α mRNA expression in the kidney
4
3
2
1
0
*
*
Sham
IR
Sham
IR
1h
6h
Fold change of IL-6 mRNA expression in the kidney
25
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IR
1h
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(B)

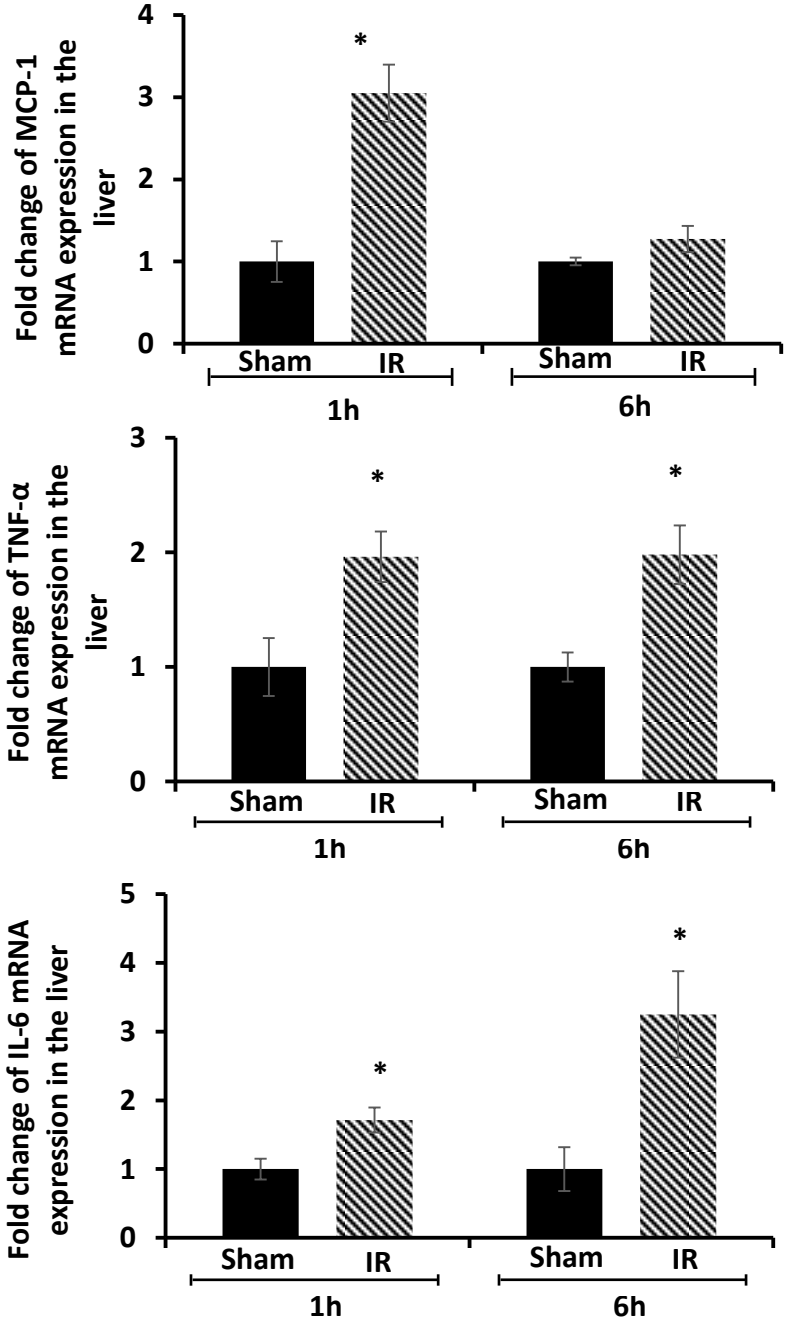
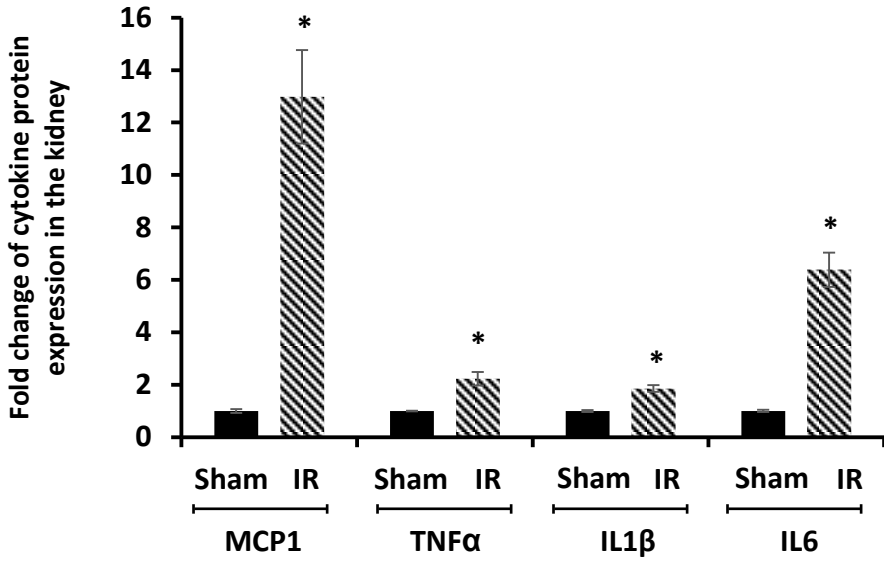


Figure 4.22 Effect of kidney ischemia-reperfusion on cytokine mRNA expression in the kidney and liver

The left kidney of rats was subjected to 45 min ischemia followed by 1 h and 6h of reperfusion (IR). As a control, rats were subjected to a sham-operation without inducing ischemia (Sham). The mRNA expressions of MCP-1, TNF-α and IL-6 were determined in the kidney (A) and liver

(B) by real-time PCR. Results are expressed as fold change to the Sham (n = 5 for each group). *$p < 0.05$ when compared with the value obtained from the sham-operated group.

(A)



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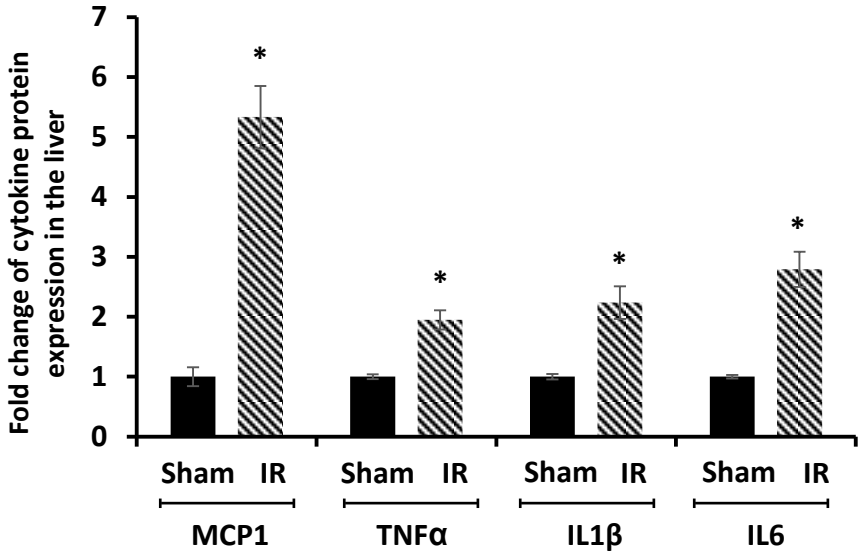


(C)

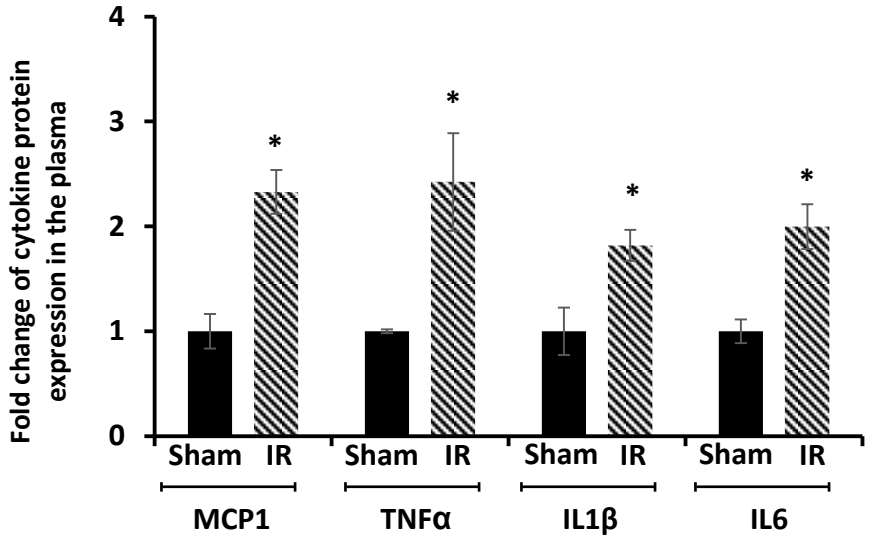


Figure 4.23 Effect of kidney ischemia-reperfusion on cytokine protein expression in the kidney, liver and plasma

The left kidney of rats was subjected to 45 min ischemia followed by 6h of reperfusion (IR). As a control, rats were subjected to a sham-operation without inducing ischemia (Sham). The protein expressions of MCP-1, TNF-α, IL-1β and IL-6 were measured in the kidney (A), liver (B) and plasma (C). Results are expressed as fold change to the Sham (n = 5 for each group). *p < 0.05 when compared with the value obtained from the sham-operated group.

