Regulation of oxidative stress and inflammation in ischemia/reperfusion-induced acute kidney injury

By

Pengqi Wang

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Department of Animal Science

University of Manitoba

Winnipeg

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ABSTRACT

Renal ischemia/reperfusion (I/R) is a main cause of acute kidney injury (AKI) and delayed graft function after renal transplantation. Previous studies in human and experimental models have identified that inflammation and oxidative stress are two key players in renal I/R injury. However, the underlying mechanisms remain speculative. The overall objective of the study was to investigate the biochemical and molecular mechanisms of I/R-induced renal injury and the effect of tyrosol supplementation on I/R-induced kidney oxidative stress damage.

In the present study, renal I/R was induced in Sprague-Dawley rats and in a human kidney proximal tubular cell line. A significantly elevated expression of pro-inflammatory cytokine expression (MCP-1, IL-6) was observed. There was a significant decrease in mRNA and protein levels of two hydrogen sulphide (H_2S)-producing enzymes, CBS and CSE, with a concomitant reduction of glutathione and H_2S production. In the cell culture model, hypoxia–reoxygenation of proximal tubular cells led to a decrease in CBS and CSE expression and an increase in pro-inflammatory cytokine expression. Supplementation of glutathione or H_2S donor (NaHS) effectively abolished cytokine expression in tubular cells.

Experiments were conducted to detect oxidative stress markers. It was demonstrated that there was a significant increase in peroxynitrite formation and lipid peroxidation in the kidney after I/R insult, which might be caused by the elevation in nitric oxide (NO) metabolites and inducible nitric oxide synthase (iNOS). Administration of tyrosol, a natural phenolic compound, reduced peroxynitrite formation, lipid peroxidation and the level of NO metabolites via inhibiting NF-κB activation and iNOS expression. Tyrosol treatment improved kidney function and had a protective effect against I/R-induced AKI.

The present study has clearly demonstrated that (1) there is a reduction of H_2S production via inhibition of CBS and CSE expression, which contributes to increased pro-inflammatory cytokine expression in the kidney and in tubular cells upon I/R insult; (2) restoration of endogenous H_2S production would be of therapeutic value in regulating inflammatory response in I/R-induced kidney injury; (3) tyrosol treatment has a beneficial effect against renal I/R-induced oxidative stress, in part, through its inhibition on NF-κB activation and iNOS-mediated NO production.

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3. Wu N, Sarna LK, Hwang SY, Zhu Q, **Wang P**, Siow YL, O K. Activation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase during high fat diet feeding. *Biochim Biophys Acta*. 2013 May 4. doi:pii: S0925-4439(13)00148-8.

4. **Wang P**, Zhu Q, Wu N, Siow YL, Aukema H, O K. Tyrosol attenuates ischemia-reperfusion induced kidney injury via inhibition of inducible nitric oxide synthase. *J Agric Food Chem*. 2013 Apr 17;61(15):3669-75.

5. Sarna L, Wu N, **Wang P**, Hwang SY, Siow YL, O K. Folic acid supplementation attenuates high fat diet induced hepatic oxidative stress via regulation of NADPH oxidase. *Can J Physiol Pharmacol*. 2012 Feb;90(2):155-65.

Abstracts Published:

1. **Wang P**, Isaak C, Siow YL, O K. Reduced endogenous hydrogen sulfide generation contributes to upregulation of pro-inflammatory cytokines in acute kidney injury. Congress of Chinese Society of Nephrology 2015, Zhuhai, China, 2015

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3. **Wang P**, Isaak C, Siow YL, O K. Acute kidney injury decreased endogenous hydrogen sulfide production and increased cytokine expression: Impact on cardiovascular system. Canadian Cardiovascular Congress 2014, Vancouver, BC, 2014

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5. Sid V, Sarna L, Hwang SY, **Wang P**, Siow YL, O K. Folic acid supplementation reduces hepatic inflammation caused by a high fat diet. Canadian Student Health Research Forum 2014, Winnipeg, Manitoba, 2014

6. Wu N, Sarna L, Hwang SY, Zhu Q, **Wang P**, Siow YL, O K. Regulation of HMG-CoA reductase in diet-induced non-alcoholic fatty liver disease. Canadian Cardiovascular Congress, Montreal, Quebec, 2013

7. **Wang P**, Zhu Q, Wu N, Siow YL, Aukema H, O K. Attenuation of renal ischemia reperfusion injury by phenolic compound. World Congress of Nephrology 2013, Hong Kong, China, 2013

8. Sarna L, Wu N, **Wang P**, Hwang SY, Siow YL, O K. High fat diet induced hepatic oxidative stress is mediated via activation of NAPDH oxidase. 7th Canadian Oxidative Stress Consortium, Thunder Bay, Ontario, 2012

9. Sarna L, **Wang P**, Wu N, Siow YL, O K. Diet Induced Obesity Disrupts Hepatic Redox Homeostasis Leading to Oxidative Stress in Mice: Protective Effect of Natural Compounds. Experimental Biology Meeting, Washington DC, 2011

10. Echeverry H, Yitbarek A, Munyaka P, Alizadeh A, **Wang P**, O K, Rodriguez-Lecompte C, Camelo-Jaimes G. Low inclusion of a blend of organic trace minerals (Zn, Cu, Fe, and Mn) to broiler chickens diet is able to regulate oxidative stress. American Association of Avian Pathologists Meeting, St. Louis, MO, 2011

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ABBREVIATIONS

ALT	Alanine aminotransferase
AKI	Acute kidney injury
AKIN	Acute kidney injury network
ANOVA	Analysis of variance
AOAA	Aminooxyacetic acid
AP-1	Activator protein-1
AST	Aspartate aminotransferase
CAT	Cysteine aminotransferase
CBS	Cystathionine-β-synthase
CKD	Chronic kidney disease
CRP	C-reactive protein
CSE	Cystathionine-γ-lyase
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
ESRD	End-stage renal disease
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
Hcy	Homocysteine
HK-2	Human kidney cortex proximal tubular cells
HR	Hypoxia–reoxygenation
ICAM	Intercellular adhesion molecule
IL	Interleukin
KDIGO	Kidney disease improving global outcomes
Keap1	Kelch-like ECH-associated protein-1
KIM	Kidney injury molecule-1
LDL	Low-density lipoprotein

MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein-1
MDA	Malondialdehyde
Met	Methionine
MST	Mercaptopyruvate sulfurtransferase
NaHS	Sodium hydrosulfide
NGAL	Neutrophil gelatinase-associated lipocalin
NF-κB	Nuclear factor-κB
NO	Nitric oxide
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
Nrf-2	NF-E2-related factor 2
PAG	DL-propargylglycine
PCR	Polymerase chain reaction
PGE2	Prostaglandin E_2
PLP	Pyridoxal-5'-phosphate
RIFLE	Risk, Injury, Failure, Loss, and End-stage renal disease
mRNA	Messenger ribonucleic acid
ROS	Reactive oxygen species
RRT	Renal replacement therapy
SCr	Serum creatinine
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TNF-α	Tumor necrosis factor-α
VCAM	Vascular cell adhesion molecule

Chapter 1. INTRODUCTION

1.1 Acute kidney injury (AKI)

1.1.1 Definition, classification and prevalence

1.1.1.1 Definition of AKI

Acute kidney injury (AKI), previously known as acute renal failure (ARF), is a syndrome characterized by an abrupt (hours to days) deterioration in kidney function. AKI includes a broad range of the complications, from less severe forms of injury such as subtle biochemical and structural changes, to more advanced injury in which renal replacement therapy (RRT) may be required (Lewington & Kanagasundaram, 2011). The kidneys play a critical role in maintaining electrolyte (sodium, potassium, bicarbonate) and acid/base balances, regulating the body fluid volume, and excreting metabolic toxic end products (Bellomo, Kellum, & Ronco, 2012). Characterized by a rapid progressive loss of renal function, AKI is typically diagnosed either by an accumulation of nitrogenous waste products, such as serum creatinine, or by the detection of oliguria via the measurement of urine output (Mehta & Chertow, 2003). Other common clinical and laboratory manifestations include the accumulation of metabolic acids and an increase in potassium and phosphate concentrations (M. Rahman, Shad, & Smith, 2012).

1.1.1.2 Classification of AKI

Estimates of the incidence of AKI have evolved along with the definition of AKI. The validation of standardized definitions and staging criteria for AKI is important in clinical practice and in the conduct of research (Thomas et al., 2014). The definition of AKI has evolved rapidly since 2004, when the Acute Dialysis Quality Initiative (ADQI) Workgroup published a landmark consensus definition and classification known as the RIFLE (Risk,

Injury, Failure, Loss, and End-stage renal disease) (Bellomo, Ronco, Kellum, Mehta, & Palevsky, 2004). 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). The AKIN criteria modified the RIFLE criteria, making it more sensitive and reliable. More recently, the Kidney Disease Improving Global Outcomes (KDIGO) group published a clinical practice guideline for acute kidney injury, which merged the RIFLE criteria and the AKIN definition ("KDIGO clinical practice guidelines for acute kidney injury," 2012) (Table 1-1). Those recent advances in the standardization of the AKI definition and diagnostic criteria may be helpful in estimating incidence of AKI, increasing the sensitivity and specificity of diagnosis, helping researchers identify appropriate participants and determine optimal timing for trials, and eventually helping clinicians to easily follow evidence-based clinical practice guidelines.

Table 1-1 Classification and staging of AKI defined by RIFLE, AKIN and KDIGO criteria

Severity / Scheme	Increasing severity					
RIFLE	**Stage**	**Risk**	**Injury**	**Failure**	**Loss**	**End-stage kidney disease**
	SCr[1] or GFR[2] criteria	↑ SCr ×1.5 or ↓ GRF >25%	↑ SCr ×2 or ↓ GRF >50%	↑ SCr ×3 or ↓ GRF >75% or ↑ SCr ⩾0.5 mg/dl (44.2 μmol/l) if baseline SCr ⩾4 mg/dl (353.6 μmol/l)	Complete loss of renal function for >4 weeks	Complete loss of renal function for >3 months
	UO[3] criteria	<0.5 ml/kg/h for >6 h	<0.5 ml/kg/h for >12 h	<0.3 ml/kg/h for >24 h or anuria for >12 h		
AKIN	**Stage**	**1**	**2**	**3**		
	SCr criteria	↑ SCr ×1.5 or ↑ SCr ⩾ 0.3 mg/dl (26.4 μmol/l)	↑ SCr ×2	↑ SCr ×3 or ↓ GRF >75% or ↑ SCr ⩾0.5 mg/dl(44.2 μmol /l) if baseline SCr ⩾4 mg/dl (353.6 μmol /l)	Stage 3 also includes any patients who require renal replacement therapy (RRT) independenly of the stage (defined by SCr and/or UO) they are in at the point of RRT initiation	
	UO criteria	<0.5 ml/kg/h for >6 h	<0.5 ml/kg/h for >12 h	<0.3 ml/kg/h for >24 h or anuria for >12 h		
KDIGO	**Stage**	**1**	**2**	**3**		
	SCr criteria	↑ SCr ×1.5 within 7 days or ↑ SCr ⩾0.3 mg/dl (26.4 μmol/l) within 48 h	↑ SCr ×2	↑ SCr ×3 or ↑ SCr ⩾4 mg/dl (353.6 μmol/l)	Stage 3 also includes any patients who require renal replacement therapy (RRT)	
	UO criteria	<0.5 ml/kg/h for >6 h	<0.5 ml/kg/h for >12 h	<0.3 ml/kg/h for >24 h or anuria for >12 h		

[1] SCr-serum creatinine. [2] GFR-glumerular filtration rate. [3] UO-urine output.