4.4.3 Renal ischemia-reperfusion activated transcriptional factor NF-κB in the liver

We previously observed that renal IR caused an activation of NF-κB, a main transcription factor for cytokine expression (Sung et al., 2002). To investigate whether renal IR also activated NF-κB in a distant organ, liver nuclear proteins were prepared and activation of NF-κB was examined by EMSA. There was a significant increase in the NF-κB/ DNA binding activity in the liver of rats at 1h and 6h after renal IR (Figure 4.24). These results suggested that renal ischemic injury could directly trigger NF-κB activation in a distant organ.

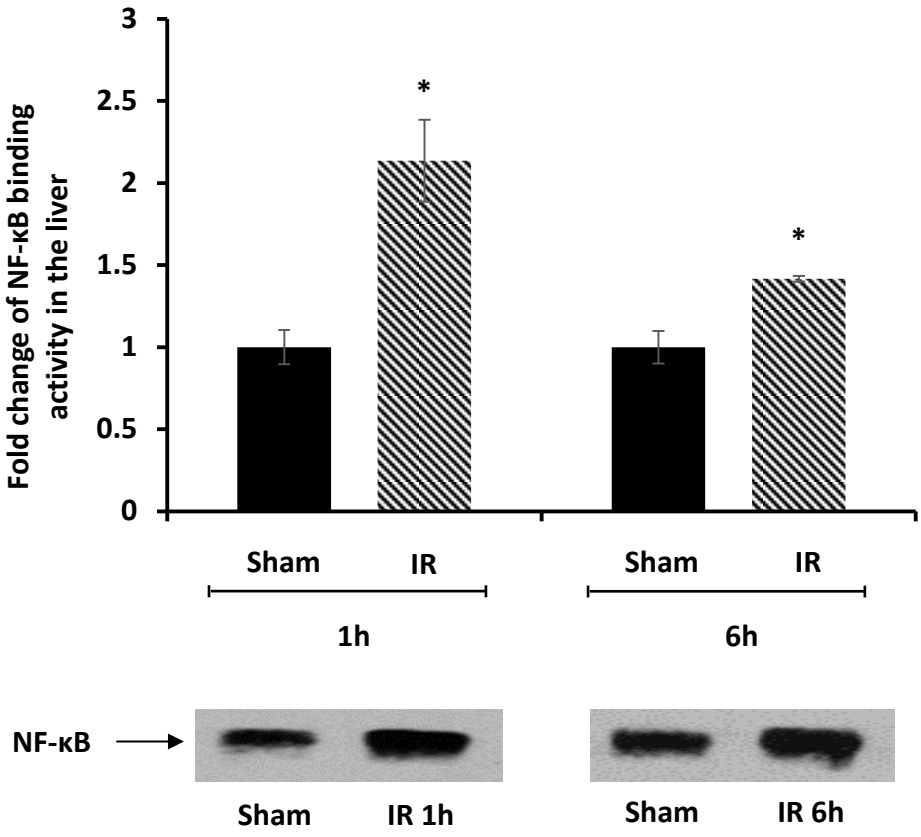


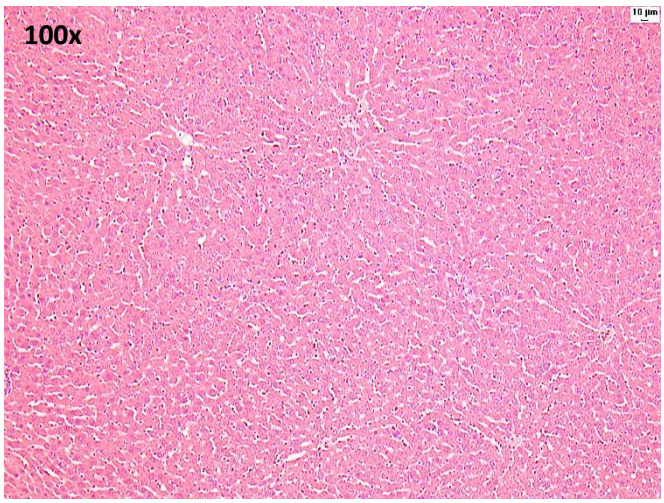
Figure 4.24 DNA binding activity of NF-κB in the liver

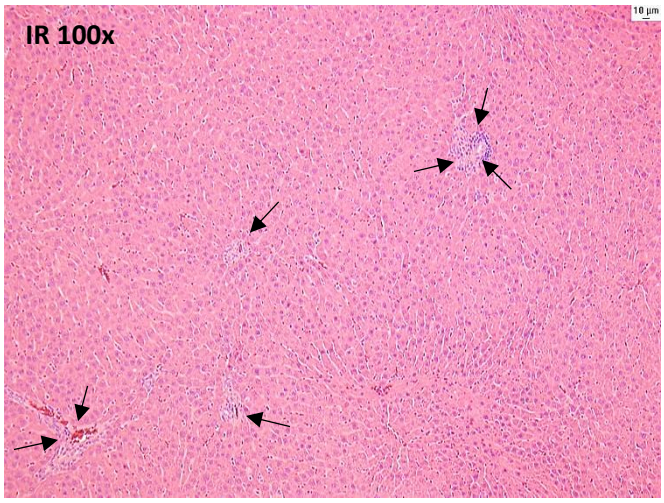
The left kidney of rats was subjected to 45 min ischemia followed by 1 h and 6h of reperfusion (IR). As a control, rats were subjected to a sham-operation without inducing ischemia (Sham). The DNA binding activity of NF-κB in the liver was determined by EMSA. Results are expressed as fold change to the Sham (n = 5 for each group). *$p < 0.05$ when compared with the value obtained from the sham-operated group.

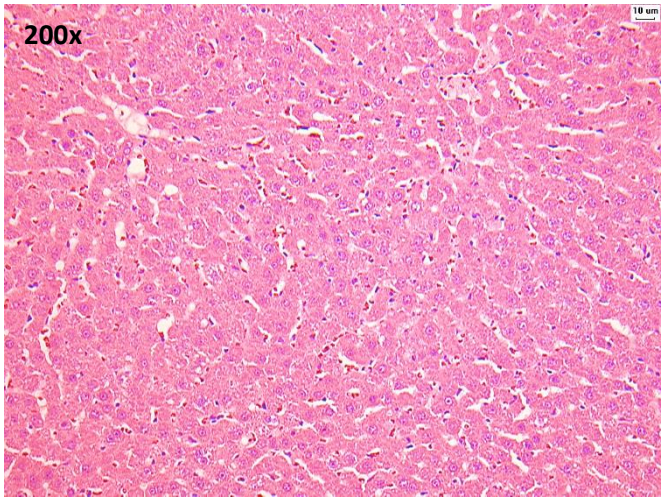
4.4.4 Renal ischemia-reperfusion changed kidney and liver histopathology, increased MPO activity and NGAL level

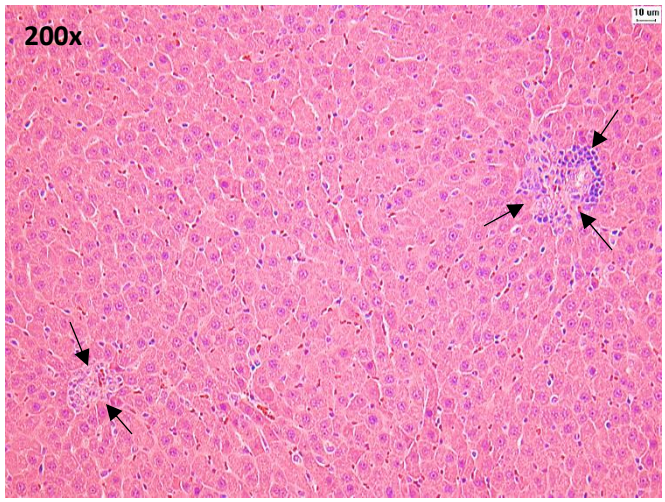
The liver morphology was examined by H&E staining. There was deposition of inflammatory foci (characterized by dense aggregates of cells) in the liver of rats subjected to renal IR (Figure 4.25). Accumulation and infiltration of leukocytes can be observed around the hepatic sinusoids. MPO activity was measured to determine the presence of neutrophils in the liver. The hepatic MPO activity significantly increased as early as 1h after renal IR and remained elevated at 6h after renal IR (Figure 4.26). Studies have shown that elevation of blood neutrophil gelatinase-associated lipocalin (NGAL) levels positively correlate with kidney injury (Akcay et al., 2009, Sharfuddin and Molitoris, 2011, Isaak et al., 2017) as well as with liver injury (Ariza et al., 2016, Yoshikawa et al., 2017). Compared to the sham-operated group, rats subjected to renal IR had a significantly higher level of NGAL in the kidney and in the liver (Figure 4.27A, 4.27B). Consistent with these results, there was also a marked elevation of NGAL levels in the plasma (Figure 4.27C). The H&E staining in the kidney showed a damaged structure with tubular necrosis, glomerulus enlargement and interstitial congestion of red blood cells in rats subjected to renal IR (Figure 4.28).

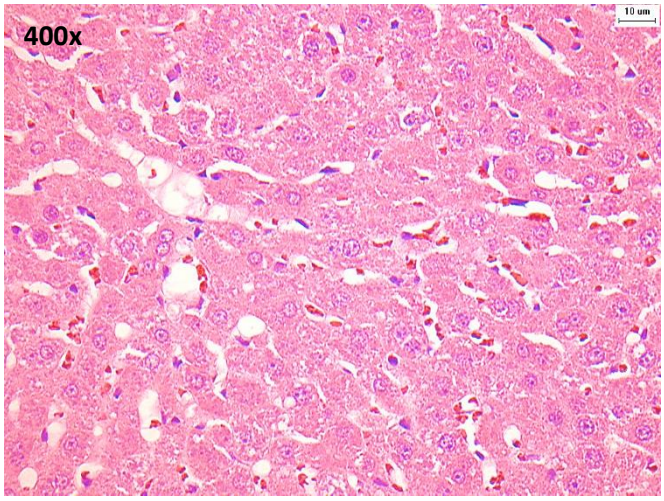
Sham **IR**











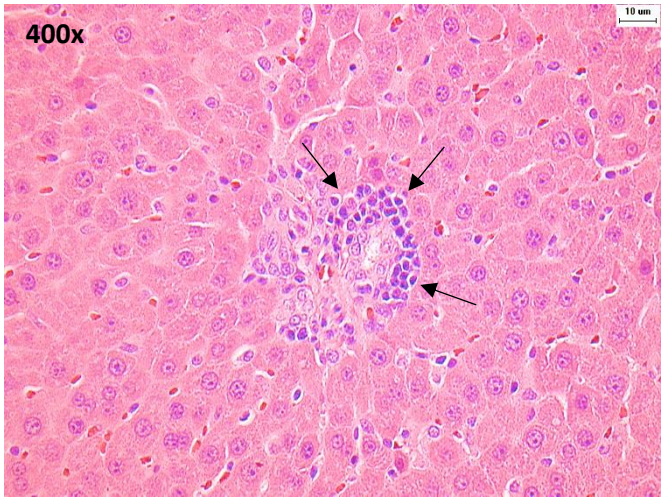


Figure 4.25 Effect of kidney ischemia-reperfusion on pathohistological change of the liver

The left kidney of rats was subjected to 45 min ischemia followed by 6 h of reperfusion (IR). As a control, rats were subjected to a sham-operation without inducing ischemia (Sham). The gross appearance of a mid-transverse plane of liver was examined by hematoxylin and eosin (H&E) staining and analyzed at ×100, ×200 and ×400 magnification. Livers of the IR group showed infiltration of leukocytes around the sinusoids (arrow) compared with the Sham group.

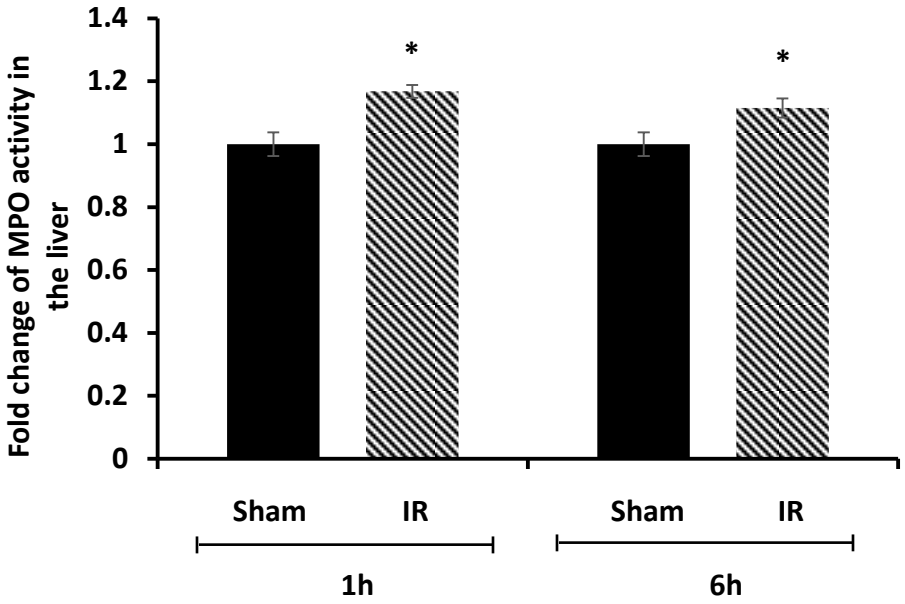
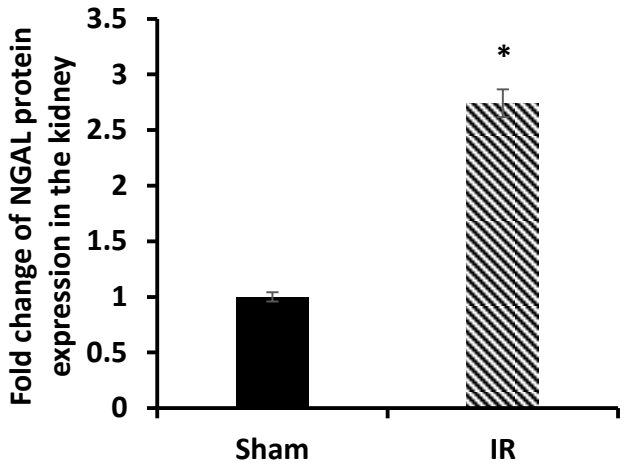


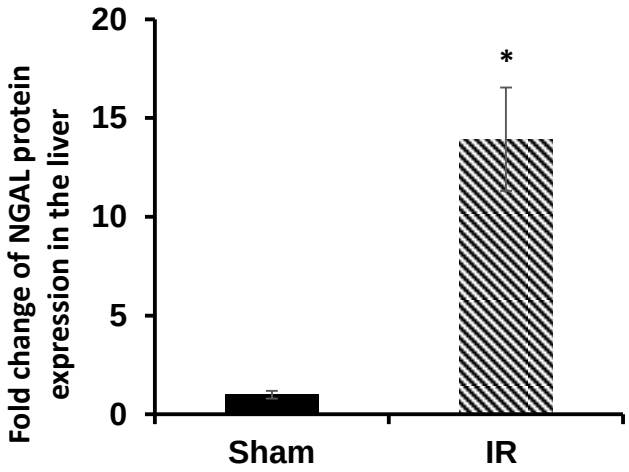
Figure 4.26 Effect of kidney ischemia-reperfusion on MPO activity in the liver

The left kidney of rats was subjected to 45 min ischemia followed by 1 h and 6h of reperfusion (IR). As a control, rats were subjected to a sham-operation without inducing ischemia (Sham). The myeloperoxidase (MPO) activity were determined in the liver. Results are expressed as fold change to the Sham (n = 5 for each group). *$p < 0.05$ when compared with the value obtained from the sham-operated group.