1.1.1.3 Prevalence of AKI

A recent systematic review and meta-analysis included 312 cohort studies and 49 million patients (mostly from high-income countries) (Susantitaphong et al., 2013). Using KDIGO definition, this analysis showed that AKI occurred in one in five adults and one in three children associated with an acute hospitalization. A study from the United States with a large population (n = 3,787,410) gave incidence rates of 384.1 per 100,000 person years for AKI not requiring dialysis (defined using relative changes in SCr levels) and 24.4 per 100,000 person years for AKI requiring dialysis (defined using integrated administrative data). The authors confirmed the finding of a rising incidence of AKI over time. It was reported that between 1996 and 2003, there was a significantly increased incidence of non-dialysis AKI and dialysis requiring AKI from 322.7 to 522.4 (38%) and 19.5 to 29.5 per 100,000 person years (33%), respectively (Waikar, Curhan, Wald, McCarthy, & Chertow, 2006). In the United States, older, male and black Americans are associated with higher incidence of AKI (R. K. Hsu, McCulloch, Dudley, Lo, & Hsu, 2012). Although the majority of cases on the epidemiology of AKI are from developed countries, in developing nations, AKI is increasingly recognized as a major contributor to morbidity, mortality and health care resource strain. To put the economic burden into perspective, it is estimated that in the United Kingdom, the cost of AKI to the National Health Service (NHS) is between £434 - £620 million per year (NICE, 2013). In developing countries, the epidemiology of AKI is different from that in developed countries. In developing nations, AKI is a disease that might be more likely to occur in youth and children because of a host of socioeconomic and environmental influences (Riley et al., 2013; Seedat & Nathoo, 1993). Although overall mortality of AKI in developing countries seems to be lower than that in developed countries,

the mortality in youth and children is high (Agarwal, Kirubakaran, & Markandeyulu, 2004; Phadke & Dinakar, 2001) .

AKI is a complication that occurs frequently in hospitalized patients and also has a poor prognosis with the mortality ranging from 10%-80%, depending on the patient population studied. Studies in the developed world report AKI from 1% (community-acquired) up to 7.1% (hospital-acquired) of all hospital admissions (Uehlinger et al., 2005; Vinsonneau et al., 2006), with overall in-hospital mortality around 20%, and up to 50% in ICU patients (C. Y. Hsu et al., 2007). It has been estimated that AKI is responsible for approximately 2 million deaths every year worldwide (Murugan & Kellum, 2011). Those patients who survive an episode of AKI are at higher risk for developing chronic kidney disease (Coca, Singanamala, & Parikh, 2012).

1.1.2 Clinical features and diagnosis of AKI

1.1.2.1 Clinical features of AKI

Symptoms of AKI vary by the underlying cause, severity of renal impairment and associated diseases. Most patients with mild to moderate acute kidney injury may have no symptoms, and diagnosis is based on laboratory testing, sometimes followed by renal biopsy (M. Rahman et al., 2012). AKI patients can display oliguria (<400 ml urine/24 hours), anuria (<100 ml urine/24 hours) or normal urine volumes (Docimo, 2006). Some extrarenal manifestations include anorexia, nausea, vomiting, weakness, fatigue or edema. In addition, features of AKI may also include platelet dysfunction, bleeding disorders and neurologic dysfunction (Samimagham, Kheirkhah, Haghighi, & Najmi, 2011). A variety of causes may

lead to AKI and they commonly include pre-renal, intrinsic (intrarenal), and post-renal causes according to their origins (Basile, Anderson, & Sutton, 2012) (Figure 1-1)

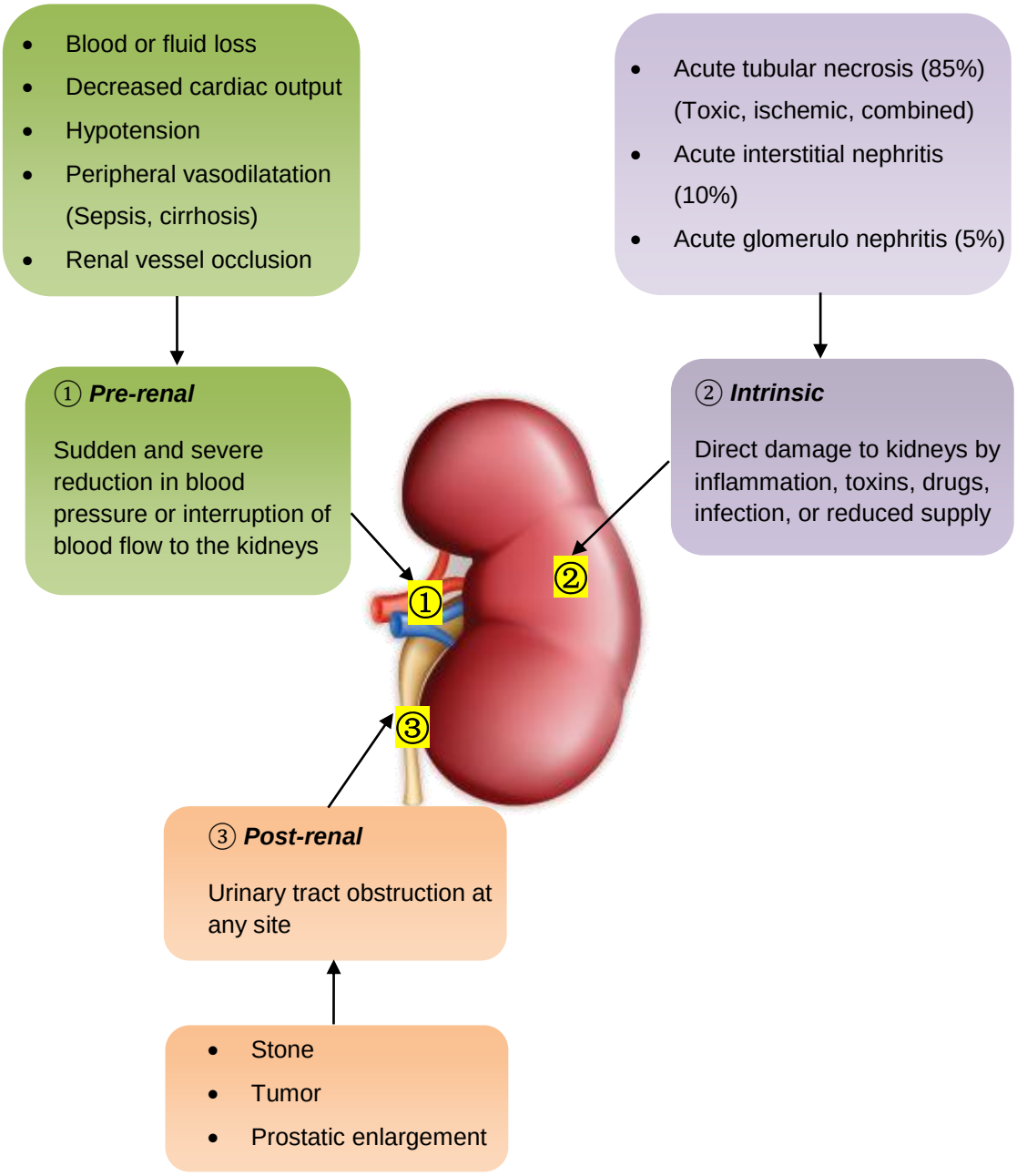

Figure 1-1 Causes of acute kidney injury

The causes of AKI can be divided into three broad categories: pre-renal, intrinsic renal, and postrenal.

1.1.2.2 Diagnosis of AKI

A careful patient history review, thorough physical examination, and urine test are crucial components of the approach to determine the cause of AKI (Kellum & Lameire, 2013). A full current and past medication history should be obtained to identify the use of nephrotoxic drugs or systemic illnesses that can lead to decreased renal function. Physical examination should also include assessment of patients' intravascular volume status and any skin manifestations which are signs of systemic illness. Laboratory tests include an assessment of renal function by measuring serum creatinine level and a careful examination of the urine. Kidney imaging can help to identify any obstruction (M. Rahman et al., 2012). Creatinine is currently the most widely used marker of renal function, with some limitations, such as the poor predictive accuracy for early stage AKI and the dependence on nonrenal factors (age, sex, muscle mass, infection, medication) (Coca, Yalavarthy, Concato, & Parikh, 2008). Therefore, there has been much interest in identifying early reliable biomarkers of AKI. Over the past few years, several novel biomarkers of AKI have been identified and have shown promise in evaluation of ischemic renal injury in experimental animals, as well as in clinical studies of AKI. Those biomarkers include cystatin C (Herget-Rosenthal et al., 2004), kidney injury molecule-1 (KIM) (Han, Bailly, Abichandani, Thadhani, & Bonventre, 2002), interleukin-18 (IL-18) (Parikh, Abraham, Ancukiewicz, & Edelstein, 2005), neutrophil gelatinase-associated lipocalin (NGAL) (Mishra et al., 2003), and liver-type fatty acid binding protein (L-FBP) (Portilla et al., 2008), to name a few. More studies are required for further validation of multiple AKI biomarkers. In the future, the combination of current biomarkers and newer biomarkers will be useful for the diagnosis of AKI and for improving the ability of the RIFLE, AKIN or KDIGO criteria to define AKI.

1.1.3 Pathophysiology of AKI

The mechanisms involved in the etiology of AKI include (1) renal vascular endothelium damage with the loss of ability to regulate vascular tone, perfusion, permeability and inflammation/adhesion; (2) renal tubular epithelial cell injury, which is caused by hypoxia and by the generation of reactive oxygen species during reperfusion; (3) inflammation, which is triggered by ischemia/reperfusion injury, inflammatory cytokines, and immune cell attachment and migration; and (4) direct nephrotoxic effects of nephrotoxins (Lattanzio & Kopyt, 2009). Endothelial cells constitute the inner lining of the vessels and work as a major regulator of vascular homeostasis. They contribute to vascular tone, the regulation of blood flow to local tissue beds, the modulation of coagulation and inflammation, and endothelial permeability. Following the initial insult of AKI, vascular alterations contribute to renal dysfunction by increasing endothelial permeability, platelet aggregation, leukocyte adhesion, and loss of the regulation of vascular tone and by generating inflammatory mediators (Molitoris & Sutton, 2004). Histopathologically, it has been documented that there is vascular congestion, edema formation, diminished blood flow, and infiltration of inflammatory cells (Sutton et al., 2003). In addition to endothelial cells, the renal tubular epithelium is a major site of cell injury and death during AKI. During the initiation phase the onset sublethal injury occurs, characterized by the loss of cell polarity and brush borders, disruption of the junction integrity and cell-matrix adhesion. When the injury progresses to the extension phase, multiple interrelated events lead to further damage to the epithelial cell, causing cast formation and obstruction, cell death, dedifferentiation of viable cells, epithelial proliferation, and restitution of a normal epithelium (Devarajan, 2006). These result in impaired kidney function (Bonventre & Yang, 2011). The functional changes in the vascular

endothelial cells and/or in the tubular epithelium will initiate the recruitment of leukocytes. The leukocytes, including neutrophils, macrophages, natural killer cells, and lymphocytes, infiltrate into the injured kidneys. The injury induces the release of inflammatory mediators such as cytokines and chemokines from endothelial and epithelial cells, which contributes to renal damage by recruiting inflammatory cells to the kidney (Bonventre & Yang, 2011). Furthermore, there is an imbalance between the production of vasoconstrictors (such as endothelin-1, angiotensin II) and vasodilators (such as NO), resulting in intra-renal vasoconstriction and eventually causing continued hypoxia and cell damage (Legrand, Mik, Johannes, Payen, & Ince, 2008) (Figure 1-2). Despite advances in the understanding of AKI, some underlying signaling pathways remain unclear. Further studies are required to understand better the pathophysiology of AKI.