(A)



(B)



(C)

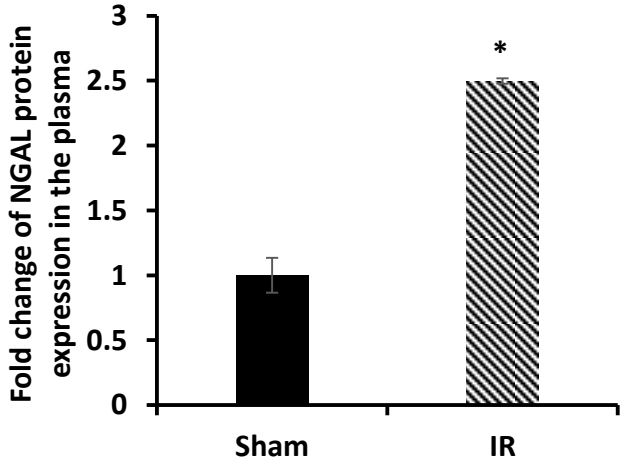
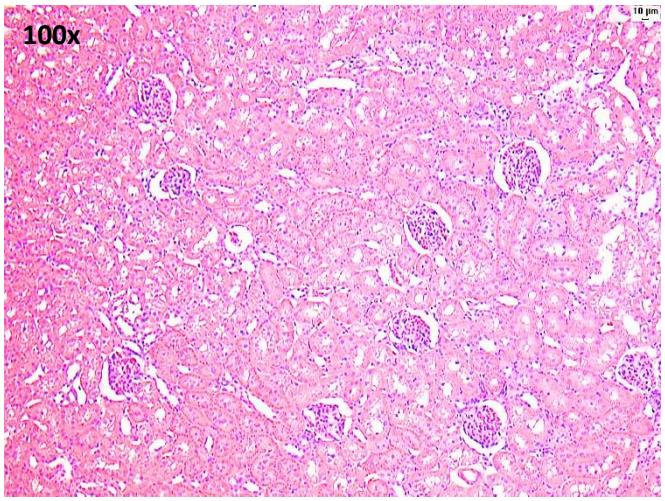
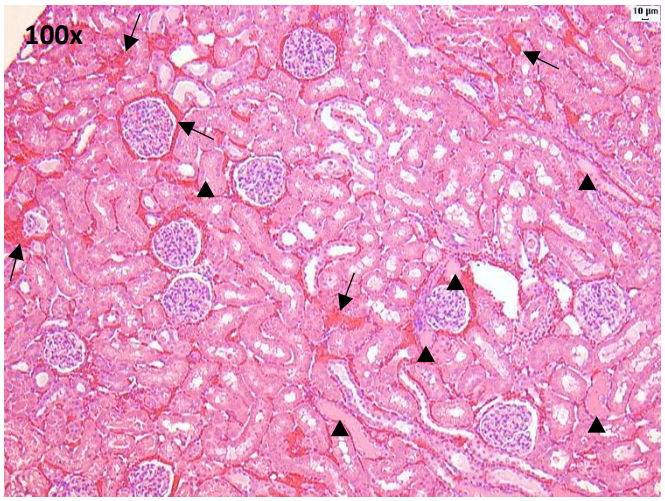


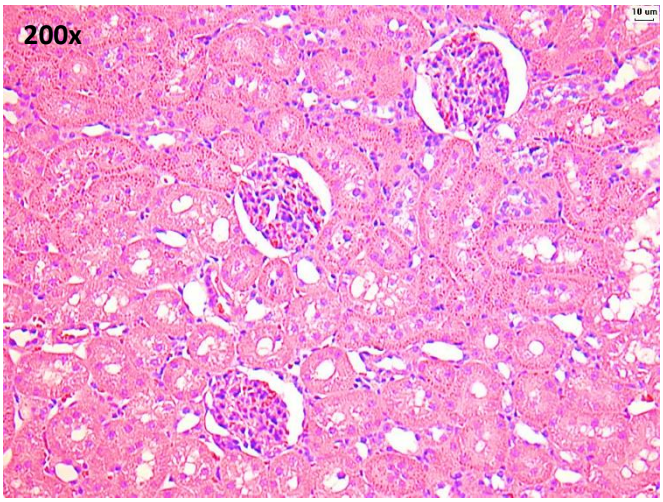
Figure 4.27 Effect of kidney ischemia-reperfusion on NGAL protein expression in the kidney, liver and plasma

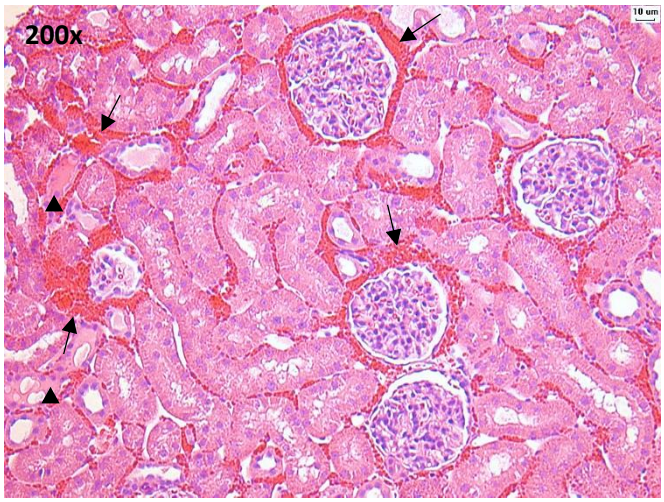
The left kidney of rats was subjected to 45 min ischemia followed by 6h of reperfusion (IR). As a control, rats were subjected to a sham-operation without inducing ischemia (Sham). The protein expressions of Neutrophil gelatinase-associated lipocalin (NGAL) were measured in the kidney (A), liver (B) and plasma (C). Results are expressed as fold change to the Sham (n = 5 for each group). *$p < 0.05$ when compared with the value obtained from the sham-operated group.

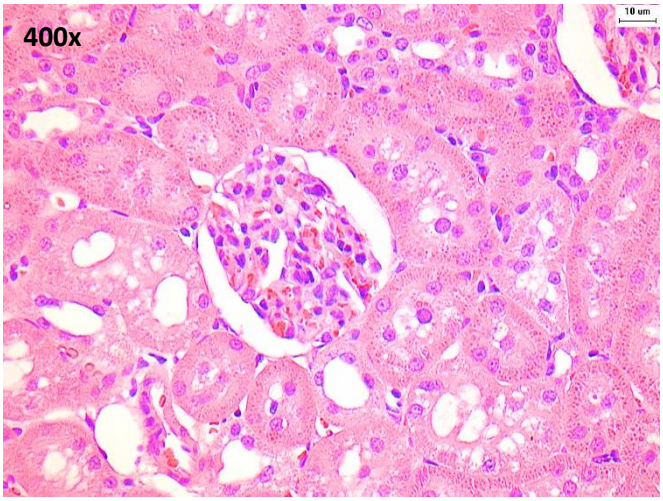
Sham **IR**











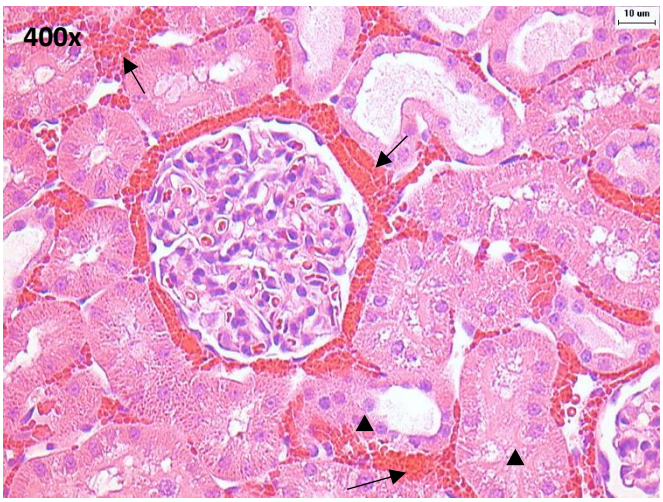


Figure 4.28 Effect of kidney ischemia-reperfusion on pathohistological change of the kidney

The left kidney of rats was subjected to 45 min ischemia followed by 6 h of reperfusion (IR). As a control, rats were subjected to a sham-operation without inducing ischemia (Sham). The gross appearance of a midtransverse plane of kidney was examined by hematoxylin and eosin (H&E) staining and analyzed at ×100, ×200 and ×400 magnification. Kidneys of the IR group showed tubular necrosis (triangle), interstitial congestion of red blood cells (arrow) and glomerulus enlargement compared with the Sham group.