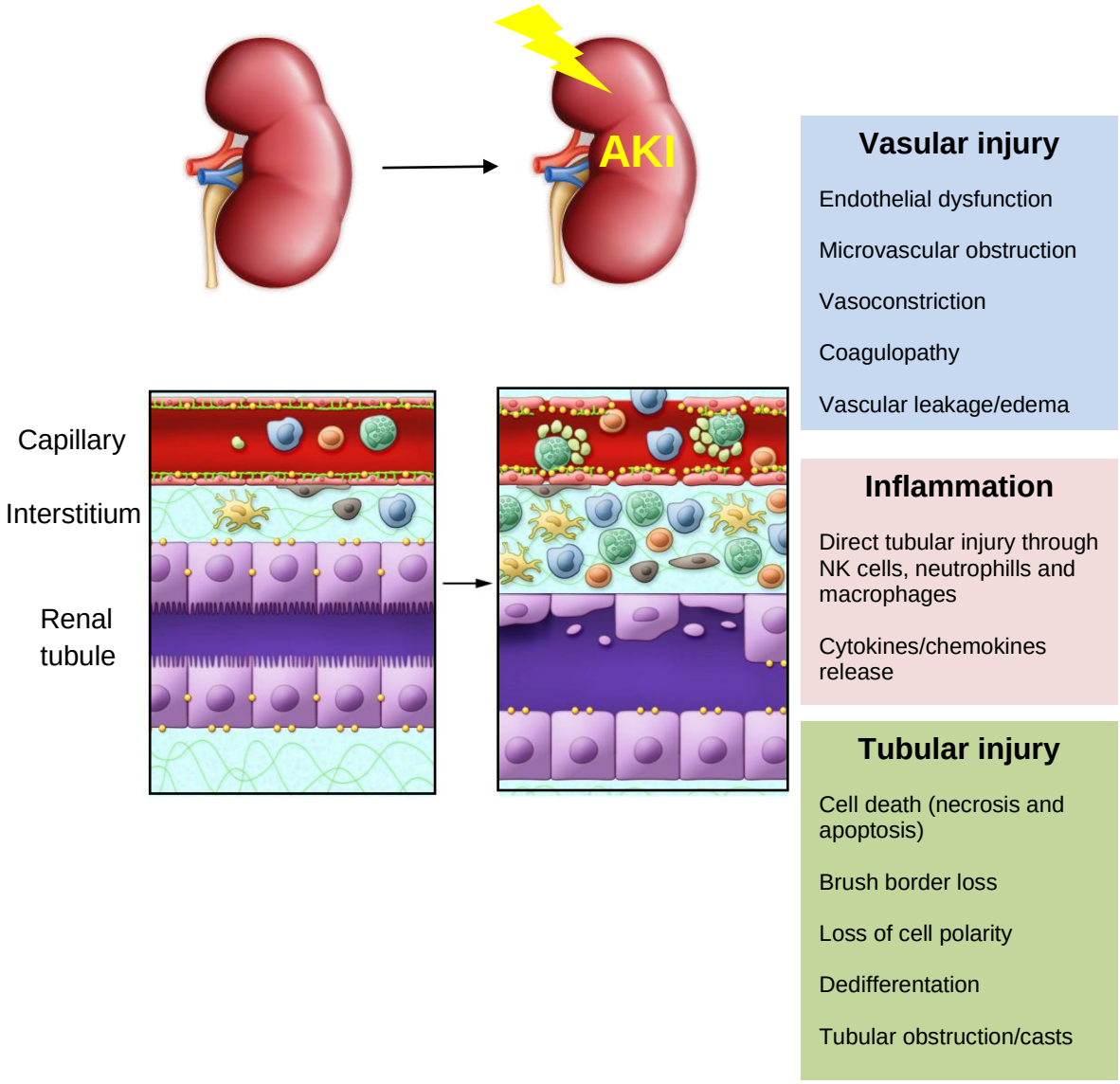

Figure 1-2 Pathophysiology of acute kidney injury

AKI leads to the alternations in three major structures of the kidney: 1) the renal tubule, 2) the capillary and 3) the interstitium. Components of the pathophysiology include vascular damage, inflammatory responses as well as tubular injury and their consequences. (Figure adapted from Bonventre et.al 2011 and Tögel et.al 2014)

1.1.4 Management of AKI

Management of AKI depends on the underlying cause and severity of AKI, and treating the primary problem will cure the AKI once it has been identified. Correction or treatment for the underlying cause of AKI should begin at the earliest indication of the disorder. The main goal of AKI management is, as far as possible, to maintain fluid and electrolyte homeostasis and to correct the biochemical abnormalities (Fry & Farrington, 2006). Complications, such as metabolic acidosis, high potassium levels and uremia, can be prevented in some cases by actions which range from restricting fluid to using renal replacement therapy (RRT) (M. Rahman et al., 2012). Once AKI is established, management is primarily supportive, including RRT, nutritional support, avoidance of nephrotoxins, and blood pressure and fluid management. Nutritional support is recommended since protein-energy wasting is common in AKI patients and represents a major negative predictive factor (Fiaccadori, Parenti, & Maggiore, 2008). An essential requirement for AKI management would be close collaboration among physicians, nurses, dietitians, specialists, nephrologists, and other subspecialists involved in the care of the patient.

1.1.5 Experimental AKI models

The utilization of experimental models plays a critical role in advancing the understanding of AKI pathogenesis and the development of potential therapeutic strategies. There are two categories of experimental models of AKI: *in vivo* and *in vitro*. Each category can be subdivided according to the methods used to simulate AKI. The methods of inducing AKI include ischemia/reperfusion, sepsis, and nephrotoxic agents (A. P. Singh et al., 2012).

1.1.5.1 Animal model of AKI

For understanding the renal tubule response in the context of the whole kidney and the whole organism, animal models are required for AKI studies. There is a complex interplay of several hemodynamic, humoral and toxic factors in the pathogenesis of human AKI. The need for whole animal studies to unravel these complexities and to develop therapeutic modalities has been partially met by the development of "single insult" models or AKI induced by I/R. Clamping of the renal artery or the pedicle for various periods (e.g., 30-60 minutes), followed by reperfusion established for minutes to days before kidney harvesting, is a well-established model and is commonly used in the studies of AKI (Chatterjee, di Villa Bianca et al., 2003; D. Singh & Chopra, 2004; Takeda et al., 2006). As a simple and reproducible study model, I/R can achieve a graded response easily, and there is a good correlation between functional injury and pathology (Luyckx & Bonventre, 2008).

1.1.5.2 *In vitro* model of AKI

In addition to the animal model, investigators have utilized proximal tubule cell culture, isolated proximal tubules, and isolated perfused kidneys *in vitro*. Cultured tubular epithelial cells, obtained from primary cultures or established cell lines, are the simplest and have been widely used to study tubular epithelial cell injury in AKI (Breggia & Himmelfarb, 2008; Hagemann, Thomasova, Mulay, & Anders, 2013). Some sublethal tubular epithelial cell injuries during AKI, such as loss of brush border and cell swelling, could be mimicked in cultured cells that are subjected to hypoxia-reoxygenation (Racusen, Fivush, Li, Slatnik, & Solez, 1991). The proved observations allowed the investigators to proceed with mechanistic studies. However, as a single tubular epithelial cell type, the proximal tubule cell has some

limitations as a model of AKI. For example, cultured cells proliferate more rapidly and rely more on glycolysis than on mitochondrial metabolism, in contrast to tubular epithelial cells *in vivo* (Tang, Suresh, & Tannen, 1989). Nevertheless, cell culture models still remain a valuable tool for investigating the molecular pathways of injury and repair, providing insights into and direction for study of more complex models that may be more applicable to human AKI.

1.2 Renal ischemia/reperfusion injury

I/R injury represents a pathological condition that is characterized by an initial undersupply of blood to an area or organ (ischemia) followed by a restoration of perfusion and concomitant reoxygenation (reperfusion) (Yellon & Hausenloy, 2007). Experimentally and clinically prevalent findings suggest that I/R initiates a wide and complex array of pathophysiological events that may aggravate local injury as well as induce impairment of remote organ function. I/R is an important and common cause of AKI. It results from a generalized or localized impairment of oxygen and nutrient delivery to, and removal of waste product from, renal cells, leading to the mismatch of local tissue oxygen supply and demand and the accumulation of waste products of metabolism. I/R injury may occur in diverse medical and surgical settings, including systemic hypotension, hypovolemic shock, cardiac arrest, and surgical procedures involving clamping of the aorta and/or renal arteries such as surgery for supra- and juxtarenal abdominal aortic aneurysms and kidney transplantation (Chatterjee, 2007; Ellenberger et al., 2006). I/R-induced AKI has been developed and widely used as a model of acute kidney injury in rodents. There have been significant advances in our understanding of the etiology and pathophysiology of AKI, thus paving the way to the

development of novel molecularly targeted therapeutic strategies and preventing AKI-related morbidity and mortality (A. P. Singh et al., 2012).

1.3 Oxidative stress in the kidney

1.3.1 Pathogenesis of oxidative stress

1.3.1.1 Reactive oxygen species

Oxidative stress is a molecular event defined by reactive oxygen species (ROS) accumulation at the cellular and tissue level (Betteridge, 2000). It is the imbalance between the production of ROS and the capacity of antioxidants. ROS are oxygen-containing highly reactive ions and free radicals which contain atoms with an unpaired electron in the outer orbit, including superoxide anion (O_2^-), hydroxyl radical (·HO), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and nitric oxide (NO) (Fubini & Hubbard, 2003). Free radicals steal an electron from an adjacent compound to become stable. However, the adjacent compound will then become a new free radical which will steal an electron from another nearby molecule or cellular structure. In this case, there is a chain reaction causing constant cellular damage to millions of nearby cells (Lobo, Patil, Phatak, & Chandra, 2010). There are endogenous and exogenous sources of ROS production. The major endogenous sources of ROS are localized to mitochondria and can be related to the respiratory chain, substrate dehydrogenases in the matrix, monoamine oxidase and cytochrome P450 (Chernyak et al., 2006). ROS can also be produced from exogenous sources, such as pollution, cigarette smoke, excessive alcohol consumption, radiation and medication.

1.3.1.2 Antioxidants

An antioxidant is defined as a substance that, when present at low concentrations compared to those of an oxidizable compound (e.g. DNA, proteins, lipids or carbohydrates), significantly prevents or delays oxidative damage caused by the presence of ROS (Bouayed & Bohn, 2010). There are enzymatic and non-enzymatic antioxidants that can ameliorate the oxidative damage (Sies, 1997). The enzymatic antioxidant includes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR). The non-enzymatic antioxidant system consists of low molecular weight molecules, such as vitamin E (γ-tocopherol), vitamin C (L-ascorbic acid), glutathione (GSH), and some proteins, such as transferrins, albumin, and lactoferrin. In the body, the antioxidant defense system is responsible for combating overproduced ROS, therefore achieving the balance between the production of ROS and the antioxidant defense mechanism.

As shown in Figure 1-3, most of the O_2 is consumed through its four-electron reduction to water by cytochrome c oxidase. During the transfer of electrons along the electron transport complexes, a small proportion of O_2 (2-4%) is converted to superoxide (O_2^-) by univalent reduction (by single electrons) following the electron leak along the electron transport chain. O_2^- is converted by SOD to H_2O_2 that, in turn, is reduced to H_2O by glutathione peroxidase (GPx) using GSH as a substrate. Catalase is another enzyme that breaks H_2O_2 into H_2O and O_2. Oxidized glutathione (GSSG) is reduced with the conversion of NADH to NAD^+. ·HO is generated by the reduction of H_2O_2 in the presence of endogenous iron by means of the Fenton reaction. Furthermore, when both O_2^- and NO are synthesized within a few cell

diameters of each other, they will combine spontaneously to form peroxynitrite ($ONOO^-$) by a diffusion-limited reaction.

When there is an imbalance between the production of ROS and the antioxidant defense system, oxidative stress occurs. Enhanced oxidative stress results in the direct oxidative damage to biomolecules, causing the modification of cellular targets and loss of their functions. This damage can include single or double strand of DNA breaks, the loss of cellular ATP storage due to mitochondrial inner membrane destruction, initiation of lipid peroxidation in membrane phospholipids and ROS-responsive protein modification (Pacifici & Davies, 1991). Oxidative stress can also regulate some signaling pathways indirectly, which will be described later (Section 1.3.2.2).