4.5 Discussion

In the present study, we observed injury in both kidney and liver as manifested by increased creatinine and AST levels along with histological changes in rats that were subjected to renal IR. An inflammatory response is considered as one of the important mechanisms in renal IR-induced distant organ injury. In the present study, increased proinflammatory cytokine mRNA expression was detected in the kidney and liver shortly after the onset of renal IR. In correspondence, the levels of proinflammatory cytokines (MCP-1, TNF-α, IL-1β, IL-6) were significantly elevated in the kidney, liver and plasma. These results suggest that renal IR can stimulate cytokine production locally during the acute phase as well as in distant organs such as in the liver, which augments systemic inflammation and may, in turn, exacerbate kidney injury.

Previous studies conducted by other investigators and our laboratory have shown an increased cytokine expression in the kidney upon IR (Sung et al., 2002, Lee et al., 2011, Park et al., 2011, Wang et al., 2014). Animal models of AKI have shown that acute impairment of renal function also affects hepatic inflammatory responses (Golab et al., 2009). AKI increased the expression of hepatic TNF-α, IL-6 and IL-10 cytokine concentrations along with apoptosis and elevated oxidative stress markers as a result of renal ischemia in rats (Park et al., 2011). It has been suggested that the remote effect of AKI is due to circulation of humoral mediators (Golab et al., 2009). Recent evidence showed that increased production and reduced clearance of proinflammatory cytokines in the kidney could lead to a rise of cytokines in the circulation as well as in distant organs (Lane et al., 2013). However, the origin(s) of increased proinflammatory cytokines in the circulation and distant organs has not been well established. In the present study, higher levels of proinflammatory cytokines (MCP-1, TNF-α, IL-6,) were detected in the kidney,

liver and plasma of rats upon renal IR. Early up-regulation of these proinflammatory cytokines was observed in the liver of rats subjected to 1h of reperfusion.

MCP-1 is a potent chemokine for the recruitment of monocytes to the tissues by stimulating monocyte chemotaxis (Sung et al., 2002). Once infiltrated into the tissues, monocytes differentiate into macrophages which are capable of producing proinflammatory cytokines and reactive oxygen species (ROS). In the present study, renal IR increased hepatic MCP-1 gene expression at 1h after renal IR. It is plausible that renal IR stimulates proinflammatory cytokine expression locally in the liver at an early stage, which in turn, could lead to local and systemic inflammatory response. Our results also showed that the expression of MCP-1 may be time-dependant and tissue-specific. Previous study reported that the mRNA expression of MCP-1 started to show a significant increase after 2h of reperfusion in the kidney and reached the highest value at around 6h (Sung et al., 2002). In the present study, an elevation of MCP-1 mRNA and protein levels was detected in the kidney subjected to IR for 6h. An elevation of MCP-1 mRNA expression in the liver was detected as early as 1h after renal ischemia-reperfusion followed by an increase in MCP-1 protein level at 6h after reperfusion. TNF-α is a cell signaling protein involved in systemic inflammation and is one of the cytokines that contributes to the acute phase reaction (Lee et al., 2011). TNF-α can induce other pro or anti-inflammatory cytokines, such as IL-6, IL-8, and IL-10, through the activation of MAP kinase and NF-κB. This may, in turn, affect other organs (Godet et al., 2006, Golab et al., 2009). IL-6 is a proinflammatory cytokine that has been shown to mediate ischemic AKI and distant organ injury in mice with AKI (Park et al., 2011, Wang et al., 2014). Impaired IL-6 metabolism in the kidney may lead to increased serum IL-6 and contributes to systemic effect and mortality (Lee et al.,

2011). Our results showed that the hepatic mRNA expression of TNF-α and IL-6 were both increased upon 1h of renal reperfusion and were further increased after 6h of renal reperfusion, suggesting an acute local and systemic inflammatory response.

The expression of proinflammatory cytokines is mainly regulated at the level of gene transcription. The transcription factor NF-κB plays an important role in regulating inflammatory response (Bonventre and Zuk, 2004, Lawrence, 2009). In the present study, a rapid activation of NF-κB was detected in the liver, which corresponded with an increased expression of proinflammatory cytokines (MCP-1, TNF-α, IL-6). Our previous studies showed that renal IR activated NF-κB in the kidney, which was considered as one of the mechanisms responsible for the IR-induced inflammatory response and oxidative stress in the kidney (Sung et al., 2002, Wang et al., 2014). Results from the present study indicated that renal IR could also elicit an inflammatory response in the liver through activation of NF-κB and increased cytokine expression. However, the mechanisms by which renal ischemic injury triggers NF-κB activation in distant organs remain to be investigated.

Neutrophil gelatinase-associated lipocalin (NGAL) is a small (25 kd) polypeptide that belongs to the lipocalin protein family, which is produced by epithelial cells and neutrophils in response to epithelial damage and has been used for early diagnosis of AKI (Devarajan, 2008, Akcay et al., 2009, Sharfuddin and Molitoris, 2011). Elevation of NGAL protein has been detected in the kidney and in the urine upon ischaemic or nephrotoxic insults (Mishra et al., 2003). Genomic and proteomic analyses have shown up-regulated gene expression and increased protein levels of NGAL in the kidney after ischemic AKI (Mishra et al., 2003, Mårtensson and

Bellomo, 2014). A recent study has shown that NGAL is a biomarker for prognosis in cirrhosis and correlates with liver failure and systemic inflammation (Ariza et al., 2016). Elevated levels of hepatic NGAL may serve as an indicator for liver injury, and it could be produced locally by hepatocytes or by infiltrated neutrophils, indicating a local and systemic cellular damage in the liver upon AKI.

The morphological changes in the kidney after renal IR have been extensively studied (Sung et al., 2002, Golab et al., 2009, Wang et al., 2013). Our results agreed with previous observations that tubular necrosis and red blood cell congestion occurred upon renal IR. Histopathological changes in the liver after ischemic and non-ischemic AKI were characterized by leukocyte infiltration and congestion (Golab et al., 2009, Park et al., 2011). It has been reported that AKI increased vascular permeability in the liver, inducing neutrophil and T-lymphocyte infiltration (Miyazawa et al., 2002). Our observation was agreed with previous findings that in the hepatic sinusoids there was an accumulation of infiltrated leukocytes (mainly neutrophils) in rats that were subjected to 6h of renal IR. The adherence of neutrophils to the vascular endothelium is the first step in the extravasation of these cells into injured tissue such as the liver. After adherence and chemotaxis, infiltrating neutrophils can release ROS that damages other cells, contributes to the pathophysiology of ischemic tissue injury (Linas et al., 1988, Miyazawa et al., 2002, Akcay et al., 2009). In our study, MPO activity was measured to determine the presence of neutrophils in the liver. MPO is a peroxidative enzyme that is abundantly and highly expressed in neutrophil granulocytes, and it has been recognized as a marker in vascular diseases such as myocardial infarction (Klebanoff, 2005). Increased MPO activity observed in the present study further

verified the increased number of neutrophils observed in the liver of rats that were subjected to renal IR, indicating a hepatic inflammation.