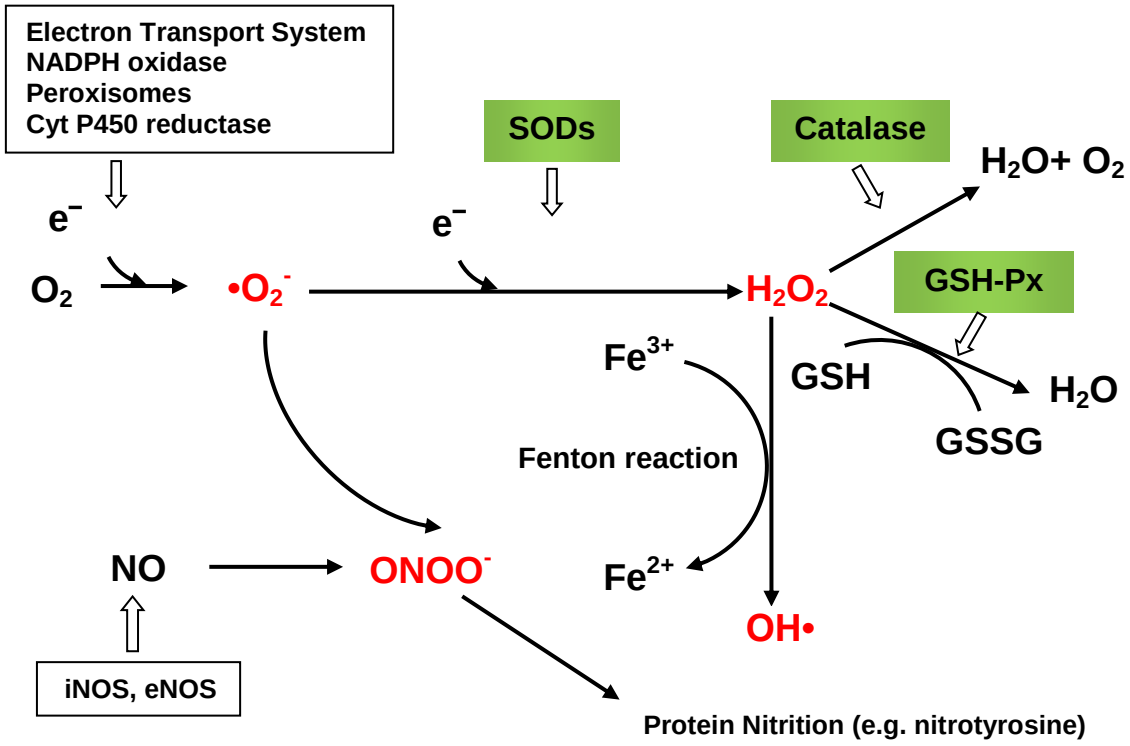

Figure 1-3 ROS and major antioxidants

Simplified schematic illustration of common forms of reactive oxygen and nitrogen species and major antioxidant

(Abbreviations: SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase)

1.3.2 Role of oxidative stress in the kidney

1.3.2.1 Sources of ROS in the kidney

ROS are produced during normal cellular metabolism. In an aerobic environment, living organisms are exposed to ROS continuously and inevitably. During the physiological cellular processes, basal levels of ROS are detected in tissues. Electron transport chains all possess the potential to "leak" electrons to oxygen resulting in superoxide formation. Mitochondrial ROS are produced by electron leakage from electron transport chain complexes during normal respiration, particularly from Complex I and Complex III (Chen, Vazquez, Moghaddas, Hoppel, & Lesnefsky, 2003). In human phagocytes, certain enzyme activities generate a superoxide anion and, via an oxidative burst, ROS are released to attack and kill cells infected with viruses, or bacteria, although surrounding tissue can also be affected during this process (Cooke, Evans, Dizdaroglu, & Lunec, 2003). Peroxisomes provide compartmentalization of various oxidative reactions leading to reactive species, and a lack of functional peroxisomes can lead to detrimental effects to the cell. However, under certain conditions these products may be released. ROS may also be generated by ionizing or ultraviolet radiation or by exposure to certain chemicals. ROS may interact with cellular biomolecules (namely proteins, lipids, and DNA), leading to modification and potentially serious consequences for the cell.

Oxidative stress caused by elevated ROS has been linked to a number of pathologies, such as hypertension, atherosclerosis, diabetes, neurodegeneration and kidney diseases (Madamanchi, Vendrov, & Runge, 2005; Uttara, Singh, Zamboni, & Mahajan, 2009). However, more accumulating evidence suggests that ROS also act as signaling molecules in

the regulation of normal physiological conditions, a process termed redox biology. It appears that the compartmentalization, specificity and selectivity of ROS regarding their targets are factors that determine the role of ROS, i.e. oxidative stress or redox biology (Schieber & Chandel, 2014).

1.3.2.2 Targets of oxidative stress in the kidney

High levels of ROS lead to cell or tissue damage by directly attacking biological molecules, regulating the expression of antioxidant enzymes and transcriptional factors. Three main targeted biological molecules are lipids, proteins and DNA. Oxidative stress generates multiple oxidation products of different biomolecules. Membrane lipids are the first target of ROS over-production. ROS-induced lipid peroxidation results in the alteration in membrane fluidity, decrease membrane potential and increase its permeability to hydrogen and other ions, and cause the eventual rupture of the cells. Specific products of lipid peroxidation have also been used as indicators of ROS-mediated oxidative stress. The measurement of malondialdehyde (MDA), a product of lipid peroxidation, by a thiobarbituric acid-reacting substances (TBARS) assay is a widely used test (Gutteridge & Halliwell, 1990). ROS can cause direct modification of proteins through disulphide bond formation, nitrosylation, carbonylation, and glutathionylation (England & Cotter, 2005). The indirect attack results from the reaction between proteins and secondary by-products of fatty acid peroxidation (Berlett & Stadtman, 1997). The oxidative damage of DNA includes double-strand breaks, modifications on base and nucleotide, particularly of guanosine (Burney, Niles, Dedon, & Tannenbaum, 1999). In addition to the attack on biomolecules, ROS are also linked with various cellular signaling pathways. Other than the regulation achieved by classical cytosolic

signaling pathways, such as the family of mitogen-activated protein kinases (MAPK) (e.g. c-Jun N-terminal kinase, and p38 kinase family), growing evidence suggests that a number of transcription factors can directly or indirectly alter their transcriptional activities in response to the cellular redox conditions, such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1), hypoxia-inducible factor-1 (HIF-1), p53 and NF-E2-related factor 2 (Nrf-2) (Trachootham, Lu, Ogasawara, Nilsa, & Huang, 2008). Through the protein modification and transcriptional regulation, oxidative stress could regulate the expression of antioxidant enzymes, as well.

In general, like other organs, ROS generation plays an important protective and functional role in the kidneys. ROS, at a physiological level, are involved in host defense to bacterial pathogens and ROS-mediated responses, including regulating vascular tone, monitoring erythropoietin production, and signal transduction from membrane receptors (Droge, 2002). The kidney is a highly vulnerable organ to damage caused by oxidative stress. Oxidative stress plays critical role in the pathophysiology of several kidney diseases. The lipid composition of the kidney contains abundance of long-chain-polyunsaturated fatty acids, which may become a vulnerable target for reactive oxygen attack leading to oxidative damages (Ozbek, 2012). Proximal tubular cells are rich in mitochondria and have high transport rates, driven primarily by the Na^+/K^+ ATPase (Gyory & Kinne, 1971). There is a relatively high energy demand that is predominantly reliant upon oxidative phosphorylation. Therefore, among the renal cells, proximal tubular cells are most sensitive to oxidative stress injury (Epstein, 1997).

1.3.3 Biomarkers of oxidative stress in the kidney

Uncontrolled oxidative stress in the kidney contributes to functional oxidative modifications of cellular protein, lipid and DNA and consequences of oxidative modification play an important role in renal damage and diseases. Measurements of oxidative modified protein, lipid and DNA in biological samples (plasma, urine and biological tissues) may assist in the elucidation of the pathophysiological mechanisms of the oxidative stress-related renal damage, the prediction of disease prognosis and the early diagnosis and selection of adequate treatment in the early stage of disease. These oxidized molecules serve as biomarkers in various diseases, including renal diseases (Table 1-2). There are advantages and disadvantages of the selected oxidative stress biomarkers. MDA, as a lipid peroxidation marker, is easy to be quantified using the TBARS assay. However, it is well documented that the TBARS assay is non-specific, and sample preparation can influence results (Meagher & FitzGerald, 2000). Oxidized LDL has been found to be a predictive biomarker of future coronary artery disease (CAD) in healthy population and correlates with increasing clinical severity. The disadvantage of oxidized LDL as a oxidative stress biomarker is that the reduction by antioxidant therapy is not matched by the reduction in cardiovascular disease (CVD) severity (Meisinger, Baumert, Khuseyinova, Loewel, & Koenig, 2005). As a protein oxidation marker, nitrotyrosine appears to be independent of traditional CAD risk factors. However, the assay for measurement of nitrotyrosine is expensive (Weber et al., 2012). The specificity and sensitivity of the assays used to measure oxidative stress biomarkers need to be improved.

Table 1-2 Oxidative stress markers

Target molecules	Oxidative stress biomarkers
Lipids	MDA (Malondialdehyde) Oxidized LDL (Low-density lipoprotein) F2-isoprosanes Isolevulglandins HNE (4-hydroxy-2-nonenal) LOOHs (Lipid hydroperoxide)
Proteins	Nitrotyrosine Carbonylated proteins AOPP (Advanced oxidative protein products)
Carbohydrates	Reactive carbonyl compounds AGE (Advanced glycosylation end products)
DNA	8-hydroxyguanine

Based on the ideas of "Free Radicals in Human Health and Disease" edited by Vibha Rani et.al

1.3.4 Oxidative stress in renal I/R kidneys

In kidney tissues subjected to I/R insult, renal ischemia initiates a series of events and causes cell injury and dysfunction, which tends to be reversible if the ischemia period is short. If the ischemia or reperfusion is of sufficient magnitude and duration, irreversible cellular damage occurs. Oxidative stress plays a major role in the pathological changes associated with renal I/R. Briefly, renal I/R-induced oxidative stress damage can be divided into two phases. During the ischemia phase, due to the lack of oxygen supply, anaerobic glycolysis prevails, which produces acidosis and leads to a decrease in cellular pH. As ATP is progressively depleted during ischemia, the dysfunction of Na^+/K^+ ATPase results in a rise in intracellular calcium (Ca^{2+}) concentration. The calcium overload activates a calcium-triggered and protease-dependent conversion of the native xanthine dehydrogenase (XD) to xanthine oxidase (XO) (Linas, Whittenburg, & Repine, 1990). At the same time, degradation of ATP during ischemia provides an oxidizable substrate, hypoxanthine, which generates superoxide anion and hydrogen peroxide, catalyzed by XO (Kalogeris, Baines, Krenz, & Korthuis, 2012). Low antioxidant activity has been shown in the renal ischemia phase (Aragno et al., 2003). In the subsequent reperfusion phase, these reactions proceed with a sudden increase in oxygen radicals. With the restoration of blood flow, reperfusion of the ischemic tissue triggers a cascade of events that exacerbate oxidative stress injury. There are increased lipid peroxidation products in the early reperfusion phase. Uncontrolled ROS production leads to endothelial cell damage and consequently to impaired endothelial function. This results in a local imbalance of vasoactive substances, with enhanced release of vasoconstrictors and decreased abundance of vasodilators. A persistent renal vasoconstriction that reduces overall kidney blood flow to approximately 50% of normal level has long been considered to

contribute to further tubular cell damage (Lameire, Van Biesen, & Vanholder, 2005). Oxidative stress is the main contributor to tubular cell damage due to lipid peroxidation and DNA mutation in renal tubular cells, causing renal dysfunction. In addition, there are several signaling pathways involved in the reperfusion phase, such as activation of transcriptional factors AP-1 and NF-κB, which can regulate the gene expression and cause oxidative stress renal injury.