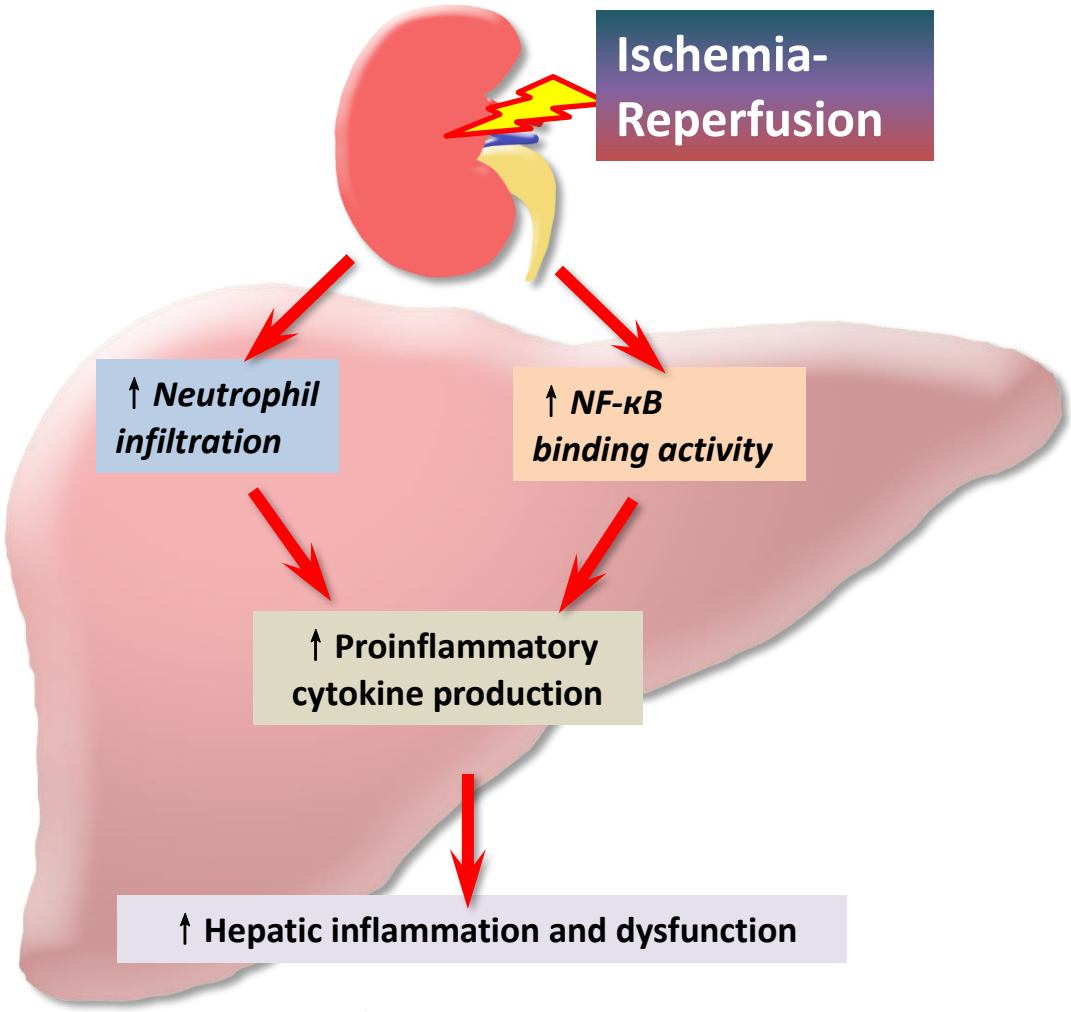


Figure 4.29 Proposed mechanism of kidney ischemia-reperfusion induced hepatic inflammation and dysfunction

Renal ischemia-reperfusion (IR) induced inflammation in the liver through (1) an increase in the neutrophil infiltration which elevates hepatic proinflammatory cytokines; and (2) an increase in the binding activity of the hepatic NF-κB which is a transcription factor responsible for proinflammatory cytokine production.

In conclusion, renal IR not only causes kidney injury but also results in liver dysfunction. Inflammation is a hallmark of kidney and distant organ injury, which is associated with the high morbidity and mortality of AKI. Infiltrated neutrophils may contribute to the elevation of proinflammatory cytokines leading to hepatic inflammation. Although increased cytokine production in the kidney is regarded as one of the important mechanisms leading to systemic and distant organ injury, our results clearly demonstrate that renal IR can directly induce acute production of cytokines in the liver, which in turn, may contribute to a systemic inflammatory response and exacerbate kidney injury. Activation of NF-κB may be involved in the renal IR-induced cytokine production and inflammatory response in the liver (Figure 4.29). Better understanding of local and distant organ responses to AKI will help identify new therapeutic targets and ultimately improve clinical outcomes.

4.6 Acknowledgements

This study was supported, in part, by the Natural Sciences and Engineering Research Council of Canada and the St. Boniface Hospital Foundation.

Chapter V. General Discussion and Future Perspectives

5.1 General discussion

The present study investigated 1) the role of oxidative stress and inflammatory responses in renal IR-induced liver injury; and 2) the mechanisms involved. Previous studies from our laboratory have reported that renal IR induces oxidative stress and inflammatory responses in the kidney, causing kidney injury (Sung et al., 2002, Prathapasinghe et al., 2007, Wang et al., 2014). Recent studies have identified that renal IR can also induce distant organ injury (Kelly, 2003, Hassoun et al., 2007, Liu et al., 2008, Grams and Rabb, 2012, Lane et al., 2013, Druml, 2014, Ologunde et al., 2014). The liver is an important metabolic organ responsible for redox balancing, immune regulation and detoxification. However, the mechanisms of oxidative stress and inflammatory response in the liver upon renal IR injury are not well understood.

Oxidative stress occurs when the antioxidant defense mechanism is impaired or when there is an overproduction of free radicals. This is considered as one of the important mechanisms responsible for IR-induced local and distant organ injury. Among the antioxidants, glutathione is a major endogenous non-enzymatic antioxidant against oxidative stress (DeLeve and Kaplowitz, 1991, Lu, 1999), and the liver is highly efficient for its synthesis. The first part of our research investigated hepatic dysfunction and oxidative stress related to decreased hepatic glutathione production upon renal IR. Our results have clearly demonstrated that: 1) renal IR reduced hepatic production of glutathione, which is an important endogenous antioxidant; 2) renal IR down-regulated the expression of glutamate-cysteine ligase in the liver, which is a key enzyme that regulates glutathione biosynthesis; 3) the down-regulation of enzymes that are responsible for glutathione biosynthesis is mediated by the inhibition of transcription factor Nrf2; and 4) down-regulation of CSE expression in the transsulfuration pathway has limited the availability of cysteine, which is an essential precursor for glutathione biosynthesis.

The inflammatory response is another important mechanism responsible for IR-induced local and distant organ injury. This is mediated by a number of proinflammatory mediators, such as cytokines, chemokines and inflammatory signaling pathways. The second part of our research investigated the inflammatory response related to increased hepatic proinflammatory markers upon renal IR and the mechanisms involved. Our results have demonstrated that: 1) renal IR can directly induce the local production of proinflammatory cytokines in the liver during the acute phase, which is mediated by the activation of transcription factor NF-κB; 2) the levels of inflammatory biomarkers such as proinflammatory cytokines and NGAL are elevated in the liver upon renal IR, along with histological changes and an increased MPO activity, which serve as indicators for neutrophil infiltration.

5.1.1 Renal IR induced oxidative stress and histological changes in the liver

In the present study, we have observed damages in both kidney and liver as indicated by increased creatinine and AST levels along with histological changes in rats that were subjected to renal IR. The increased level of plasma creatinine and aminotransferase are common indicators for kidney and liver injury. Histological evidence further confirmed that renal IR impaired kidney structure with tubular necrosis and interstitial congestion. Histological changes in the liver were characterized by leukocyte infiltration and congestion upon renal IR. It has been reported that the vascular permeability of liver is increased upon kidney injury, which induces neutrophil and T-lymphocyte infiltration (Miyazawa et al., 2002). The infiltrated neutrophils release ROS that could damage other cells and contribute to the pathophysiology of ischemic tissue injury (Linas et al., 1988, Miyazawa et al., 2002, Akcay et al., 2009).

Increased hepatic MDA level and plasma homocysteine level, along with decreased plasma and hepatic glutathione levels are indicators for renal IR-induced oxidative stress in the liver. The MDA level serves as a biomarker for lipid peroxidation, which was significantly increased in the liver of rats subjected to renal IR, indicating oxidative stress. The elevation of homocysteine levels in the plasma leads to a condition known as hyperhomocysteinemia (Clarke et al., 1991, McCully, 1996, Edirimanne et al., 2007, Prathapasinghe et al., 2007). Our previous studies have reported that hyperhomocysteinemia is directly linked to IR-induced kidney injury (Prathapasinghe et al., 2007, Prathapasinghe et al., 2008). Glutathione is a major non-enzymatic antioxidant that can be endogenously synthesized from the liver. A compromise in glutathione production may dampen the ability of the body to cope with oxidative stress locally and systemically.

5.1.2 Renal IR-induced hepatic glutathione depletion

Our study, for the first time, identified the regulatory mechanisms that caused hepatic glutathione depletion upon renal IR. The glutathione biosynthesis and transsulfuration pathway are two major pathways responsible for endogenous glutathione production in the liver.

In the present study, we have observed that renal IR caused a significant decrease in the expression of hepatic glutamate-cysteine ligase (Gcl), which is a key enzyme responsible for glutathione synthesis. We further revealed that the down-regulation of Gcl is mediated by an inhibition of Nrf2, which is a master regulator that can induce the transcription of antioxidant enzymes including those responsible for glutathione synthesis (Noel et al., 2015, Shelton et al., 2015). Increased ROS production and oxidative stress may contribute to the decreased hepatic nuclear Nrf2 protein level observed in our study. Mechanistic studies have shown that Nrf2 is

inhibited under intermediate to high level of oxidative stress, whereas other transcription factors such as NF-κB or AP-1 may be activated and thus induce inflammation and apoptosis (Stefanson and Bakovic, 2014). Our in vitro study confirmed that transfection of Nrf2 siRNA led to a decrease of glutamate-cysteine ligase (Gclc, Gclm) expression in hepatocytes. It is possible that renal IR impaired Nrf2-dependent Gcl expression, leading to a decrease in hepatic glutathione synthesis upon renal IR.

The transsulfuration pathway serves as the source of *de novo* synthesized cysteine in mammalian cells, and cysteine is a precursor for glutathione synthesis (Lu, 1999, Chen et al., 2013, Lu, 2013). The transsulfuration pathway is catalyzed by two enzymes, CBS and CSE. These two enzymes are highly expressed in the liver and contributes to high rate of transsulfuration activity and high capacity for glutathione biosynthesis (Lu, 1999). Our results showed that there is a decreased expression of hepatic CSE upon renal IR, which could further lead to a decreased *de novo* synthesis of cysteine and limit the glutathione biosynthesis in the liver. Inhibition of CSE expression in HepG2 cells has confirmed a reduction in intracellular glutathione production in our study. Downregulation of CSE could also impair homocysteine metabolism which leads to hyperhomocysteinemia and induce injury. Other than catalyzing the transsulfuration pathway, CBS and CSE are also responsible for hydrogen sulfide production through desulfurization reactions. Hydrogen sulfide is a gasotransmitter that has antioxidant and anti-inflammatory properties (Kimura and Kimura, 2004, Whiteman et al., 2005, Elrod et al., 2007, Lee et al., 2014). We have observed significantly lowered hydrogen sulfide production in the liver of rats subjected to renal IR. Reduced hepatic glutathione production, hyperhomocysteinemia and low hydrogen sulfide generation may synergistically impair the antioxidant defense mechanism and contribute to renal IR-induced oxidative stress.

5.1.3 Renal IR increased inflammatory markers and directly induced acute production of cytokines in the liver

Inflammation is a major outcome in renal IR-induced injury. In the present study, we have detected elevations of several inflammatory markers, including proinflammatory cytokines (MCP-1, TNF-α, IL-1β, IL-6), NGAL and MPO in the kidney, liver and plasma upon renal IR.

It has been reported that increased production and reduced clearance of proinflammatory cytokines in the kidney upon IR injury can lead to elevated cytokine levels in the circulation as well as in distant organs (Lane et al., 2013). However, the origin of these cytokines in the circulation and distant organs has not been well established. In the present study, increased proinflammatory cytokine expressions (MCP-1, TNF-α, IL-1β, IL-6) were detected in the kidney, liver and plasma of rats upon renal IR. Early up-regulation (1h reperfusion) of these proinflammatory cytokines was observed in the liver. The expression of proinflammatory cytokines is mainly regulated at the gene transcription level. Transcription factor NF-κB plays important roles in regulating inflammatory response and cytokine production (Bonventre and Zuk, 2004, Lawrence, 2009). We have previously identified that activation of NF-κB is one of the mechanisms responsible for IR-induced inflammation and oxidative stress in the kidney (Sung et al., 2002, Wang et al., 2014). The current study has shown a rapid activation of NF-κB in the liver, which corresponded with increased gene expressions of proinflammatory cytokines, indicating that renal IR could also induce hepatic inflammation and cytokine production through the activation of NF-κB. The present results suggest that renal IR not only stimulates cytokine production locally in the kidney, but also promotes NF-κB-mediated cytokine production in

distant organs such as in the liver, which may in turn, augment systemic inflammation and exacerbate kidney injury.

In the present study, we have also observed a significant increase in other inflammatory biomarkers, including NGAL and MPO. NGAL is a small protein that is produced by epithelial cells and neutrophils in response to epithelial damage, and it has been used as an early biomarker for AKI (Devarajan 2008, Akcay, Nguyen et al. 2009, Sharfuddin and Molitoris 2011). Studies have also shown that increased NGAL level is correlated with liver failure and systemic inflammation (Ariza et al., 2016). The increased hepatic NGAL level observed in our study may have been generated locally by hepatocytes or by infiltrated neutrophils, leading to local and systemic cellular damage in the liver upon renal IR. MPO is a peroxidative enzyme expressed in neutrophil granulocytes, which serves as another biomarker for inflammation and indicates the presence of neutrophils (Klebanoff, 2005). A marked increase of MPO activity in the present study further verified increased neutrophils and inflammation in the liver upon renal IR.

In conclusion, our study has demonstrated several pathophysiological changes, namely, increased oxidative stress and inflammatory response in the liver that are involved in renal IR-induced distant organ injury. These changes include: decreased hepatic glutathione production, decreased hepatic hydrogen sulfide production, increased systemic homocysteine level, decreased hepatic cysteine level, increased hepatic proinflammatory cytokine production, decreased the level of nuclear Nrf2 and increased NF-κB activation in the liver. We have identified novel mechanisms underlying renal IR-induced liver injury. These include 1) inhibition of Nrf2-dependant expression of antioxidant enzymes contributes to a decreased hepatic glutathione biosynthesis upon renal IR; 2) down-regulation of transsulfuration pathway and desulfurization reaction contributes to glutathione depletion and oxidative stress in the liver

upon renal IR; and 3) early stage elevation of NF-κB-mediated hepatic proinflammatory cytokine production and increased hepatic neutrophils contribute to liver inflammation and injury upon renal IR. Because AKI patients that are complicated with distant organ injury are predisposed to a worse prognosis, identification of the underlying mechanisms is urgently needed for the development of better therapeutic strategies to prevent and/or reduce AKI associated morbidity and mortality. Findings from our study suggest that restoration of the hepatic redox balance and attenuation of hepatic proinflammatory cytokine production may help reduce local and systemic oxidative stress and inflammation, which, in turn, improve distant organ function and alleviate kidney injury, leading to a better clinical outcome for AKI patients. Aside from oxidative stress and inflammatory response, other mechanisms may also be involved in distant organ injury associated with AKI. The interactions between various signalling and metabolic pathways remain to be investigated in future studies.

5.2 Future perspectives

5.2.1 To investigate different therapeutic interventions that can alleviate renal IR-induced oxidative stress and inflammation in the distant organ

Our present study has identified changes in several pathways that are involved in renal IR-induced oxidative stress and inflammation in the liver. However, no therapeutic interventions are currently available to target IR-induced distant organ injury. Restoration of glutathione, homocysteine, cysteine and hydrogen sulfide levels could be used as possible strategies against renal IR-induced oxidative stress in the liver.

Previous studies have reported that administration of N-acetylcysteine increases the production of glutathione and is effective in improving renal function in rats with acute kidney failure (DiMari et al., 1997, Conesa et al., 2001, Mehta et al., 2002). However, the protective

function of N-acetylcysteine on distant organ injury is unknown. Golab et al. (2009) administrated glutathione prior to the induction of renal IR and reported that such a treatment significantly improved liver histological structure and reduced hepatic MDA and serum ALT levels. It would be interesting to examine whether the glutathione intervention could alter the expression of enzymes in the transsulfuration pathway and desulfurization reactions as well as improve hepatic antioxidant production mediated by transcription factor Nrf2. Apart from the glutathione intervention, our group has previously reported that administration of anti-homocysteine antibodies into the kidney could eliminate IR-induced oxidative stress and cell death in the kidney and restore renal function after 1h of reperfusion (Prathapasinghe et al., 2007). It would also be interesting to investigate if such interventions exert similar protective effects on the liver during AKI. Other therapeutic interventions may include: inhibit the activation of NF-κB prior to or during renal IR, supplementation of cysteine or other exogenous antioxidants such as vitamin E or minerals during renal IR, etc. Future studies are warranted to examine the effect of different agents on the alleviation of oxidative stress and inflammation in distant organs upon renal IR.