1.3.5 Nitric oxide

Nitric oxide (NO) plays an important role in renal I/R-induced oxidative stress damage. There have been reports suggesting that the physiological and morphological changes in AKI are mediated by the NO levels in the kidneys (Goligorsky, Brodsky, & Noiri, 2002; Heemskerk, Masereeuw, Russel, & Pickkers, 2009; Ujiie, Yuen, Hogarth, Danziger, & Star, 1994). NO has been known as a toxic gas for centuries. Since its discovery as a biologically active molecule in the late 1980s, NO has been found to play an important role as a signal molecule as well as a cytotoxic or regulatory molecule in various physiological processes and pathophysiological abnormalities (Kroncke, Fehsel, & Kolb-Bachofen, 1998). NO is synthesized endogenously from L-arginine through nitric oxide synthase (NOS). There are three isoforms of NOS named after the type of cell where they were first reported: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). Several studies have investigated the expression of NOS in the kidney (Furusu et al., 1998; Roczniak, Fryer, Levine, & Burns, 1999; Ujiie et al., 1994), where all three isoforms of NOS can be found (Forstermann, Boissel, & Kleinert, 1998). eNOS is expressed in glomerular endothelial cells and the endothelium of cortical vessels. nNOS is present in the tubular

epithelial cells of the macula densa and inner medullary collecting duct. The expression of iNOS is found to be low or undetectable in normal kidney tissue, and it is mostly activated in pathological conditions. However, its cellular localization remains controversial, probably due to the differences in pathological states, experimental models and designs of experimental procedures (Choi, Nam, Jin, Kim, & Cha, 2012). It has been demonstrated that, during renal I/R injury, there is an increased NO production in kidney tissues produced by the activation of iNOS (Ling et al., 1999; Walker, Walker, Imam, Ali, & Mayeux, 2000). The direct toxicity of NO is modest, and evidence suggests that most of the cytotoxicity of NO is due to peroxynitrite ($ONOO^-$), rather than "pure" NO.

Peroxynitrite, formed from the diffusion-limited reaction between NO and superoxide anion, has been implicated as an initiator of a number of cytotoxic oxidative reactions (Jourd'heuil et al., 2001; Pacher, Beckman, & Liaudet, 2007). Peroxynitrite is a particularly powerful oxidant of aromatic molecules and organosulfur compounds that include free amino acids and peptide residues (Szabo, 2003). Peroxynitrite can nitrate aromatic amino acid residues such as tyrosine to form nitrotyrosine. The presence of nitrotyrosine on proteins can be used as a marker for peroxynitrite formation *in vivo* (Beckman, Chen, Ischiropoulos, & Crow, 1994).

1.3.6 NF-κB

Studies suggest that transcription factor nuclear factor kappa B (NF-κB) plays an important role in upregulating the expression of iNOS. Our previous study reported that the enhanced iNOS expression was mediated via NF-κB activation in kidneys (F. Zhang, Siow, & O, 2004). NF-κB is normally held in the cytoplasm in an inactive form that is associated with an

inhibitory protein called IκB. Upon stimulation by various NF-κB stimuli, IκBα (a well-studied IκB protein) is rapidly phosphorylated, leading to ubiquitination and subsequent degradation of IκBα as well as translocation of NF-κB to the nucleus (activated NF-κB). The active NF-κB binds to the NF-κB binding motifs in the promoters or enhancers of the iNOS genes and then regulates their expression. NF-κB has been suggested to play a pivotal role in the pathophysiology of I/R renal injury (Guijarro & Egido, 2001). It has been reported that iNOS-mediated NO production in the kidney during renal I/R injury was mediated via NF-κB activation, and NF-κB decoy oligodeoxynucleotides treatment was able to protect renal tissue from the effects of I/R injury and reduce the severity of AKI (Cao et al., 2004). NF-κB represents an important therapeutic target in the treatment of renal I/R-induced AKI.

1.3.7 Antioxidant treatment in AKI

As the most important defense against free radical-induced injury, antioxidants can attenuate the harmful effects of ROS by scavenging and detoxifying free radicals. Adequate dietary, enzymatic and non-enzymatic antioxidant supplementations have been investigated for the prevention and treatment of oxidative stress-related diseases. There has been much interest in determining whether antioxidant therapy can attenuate renal I/R injury.

Numerous substances such as novel antioxidants, antioxidant enzyme mimetics, vitamins, amino acids, and natural antioxidant agents, have been tested in various clinical settings and in experimental AKI animal models (Chatterjee, 2007). Several ROS-scavenging agents have provided promising results in renal I/R animal models. Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a potent scavenger of hydroxyl and peroxyl radicals, was reported to ameliorate I/R injury in the rat (Doi, Suzuki, Nakao, Fujita, & Noiri, 2004). It has been

shown that the membrane permeable SOD mimetic 4-hydroxy-Tempo (tempol) is beneficial when used in *in vivo* models of renal I/R injury (Chatterjee et al., 2000). Vitamins such as vitamin C and vitamin E have been shown to have the protective effect on I/R injury (Korkmaz & Kolankaya, 2009; Uysal, Girgin, Tuzun, Aldemir, & Sozmen, 1998). Some amino acids have also provided benefits against renal I/R injury. For examples, N-acetyl cysteine (NAC) has been shown to ameliorate I/R injury of the kidney (Di Giorno et al., 2006). The administration of glutamine (a precursor of glutathione) prior to a renal I/R improved the antioxidant system and protected the renal injury (Gouvea Junior et al., 2011). Several natural antioxidant agents have been reported to afford protection against I/R-induced renal injury, including pomegranate extracts (Sancaktutar et al., 2014), garlic oil (Savas et al., 2010), resveratrol (L. Giovannini et al., 2001) and ginkgo biloba extract (Sener et al., 2005), to name a few. There are still differences in experimental model, sample size, and patient conditions which result in minor inconsistencies in response to the treatment, and successes are limited in several animal models. Although showing promise in laboratory-based experimental models of renal I/R injury, the translation to human studies either has failed or has been inconclusive (Chatterjee, 2007). The antioxidant treatments have not reduced the mortality associated with AKI in clinical studies. Currently, our understanding of the protective effect of antioxidants on renal I/R injury is still limited. Questions remain over the long-term clinical use of antioxidant treatment, and the benefits of such treatment in clinical conditions involving renal I/R need to be fully determined.

Oxidative stress can activate a variety of transcription factors, which will lead to the gene expression of inflammatory cytokines, chemokines and some anti-inflammatory molecules.

Under the inflammatory conditions, production of ROS by inflammatory cells is central to the progression of many diseases.

1.4 Inflammatory response

1.4.1 Pathogenesis of inflammatory response

1.4.1.1 Definition of inflammation

Inflammation is known as a complex and multifactorial network of interactions among soluble factors and cells. Inflammation can arise in any tissue in response to traumatic, infectious, post-ischemic, toxic or autoimmune injury (Nathan, 2002). It is characterized by the secretion of inflammatory mediators by leukocytes and parenchymal cells, the increase in vascular permeability, the infiltration of inflammatory cells, and the accumulation and/or turnover of the extracellular matrix (Liang et al., 2007). The inflammatory process normally aims at repairing the lesion, promoting tissue remodeling and restoring tissue homeostasis. A key event in the inflammatory response is the localized recruitment of inflammatory cells to the site of tissue injury or infection, which is initiated by responsive leukocytes and lymphocytes (Mazumder, Li, & Barik, 2010). The interactions between cytokines and their complementary cell-surface receptors culminate in the expression of new gene products, leading to the elimination of invading organisms and the initiation of local tissue repair (Takeuchi & Akira, 2010). However, if the targeted destruction and assisted repair mechanisms are not properly regulated, persistent inflammation may cause tissue and organ damage, even death (Nathan, 2002).

1.4.1.2 Types of inflammation

Inflammation is commonly divided into two categories: acute and chronic. Acute inflammation occurs within seconds to minutes (almost immediately), following cellular injury or infection. The period of acute inflammation is of relatively short duration, lasting from hours to days. It is characterized by the exudation of fluids and plasma proteins (edema) and by the migration of leukocytes, predominantly neutrophils (also called polymorphonuclear leukocytes), to the site of the injury (Pooler, 2009). Chronic inflammation is of longer duration than acute inflammation (weeks or months). It is associated with the presence of mononuclear cells (such as macrophages and lymphocytes), the proliferation of blood vessels, fibrosis, and tissue necrosis (Pooler, 2009). There is an overlap between two types of inflammation, and mediators are involved in the modulation of both processes.

1.4.1.3 Overview of the inflammatory response

At the most basic level, an acute inflammatory response can be triggered by a variety of stimuli, including (1) physical agents (trauma, ischemic, exposure to heat or chemicals, mechanical injury, radiation); (2) toxic chemical agents (organic and inorganic poisons); (3) microbiological agents (viruses, bacteria, parasites, fungi); (4) immunological agents (cell-mediated, immune complex, antigen-antibody reactions) (Cray, Zaias, & Altman, 2009). Inflammation has both vascular and cellular events. In response to the recognition of stimuli, changes in vascular flow and blood vessel caliber begin immediately after injury and develop at variable rates, depending on the nature and severity of the injury (Kushner, 1982). These alterations lead to the structural changes in microvasculature, which allow plasma proteins

and leukocytes (mainly neutrophils) to leave the blood circulation (increased in vascular permeability) and migrate to the site of injury. The main and most immediate effect is to elicit an inflammatory exudate locally. In addition, endothelial cells are activated, resulting in the increased adhesion of leukocyte and migration through the vascular wall (Yadav, Larbi, Young, & Nourshargh, 2003). The cellular events in inflammation are closely related to the vascular events. When the leukocytes reach the afflicted tissue site, neutrophils become activated either by direct pathogen recognition or through the actions of cytokines secreted by tissue-resident cells (Medzhitov, 2008). The inflammatory response is coordinated by a large range of mediators that form complex regulatory networks (Medzhitov, 2008). These mediators play key regulatory roles in host defense and inflammatory diseases.

1.4.1.4 Mediators in inflammation

During inflammation, many highly reactive agents are released, including cytokines, chemokines, endothelial/leukocyte adhesion molecules, reactive oxygen species, lipid mediators (leukotrienes and prostaglandins), and matrix metalloproteases.

Firstly, cytokines represent a vast array of relatively low molecular weight pharmacologically active polypeptides with endocrine, paracrine, autocrine or juxtacrine modes of action (Mann & Young, 1994). Inflammatory cytokines are produced by a wide variety of cell types and play important roles in physiological and pathological processes (Chung et al., 2009). They play pivotal roles in the activation of endothelium and leukocytes and the induction of the acute-phase response during inflammatory response. There are both pro-inflammatory and anti-inflammatory cytokines. Major pro-inflammatory cytokines

include interleukin 1 (IL-1), IL-6, IL-8 and tumor necrosis factor alpha (TNF-α), which are toxic to the cells, whereas anti-inflammatory cytokines include IL-4, IL-10, and IL-13 that exert anti-inflammatory properties on various cell types (Dinarello, 2000).

Second, chemokines are a group of small (7–15 kDa) secreted proteins within the family of chemotactic cytokines. They are mainly involved in navigating leukocytes toward the site of inflammation. Chemokines can be produced by many different cell types in response to bacteria or stimulation with pro-inflammatory cytokines (e.g., IFN-γ, IL-1) (Luther & Cyster, 2001). Chemokines mediate chemotaxis through the activation of G-protein-coupled receptors, and the receptors expressed on the surface of circulating leukocytes determine the chemokines to which the leukocytes will respond (Charo & Taubman, 2004).

Thirdly, endothelial-leukocyte adhesion molecules play a main role in the recruitment of neutrophils to the site of inflammation by mediating the adherence of leukocytes to the endothelial surfaces. Endothelial-leukocyte adhesion molecules are glycoproteins that are expressed on the surface of leukocytes. Based on the structural features, they are grouped into three major families: the selectins (L-, E-, and P-selectin), the integrins and the members of the immunoglobulin superfamily (e.g., ICAM-1 and VCAM-1) (Langer & Chavakis, 2009). Activated by an inflammatory stimulus, the endothelium expresses selectins, whose binding to their receptors on leukocytes initiates a rolling adhesion to the vessel's luminal wall. The leukocytes activate their integrins, which binds to endothelial ICAMs, permitting a firmer and stationary adhesion. Finally, the leukocytes migrate between endothelial cells into the interstitium and move toward the source of the stimulus (chemotaxis) (Rao, Yang, Garcia-Cardena, & Luscinskas, 2007).