5.2.2 To investigate the interaction between Nrf2 and NF-κB pathways in renal IR-induced distant organ injury

Transcription factors Nrf2 and NF-κB are two master regulators that regulate redox balancing and inflammatory responses, respectively. The Nrf2 pathway regulates the transcription of antioxidant and detoxification genes (Gloire et al., 2006, Stefanson and Bakovic, 2014), whereas NF-κB pathway coordinates inflammation by releasing mediators such as cytokines and chemokines (Gloire et al., 2006). In the present study, we have observed that Nrf2 and NF-κB

were both altered in the liver by renal IR. However, the mechanisms of how renal IR triggers the decrease in nuclear Nrf2 or the activation of NF-κB in the distant organs remain unknown.

Mechanistic studies have shown that Nrf2 and NF-κB act in a synergistic manner that mediate the crosstalk between oxidative stress and inflammation (Bellezza et al., 2010, Wakabayashi et al., 2010, Cuadrado et al., 2014). A mild level of oxidative stress induces Nrf2, which activates the transcription of antioxidants, whereas an intermediate level of free radicals triggers inflammatory responses through the activation of NF-κB and further produces proinflammatory cytokines (Stefanson and Bakovic, 2014). It would be interesting to investigate the crosstalk between oxidative stress and inflammation in our renal IR model. It may provide some evidence on which reaction happens first and what triggers the alteration of transcription factors Nrf2 and NF-κB locally and systemically in both kidney and distant organs.

5.2.3 To investigate other pathways and parameters affected by renal IR in the kidney and distant organs

Other pathways and parameters of interest have been identified by our collaborators and other research groups using the renal IR model, which include: changes on membrane ion channels, hypertension, lipid metabolism, etc (Rahgozar et al., 2003, Choi et al., 2018, Liu et al., 2018, Solati et al., 2018).

A recent study examined the renal IR-induced kidney and liver injury and their relationship with lipid metabolism and fatty liver (Choi et al., 2018). It was shown that renal IR caused a downregulation of constitutive androstane receptor which, in turn, led to an inhibition of hepatic very-low-density lipoprotein triglyceride (VLDL-TG) secretion and compromised liver function

(Choi et al., 2018). In future studies, it would be necessary to take into consideration changes in different pathways that are involved in renal IR-induced kidney and distant organ injuries, and to explore effective therapeutic interventions that can attenuate acute kidney injury and its associated distant organ dysfunction.

Chapter VI. References

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Appendix I

Reagents/Chemicals	*Company*
Acrylamide/bis 30% solution 37.5:1	Bio-Rad
Anti-CBS antibody	Abnova Corporation
Anti-CSE antibody	GeneTex
Anti-Gclc antibody	Abcam
Anti-Gclm antibody	Abcam
Anti-GS antibody	Abcam
Anti-Nrf2 antibody	Abcam
Anti-rabbit IgG antibody	Cell Signaling Technology
Bovine serum albumin	BSA EMD
Bromophenol blue	Sigma-Aldrich
Chloroform	Fisher Scientific
Diethyl pyrocarbonate, DEPC	Sigma-Aldrich
Dimethyl sulfoxide, DMSO	Fisher Scientific
Disodium hydrogen orthophosphate (Na_2HPO_4)	Sigma-Aldrich
Dithiothreitol, DTT	Sigma-Aldrich
DL-Homocysteine	Sigma-Aldrich
DL-Propargylglycine, PAG	Sigma-Aldrich
Dulbecco's modified Eagle's medium, DMEM	Hyclone
EMSA kit	ThermoScientific
Ethylene glycol tetra-acetic acid, EGTA	Sigma-Aldrich

Ethylenediaminetetraacetic acid, EDTA	Sigma-Aldrich
Fetal bovine serum, FBS	PAA
First strand buffer 5X	Invitrogen
Formaldehyde (36%)	Sigma-Aldrich
Hanks balanced salt solution, HBSS	Hyclone
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	Fisher Scientific
HRP-conjugated anti-mouse antibody	Cell Signaling Technology
Hydrochloric acid, HCl	Fisher Scientific
iQ-SYBR green supermix reagent	Bio-Rad
Isopropanol	Fisher Scientific
L-Cysteine	Sigma-Aldrich
Leupeptin	Sigma-Aldrich
Mercaptoethanol-β	Sigma-Aldrich
Methanol	VWR
M-MLV-Reverse transcriptase 200 U/ml	Invitrogen
MPO kit	Abcam
N, N-Dimethyl-p-phenylenediamine sulfate salt	Sigma-Aldrich
Nonidet-P	Sigma-Aldrich
Oligo(dT)12-18 primer, 0.5 mg/ml	Invitrogen
Pentobarbital solution	Sigma-Aldrich
PMSF	Sigma-Aldrich
Potassium chloride, KCl	Sigma-Aldrich
Potassium phosphate dibasic, K_2HPO_4	Sigma-Aldrich

Potassium phosphate monobasic, KH_2PO_4	Sigma-Aldrich
Protein assay reagent	Bio-Rad
Pyridoxal 5′-phosphate hydrate	Sigma-Aldrich
Rnase inhibitor 40 U/ml	Invitrogen
S-(5′-Adenosyl)-L-methionine, SAM	Sigma-Aldrich
Sodium chloride, NaCl	VWR
Sodium hydroxide, NaOH	Fisher Scientific
Sodium nitrate, $NaNO_3$	Fisher Scientific
TE (Tris-EDTA) buffer, 50X	USB
TEMED	EMD
Trichloroacetic acid	Sigma-Aldrich
Tris	Invitrogen
Triton X-100	Sigma-Aldrich
Trizol reagent	Invitrogen
Trypsin-EDTA 0.05%	Gibco
Tween 20	Fisher
Zinc acetate dihydrate	Sigma-Aldrich
Zinc sulfate heptahydrate, $ZnSO_4$	Sigma-Aldrich

Appendix II

List of publications

Full length manuscripts

1. Solati Z., A. Edel, **Y. Shang**, K. O, and A. Ravandi. 2018. Oxidized phosphatidylcholines are produced in renal ischemia reperfusion injury. PLoS ONE 13(4):e0195172.
2. **Shang Y.**, E. Khafipour, H. Derakhshani, L. K. Sarna, C. W. Woo, Y. L. Siow and K. O. 2017. Short-term high fat diet induces obesity-enhancing changes and partially restores mouse gut microbiota upon cessation of the high fat diet. Lipids, 52(6):499-511.
3. **Shang Y.**, Y. L. Siow, C. K. Isaak and K. O. 2016. Downregulation of glutathione biosynthesis contributes to oxidative stress and liver dysfunction in acute kidney injury. Oxidative Medicine and Cellular Longevity, 2016(10):1-13.

Conference abstracts

1. **Shang Y.**, Y. L. Siow, C. K. Isaak, and K. O. 2017. Acute kidney injury induces oxidative stress and distant organ dysfunction due to glutathione depletion. The FASEB Journal 31:698.4 – 2017 Experimental Biology Meeting, Chicago, IL.
2. Sid V., **Y. Shang**, Y. L. Siow, and K. O. 2017. Regulation of Hepatic Inflammation by Folic Acid in Non-alcoholic Fatty Liver Disease (NAFLD). The FASEB Journal 31:780.8 – 2017 Experimental Biology Meeting, Chicago, IL.
3. Amarakoon S., B. Jayaraman, Y. L. Siow, S. Prashar, **Y. Shang**, K. O, and M. Nyachoti. Effect of Dietary Supplementation of Plant-Based Products on Oxidative Status of Weanling Piglets. 2017 Anima Nutrition Conference of Canada. Quebec City, Canada.
4. **Shang Y**, H. Derakhshani, L. Sarna, Y. L. Siow, E. Khafipour, and K. O. 2016. Impact of dietary fat on murine gut microbiota and fatty liver. The FASEB Journal 30:1258.1 –

2016 Experimental Biology Meeting, San Diego, CA.

5. Sid V., N. Wu, L. Sarna, **Y. Shang**, J. House, Y. L. Siow, and K. O. 2016. Regulation of AMPK and hepatic metabolism by folic acid supplementation in non-alcoholic fatty liver disease. The FASEB Journal 30:870.3 – 2016 Experimental Biology Meeting, San Diego, CA.
6. Sid V., **Y. Shang**, L. Sarna, P. Wang, Y. L. Siow, and K. O. 2014. Supplementation of folic acid attenuates hepatic inflammation in high fat diet fed mice. The FASEB Journal 29:884.45.