In addition, lipid mediators are widely appreciated for their pro-inflammatory activities, such as prostaglandins (PGs), leukotrienes and platelet-activating factors, which play specific roles in the physiology of the inflammatory response (Serhan, Chiang, & Van Dyke, 2008). Biosynthesis of these lipid mediators is activated rapidly within seconds to minutes of acute challenge by leukocytes from membrane-derived arachidonic acid, which is oxidized via the cyclooxygenase and lipoxygenase (Funk, 2001). Prostaglandins are formed by most cells in our body and exert their effect by binding to specific G-protein-coupled receptors and influencing second messenger systems. As one of the most abundant PGs produced in the body, prostaglandin E_2 (PGE_2) acts as a mediator of pyrexia, hyperalgesia, and vasodilation, which increases blood flow to inflamed tissues and leads to enhanced microvascular permeability and edema (Boniface et al., 2009). Leukotrienes are made mainly by inflammatory cells, such as polymorphonuclear leukocytes, activated macrophages, and mast cells (Funk, 2001). Leukotrienes function as potent chemoattractants for neutrophils and as mediators of vascular permeability (Samuelsson, 1983). Inflammaory response is further mediated by platelet-activating factor (PAF), which is another important lipid mediator regulating leukocyte recruitment and partially influencing the vascular tone, permeability and platelet activation (Medzhitov, 2008).

An inflammatory stimulus results in the recruitment and activation of various immune cells (i.e. macrophages, dendritic cells), which produce and release ROS. ROS formed by phagocytes are the principal mediators by which inflammatory cells kill their targets at sites of inflammation (Jaeschke, 2000). Matrix metalloproteases facilitate neutrophil migration and recruitment by the degradation of the extracellular matrix (Chen et al., 2013). It has become apparent that inflammatory mediators play a crucial role in the development and/or

progression of diseases. As our understanding grows, inflammatory mediators will provide opportunities to develop novel diagnostic and therapeutic strategies.

1.4.2 Role of inflammatory response in kidney injury

Typically, there are two different types of renal injury: acute and chronic. Acute kidney injury (AKI) is commonly caused by infection, sepsis or I/R injury. Chronic kidney disease (CKD) is associated with diabetes mellitus, obesity, hypertension and autoimmunity. AKI may lead to CKD, and especially if undetected, both can progress to end-stage renal disease (ESRD) (Chawla & Kimmel, 2012). Irrespective of the different initiating events that promote renal disease, inflammation is a common underlying characteristic for both AKI and CKD (Imig & Ryan, 2013).

Inflammatory response plays an important role in renal disease process. An association between renal impairment and different mediators and markers of inflammation has been observed in different studies, even among patients with moderate renal impairment. Those inflammatory markers include fibrinogen, P-selectin, IL-6, TNF-α, soluble ICAM-1, and C-reactive protein (CRP) (Goicoechea et al., 2008; Shankar et al., 2012). Evidence suggests that there is a close correlation between the inflammatory cell numbers in kidney tissue and the degree of glomerular and tubulointerstitial lesions, as well as the loss of kidney function (Hiromura, Kurosawa, Yano, & Naruse, 1998). The importance of inflammatory mediators in the development of renal inflammation is confirmed by studies which show that their removal from the plasma by dialysis could provide a beneficial effect (De Vriese et al., 1999; Inthorn & Hoffmann, 1996).

1.4.3 Mechanism of inflammatory response in kidney

Inflammatory response is not only a local process but can be reflected systemically. It is characterized by inflammatory cell accumulation and increased expression of adhesion molecules, chemokines, and inflammatory cytokines. Regardless of the initial cause of renal insult, there is an inflammatory cell influx to a localized area in the early stages of kidney damage (Noronha, Fujihara, & Zatz, 2002). As the predominant cells, lymphocytes are mobilized to the injury site and become activated by exposure to antigens. The stimulation of lymphocytes produces lymphokines, which results in the attraction and activation of neutrophils, macrophages, and lymphocytes. During the inflammatory process, the inflammatory cells can cause direct injury to renal tubular epithelial cells through direct cell-cell contact, or indirectly by the up-regulated expression of cytokine and chemokine (Anders, Vielhauer, & Schlondorff, 2003; Bonventre & Yang, 2011).

1.4.4 Inflammatory response in renal I/R-induced injury

The mechanisms of inflammation in renal I/R injury involve various cell types and signal transduction cascades. Endothelial dysfunction plays a major role in the inflammatory responses. Following the ischemic insult and subsequent reperfusion, chemokines and adhesion molecules are released from endothelium of blood vessels in the kidney. Upregulation of chemokines and adhesion molecules results in the infiltration of inflammatory cells, including macrophages, neutrophils and lymphocyte from renal blood vessels, being directed to the renal interstitium following the chemokine concentration gradient. Inflammatory cascades that are initiated by endothelial dysfunction can be augmented considerably by several potent mediators which are produced by proximal tubule,

representing a "maladaptive response" (Ramesh & Reeves, 2004). Among various resident kidney cells, tubular epithelial cells undergo the most significant morphological changes. Rather than being a passive victim, tubular epithelial cells play a pivotal role during the process of inflammation (de Haij, Woltman, Bakker, Daha, & van Kooten, 2002). Proximal tubular epithelial cells produce many inflammatory mediators, including cytokines IL-6, IL-8 and TNF-α (Patel et al., 2005; Simmons et al., 2004). After the production and release by the proximal tubule, cytokines enter the interstitium and activate macrophages, neutrophils and lymphocytes in the kidney. The activation of inflammatory cells induces vasoconstriction by producing vasoconstrictors, which may cause further tubular and vascular damage (Akcay, Nguyen, & Edelstein, 2009). There are increased circulating pro-inflammatory cytokines such as TNF-α and IL-6 which may result in not only renal tissue damage, but also distant organ injury (S. W. Park et al., 2011).

1.4.5 Anti-inflammatory response

1.4.5.1 Anti-inflammatory mechanisms

It has been shown that inflammation can be limited by anti-inflammatory counterregulatory mechanisms, which play a fundamental role in maintaining vascular homeostasis and preventing the spread of inflammatory mediators into the bloodstream (Tracey, 2002). The anti-inflammatory mechanisms involve anti-inflammatory external signals and intracellular mediators (Tedgui & Mallat, 2001).

The external signals include IL-10, transforming growth factor-β (TGF- β), and IL-1 receptor antagonist, HDL, as well as some angiogenic growth factors (Tedgui & Mallat, 2001). As the most important anti-inflammatory cytokine found within the human immune response, IL-10

acts as a Th2 lymphocyte cytokine, as well as a potent deactivator of monocyte/macrophage pro-inflammatory cytokine synthesis (Kasama, Strieter, Lukacs, Burdick, & Kunkel, 1994). IL-10 can also directly block NF-κB/DNA binding by blocking IκB kinase activity and stimulating IκBα phosphorylation (Schottelius, Mayo, Sartor, & Baldwin, 1999). TGF-β family members are synthesized as inactive precursor and require activation before exerting their effects. TGF-β has been reported to exert anti-inflammatory actions by deactivating the macrophage, suppressing cytokine-induced expression of cellular adhesion molecules, and by restoring endothelial-dependent vasodilation (Bogdan & Nathan, 1993; Lefer, Tsao, Aoki, & Palladino, 1990; S. K. Park et al., 2000). IL-1 receptor antagonist blocked the action of IL-1α and IL-1β functional ligands by competitive inhibition at the IL-1 receptor level (Dinarello, 1997).

Several regulators are involved in the anti-inflammatory intracellular mediators, including NF-κB signaling pathway regulators, protective genes (e.g. Bcl-2, A20) and peroxisome proliferator–activated receptors (PPARs). Endogenous protective genes, such as Bcl-2 family (Bcl-2, Bcl-xL, and A1), A20, and heme oxygenase-1 (HO-1), can be expressed by vascular cells to limit the inflammatory process and injury by inhibiting NF-κB activation (Badrichani et al., 1999; Ferran et al., 1998). PPARs are a family of ligand-activated transcription factors, belonging to the nuclear receptor superfamily. PPARs exert their anti-inflammatory effects by inhibiting the induction of pro-inflammatory cytokines, adhesion molecules and extracellular matrix proteins or by stimulating the production of anti-inflammatory molecules (Kostadinova, Wahli, & Michalik, 2005). Among the anti-inflammatory intracellular mediators, NF-κB signaling pathway has been considered to play a key role in the regulation of anti-inflammatory actions of mechanical strain. As a primary transcription factor, NF-κB

regulates inducible gene expression which leads to the synthesis of a wide range of pro-inflammatory mediators, such as cytokines, chemokines and adhension molecules. NF-κB pathway is widely believed to have great potential as a molecular target for anti-inflammatory therapy (Yamamoto & Gaynor, 2001).

1.4.5.2 Anti-inflammation therapy in I/R-induced injury

Inflammation plays a key role in the progression of I/R-induced AKI and multiple cell types which derive from monocyte/macrophage lines have been implicated in the disease process. Several inflammatory mediators such as chemokines, cytokines, and adhesion molecules that derive from the renal vascular wall cause damage to kidney tissues via the activation of inflammatory pathways. Thus, targeting different inflammatory pathways might be a potential pharmacologic intervention and have efficacy in prevention and treatment for renal I/R injury. During recent years, anti-inflammatory treatment has been studied in renal I/R. Several therapeutic strategies for the prevention or treatment of renal I/R injury have been employed. These include blockade of cytokine and chemokine synthesis (e.g. IL-1α, -1β, -6, -8 and TNF-α), adhesion molecules (P-selectin, ICAM-1) (Lien, Yong, Cho, Igarashi, & Lai, 2006; K. Wu, Lei, Tian, & Li, 2014), NF-κB (Cao et al., 2004), specific MAPK (Furuichi et al., 2002), and so on. Several pharmacological agents have been shown to reduce inflammation and protect renal function in I/R-induced AKI. Statins were shown to reduce renal damage and attenuate renal dysfunction by exerting anti-inflammatory effects (Gueler et al., 2007; K. Wu et al., 2014). Sphingosine 1-phospate (S1P) maintains endothelial cell integrity and inhibits lymphocyte egress via the specific S1P1 receptor. The S1P1 receptor exerts an anti-inflammatory role by maintaining endothelial cell integrity, and trafficking of

lymphocytes. SEW2871, a S1P1 receptor selective agonist, has been shown to reduce the inflammation and protect renal function in mice I/R model (Awad et al., 2006; Lai, Yong, Igarashi, & Lien, 2007). Hypersulfated and low-molecular-weight heparins also reduced renal inflammation after I/R injury (Gottmann et al., 2007).

Although some anti-inflammatory treatment effectively ameliorates I/R injury in the experimental models, much remains unknown regarding the underlying mechanisms. The study on the potential anti-inflammatory treatment for renal I/R injury is still an area of research interest. Hydrogen sulfide (H_2S) has drawn much attention and might have the therapeutic potential because it has recently been shown to exert several anti-inflammatory effects.

1.5 Hydrogen sulfide

1.5.1 Introduction

Hydrogen sulfide (H_2S) is a colourless, flammable, and moderately water-soluble gas with a characteristic odour of rotten eggs. For many decades, H_2S has been considered primarily as a toxic gas and as an environmental hazard. However, recently H_2S was found to be produced endogenously and growing evidence has emerged for H_2S as a critical mediator of multiple physiological and pathological processes in mammalian systems (Li & Moore, 2008; R. Wang, 2002). H_2S has been identified as the newest member of a small family of gaseous, biological signaling molecules, termed gasotransmitters, after nitric oxide and carbon monoxide (R. Wang, 2002). There are several challenges of H_2S measurement, involving sample processing, detection methods used, and actual biochemical products

measured. One of the major challenges to measure H_2S in tissues and blood is the extremely short half-life of H_2S *in vivo* (Reiffenstein, Hulbert, & Roth, 1992).

1.5.2 Biosynthesis, storage and metabolism of hydrogen sulfide

Hydrogen sulfide is generated in mammals via both enzymatic and non-enzymatic pathways, although the latter only contributes small amounts to H_2S production (S. Singh & Banerjee, 2011). There are mainly three H_2S-producing enzymes which produce H_2S via transsulfuration pathways, namely cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (MST). Both CBS and CSE are cytosolic and pyridoxal-5'-phosphate (PLP) (active form of vitamin B6)-dependent enzymes whereas 3-MST is mostly present in the mitochondria and is not PLP dependent. CBS and CSE are widely expressed in cells and tissues, including liver, kidney, lungs, brain, skin fibroblasts, and blood lymphocytes (R. Wang, 2002). However, a degree of tissue specificity is apparent (Whiteman & Moore, 2009). Current evidence suggests that CBS is the main H_2S-forming enzyme in central nervous system (hippocampus, cerebellum, cerebral cortex, and brain stem) (Enokido et al., 2005), whereas CSE is the major H_2S-producing enzyme observed in the cardiovascular system (aorta, mesenteric artery, portal vein, and other vascular tissue) (Ishii et al., 2004). Moreover, it is reported recently that 3-MST and cysteine aminotransferase (CAT) are localized to neurons in the brain and to the vascular endothelium (Shibuya et al., 2009). These enzymes coordinately regulate transsulfuration pathway and control physiological H_2S levels in a complex and overlapping manner (Figure 1-4). Homocysteine (Hcy), as a H_2S precursor, is formed from methionine (Met) metabolism. From Hcy to cystathionine, CBS catalyzes the first step in the transsulfuration pathway by

using vitamin B6 as cofactor (Stipanuk & Ueki, 2010). Cystathionine is then hydrolyzed by CSE to form cysteine (Hughes, Centelles, & Moore, 2009). Cysteine is used as a substrate for both CBS and CSE to generate H_2S. The third pathway responsible for H_2S production is PLP-independent CAT/3-MST pathway, which is localized primarily in mitochondria (Shibuya et al., 2009). CAT converts cysteine to the intermediary, 3-mercaptopyruvate, which is followed by its conversion to H_2S by 3-MST. Once H_2S is formed, it is quickly broken down via chemical and enzymatic reactions. H_2S is metabolized by three existing pathways: (1) oxidation to sulfate (SO_4^{2-}), (2) methylation to dimethylsulfide, and (3) reaction with metalloproteins or disulfide-containing proteins (R. Wang, 2012). The major metabolic pathway for H_2S is the rapid multistep hepatic oxidation of sulfide to sulfate and the subsequent elimination of sulfate in the urine by the kidney (Beauchamp, Bus, Popp, Boreiko, & Andjelkovich, 1984). After H_2S is synthesized, it directly exerts its biological actions via different mechanisms. H_2S can also be stored as a form of bound sulfane sulfur or acid-labile sulfur and release sulfur later in response to a physiological signal (Ishigami et al., 2009; Ogasawara, Isoda, & Tanabe, 1994). Free H_2S is maintained at a baseline level and the concentration in the human blood and tissues shown in literature ranges broadly from 1-160 μM (Kiss, Deitch, & Szabo, 2008).

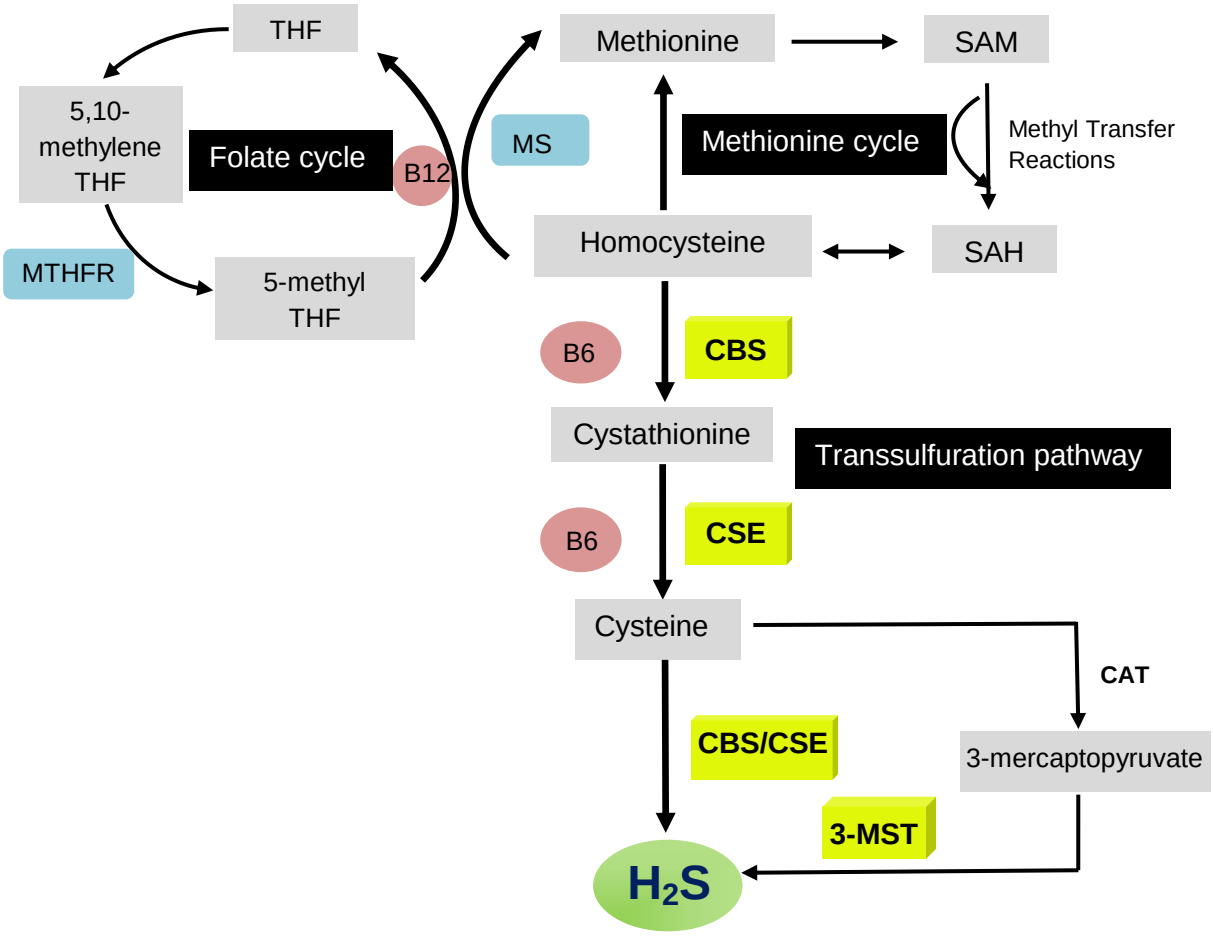

Figure 1-4 Enzymatic pathways involved in H_2S synthesis

There are three enzymatic pathways involved in the H_2S synthesis in mammalian systems. Much of the enzymatically generated H_2S is produced from L-cysteine by two pyridoxal-5'-phosphate (vitamin B6)-dependent enzymes, CBS and CSE. CBS is the first enzyme in the transsulfuration pathway, catalyzing the conversion of serine and homocysteine to cystathionine. 3-MST/CAT pathway has emerged as the third pathway for the production of H_2S. Hcy, a sulfur-containing amino acid and H_2S precursor, is formed from methionine metabolism. Homocysteine metabolism links the methionine cycle with the folate cycle.

(Abbreviations: CAT, cysteine amino transferase; CBS, cystathionine-β-synthase; CSE, cystathionine-γ-lyase; MS, methionine synthase; 3-MST, 3-mercaptopyruvate sulfur transferase; MTHFR, methylene tetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate)

1.5.3 Biological effects of hydrogen sulfide

As the most recent endogenous gasotransmitter, H_2S has been reported to exert various biological effects in physiology and disease condition. H_2S is thought to act via several pathways, some of which are illustrated in Figure 1-5 Biological effects of H2S

Firstly, H_2S is widely known to relax blood vessels through inhibition of phosphodiesterase (PDE) activity. H_2S has anti-inflammatory effect by suppressing leukocyte adhesion, leukocyte infiltration, and the consequent formation of edema (Zanardo et al., 2006). The anti-inflammatory effect of H_2S can be also promoted by inducing neutrophil apoptosis and by triggering significant changes in macrophage from pro-inflammatory M1 to the anti-inflammatory M2 phenotype. Accumulating evidence shows that H_2S has antioxidative action. As a potent one-electron chemical reductant and nucelophile, H_2S is capable of scavenging free radicals by single electron or hydrogen atom transfer directly (Carballal et al., 2010). H_2S can also attenuate oxidative stress-induced tissue damage via the inhibition of mitochondrial respiration. It can be utilized by mitochondria to generate ATP, particularly under hypoxia condition (Kimura, Goto, & Kimura, 2009). Additionally, H_2S participates in the regulation of inflammation, oxidative stress and apoptosis via the modulation of transcription factor activity. It has the ability to suppress the activation of NF-κB, leading to a reduction of pro-inflammatory cytokines (Oh et al., 2006). H_2S activates Nrf2-antioxidant response element (ARE) transcriptional pathway through the S-sulfhydration of Kelch-like ECH-associated protein-1 (Keap1) and protects cells against oxidative stress (Yang et al., 2012). H_2S also stimulates angiogenesis and contributes significantly to promoting the

healing of the injured tissues (Papapetropoulos et al., 2009). Antinociceptive effects of H_2S have also been demonstrated (Distrutti et al., 2006).

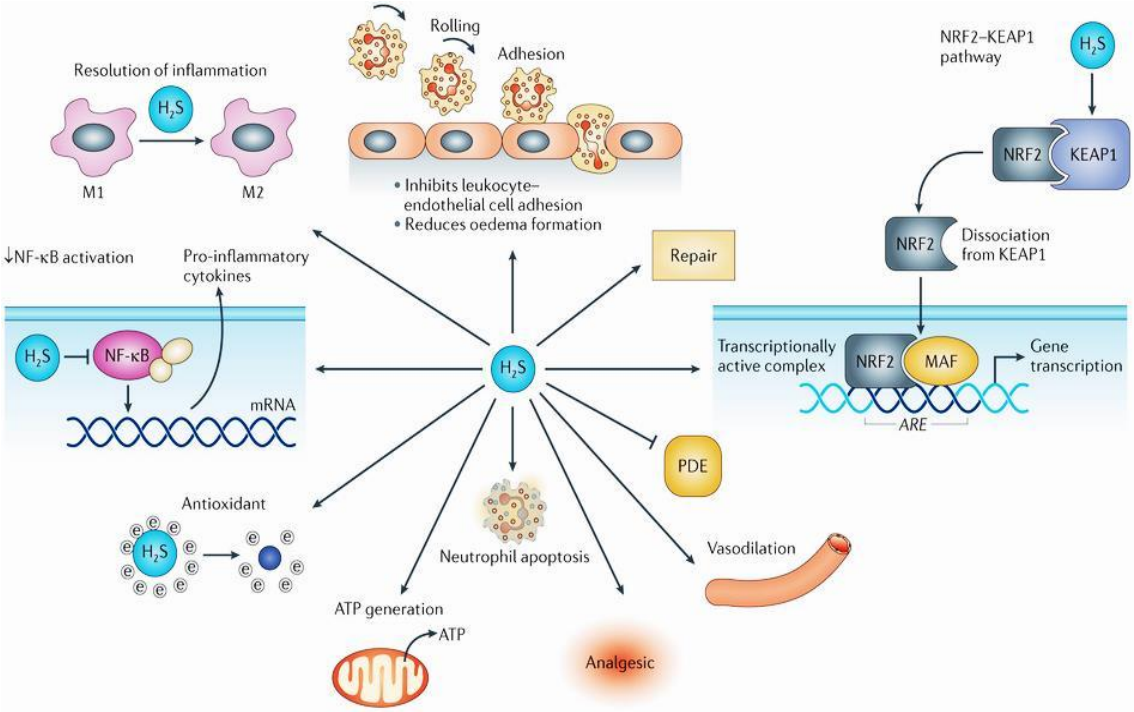

Figure 1-5 Biological effects of H_2S

H_2S suppresses leukocyte adhesion to the vascular endothelium and migration of leukocytes through to the subendothelial space, thereby reducing edema formation. It also reduces the expression of pro-inflammatory markers, through the downregulation of nuclear transcription factor-κB (NF-κB). It promotes tissue repair due to its effects as a vasodilator and its ability to be used as a substrate for ATP production. H_2S-induced vasodilation is through the inhibition of PDE. It displays anti-inflammatory activity by inducing neutrophil apoptosis and by promoting macrophage polarization from M1 to M2 phenotype. H_2S exerts its antioxidant effect by scavenging free radicals directly and activating the Nrf2/ARE signaling pathway (Figure from Wallace & Wang, 2015).

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(Abbreviations: AREs, antioxidant-response elements; KEAP1, Kelch-like ECH-associated protein 1; NF-κB, nuclear factor-κB; NRF2, nuclear factor erythroid 2-related factor 2; PDEs, phosphodiesterases)

1.5.4 Anti-inflammatory effect of hydrogen sulfide

Hydrogen sulfide exerts beneficial effects through numerous mechanisms and its role in inflammation has attracted growing interest in recent years. Although it has recently emerged as an area of intense concern for H_2S study, the exact regulatory mechanisms of H_2S in inflammation are still not well understood. It has been reported that the mechanisms of anti-inflammatory effect involve the reduction of nuclear translocation of NF-κB (H. Zhang, Zhi, Moochhala, Moore, & Bhatia, 2007), the repression of AP-1/c-fos activity (Li et al., 2007) and the inhibition of p38 MAPK pathway (Guo et al., 2013) by H_2S. Treatments using either H_2S-releasing non-steroidal anti-inflammatory drugs (NSAIDs) or H_2S donors have demonstrated anti-inflammatory activity in various models of inflammation (Li et al., 2007; Li, Salto-Tellez, Tan, Whiteman, & Moore, 2009; Sidhapuriwala, Li, Sparatore, Bhatia, & Moore, 2007; Sivarajah et al., 2009). However, the precise role for H_2S in inflammation is controversial, with pro-inflammatory effects documented as well (Bhatia, Sidhapuriwala, Ng, Tamizhselvi, & Moochhala, 2008; Mok & Moore, 2008). The effect of H_2S on inflammation may depend on the stage of inflammation, experimental model studied, different routes of H_2S administration, as well as the source and concentration of H_2S. Elucidation of the role of H_2S in inflammation will enable the development of new anti-inflammatory pharmacological agents and natural H_2S donor compounds.

1.6 Tyrosol

1.6.1 The Mediterranean diet

The Mediterranean diet is the dietary pattern that prevails in the olive growing areas of the Mediterranean regions, and is characterized by high consumption of foods of plant origin,

relatively low consumption of red meat, and high consumption of olive oil (Trichopoulou, Lagiou, Kuper, & Trichopoulos, 2000). Many of the health-promoting effects of the Mediterranean diet or its elements have been largely evidenced, resulting in a reduced risk of cardiovascular diseases and certain cancers (Knoops et al., 2004; Sofi, Cesari, Abbate, Gensini, & Casini, 2008). Olive oil, as the principal source of fat in the Mediterranean diet, has been reported to have many health benefits. The healthy effects can be attributed not only to the high proportion in monounsaturated fatty acids (i.e. oleic acid) in olive oil but also to the antioxidant property of its phenolic compounds (Frankel, 2010). Phenolic compounds are a class of chemical compounds consisting of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group (Figure 1-6). Olive oil is a rich source of phenolic antioxidants, compared with other oils. Argan oil, obtained from the pit of argania spinosa, is another vegetable oil rich in phenolic compounds (Khallouki et al., 2003). Olive oil contains at least 30 phenolic compounds and the main phenolics in highest concentration include hydroxytyrosol, tyrosol, and glycoside oleuropein (Tuck & Hayball, 2002). In recent years, tyrosol has become a compound of interest in the search for naturally occurring compounds with pharmacological qualities.

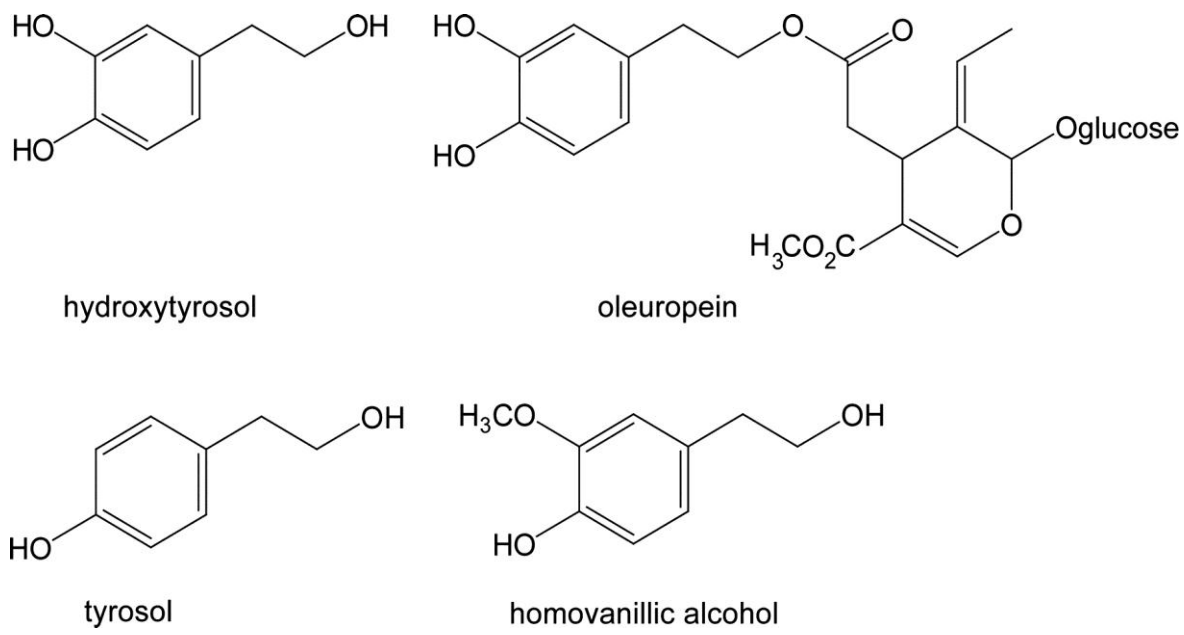

Figure 1-6 Chemical structures of phenolic compounds

(1) Hydroxytyrosol; (2) Oleuropein; (3) Tyrosol; (4) Homovanillic alcohol

1.6.2 Dietary sources of tyrosol

Tyrosol (2,4-(hydroxyphenyl)-ethanol) is a naturally occurring phenolic compound and is present in a variety of natural sources. Tyrosol appears most abundantly in olive oil in the human diet, where it has a high bioavailability. The levels of both total and individual phenolics in olive oil vary considerably among cultivars, harvests and analyzing methods. The concentration of tyrosol has been reported to be 27.45 mg/kg in extra virgin oil (Owen et al., 2004) . It was reported that tyrosol concentration ranged from 40 to 180 mg/kg oil in several types of commercial olive oils (Romero & Brenes, 2012). The presence of tyrosol has also been identified in some drinks, such as white wines, vermouth, beers and green tea (Covas et al., 2003; Frankel, Kanner, German, Parks, & Kinsella, 1993; C. Giovannini et al., 1999).

1.6.3 Bioavailability of tyrosol

Tyrosol is absorbed mainly by the small intestine in a dose-dependent manner. After olive oil ingestion, tyrosol levels increase rapidly and reach a peak concentration at around 1 hour in plasma and 2 hours in urine postprandially (Miro Casas et al., 2001; Weinbrenner et al., 2004). The main urinary metabolites are the conjugated form (glucuronidated metabolites) (Caruso, Visioli, Patelli, Galli, & Galli, 2001; Miro Casas et al., 2001). It has been reported that tyrosol concentrates mainly in the kidney after its absorption, where it may play an important role in preventing oxidative stress-induced renal injury (Loru et al., 2009).

1.6.4 Known protective effect of tyrosol against diseases

Tyrosol has also been found to exert potent protective effects against several pathogeneses by experimental evidence. It has been demonstrated to possess a wide range of antioxidant, anti-apoptotic, and anti-inflammatory effects. Tyrosol has been reported to have a neuroprotective effect in Alzheimer's and Parkinson's disease models (Dewapriya, Himaya, Li, & Kim, 2013; St-Laurent-Thibault, Arseneault, Longpre, & Ramassamy, 2011). In an oxidized LDL-stimulated macrophage model, tyrosol was shown to reduce H_2O_2 production and COX-2 over-expression (Vivancos & Moreno, 2008). The cytoprotective effect of tyrosol was also reported against I/R-induced apoptosis in H9c2 cells (Sun et al., 2012). A recent study reported that the cytoprotection of tyrosol in I/R-caused myocyte mortality was involved with the mitigation of ROS (Sun, Fan, Yang, Shi, & Liu, 2015). However, the effect of tyrosol has not been well studied in kidney disease models, and the underlying mechanisms of the protective effect of tyrosol remain unknown.

1.6.5 Other olive oil phenolic compounds

Although tyrosol has been studied in terms of its antioxidant potential and its role in different pathologies, it should be noted that there are other phenolic compounds in olive oil which also contribute to health benefits. Hydroxytyrosol has been shown to have comparable bioavailabilities and antioxidant effects (de la Torre, 2008; Rietjens, Bast, de Vente, & Haenen, 2007). The beneficial effects of tyrosol, as a natural antioxidant along with other phenolic compounds, have also been examined. Tyrosol, oleuropein, and the polyphenol extract from olive pomace oil have been shown to reduce NO levels in human endothelial cells subjected to anoxia (Palmieri et al., 2012). It has been demonstrated that tyrosol and β-

sitosterol (a minor compound in olive oil) were able to reduce PMA-induced NO release from macrophages (Moreno, 2003). Further investigation is needed to identify the mechanisms contributing to the protective effects of these phenolics.

Chapter 2. HYPOTHESES AND OBJECTIVES

2.1 Rationale and Hypotheses

Acute kidney injury (AKI) is a common problem in hospitalized patients. AKI causes end-stage renal disease (ESRD) directly, promotes the development of chronic kidney disease (CKD). Renal ischemia/reperfusion (I/R) is one of the major causes of AKI, associated with high morbidity, mortality and prolonged hospital stay. Several experimental models, both *in vitro* and *in vivo*, have been used to study the cellular mechanisms of renal I/R-induced AKI and to investigate the potential therapeutic strategies. In my research, the effect of potential treatment and the molecular basis of renal I/R injury were investigated in both cellular and animal systems. A well-established model *in vitro* and *in vivo* has been employed in our laboratory. I/R model was induced in SD rat by the clamping of the left renal pedicles, followed by reperfusion. In addition, an *in vitro* model of renal I/R was designed in a renal tubular cell line by hypoxia followed by reoxygenation.

Inflammation plays a critical role in kidney ischemia/reperfusion injury. It has been reported that renal I/R results in a robust inflammatory response in both kidney tissue and distant organs. The damage is mediated by various inflammation mediators, such as cytokines, chemokines and inflammatory signaling pathways are involved. To date, limited information is available about the regulatory mechanisms in inflammatory response after renal I/R. Our previous study has observed an elevation of a pro-inflammatory cytokines, MCP-1 in rat kidney subjected to I/R injury (Sung, Zhu, Au-Yeung, Siow, & O, 2002). Other studies also indicated that IL-6 could act as a pro-inflammatory cytokine in renal I/R injury and exacerbate the degree of renal dysfunction (Kielar et al., 2005; Patel et al., 2005). Thus, strategies exhibiting anti-inflammatory activity may be useful in conditions associated with

renal I/R injury. As a gasotransmitter, hydrogen sulfide (H_2S) has been shown to exert anti- and pro- inflammatory actions. It has been observed that ischemia followed by a short period of reperfusion (6 hours) results in a significant decrease in H_2S production in rat kidney, and supplementation of exogenous H_2S has renal protective effect in AKI (Xu et al., 2009). Despite a growing understanding of renal I/R-induced AKI, the role H_2S in I/R-induced inflammatory response in the kidney remains to be further investigated.

Besides inflammatory response, oxidative stress is also involved in the development and progression of renal I/R-induced injury. The generation of reactive oxygen species (ROS) contributes to morphological and functional renal injuries during I/R. Our previous studies reported that I/R leads to increased iNOS-mediated NO production and peroxynitrite formation in rat kidney (Prathapasinghe, Siow, Xu, & O, 2008). Nowadays, the identification of novel therapeutic interventions against kidney I/R injury has become a subject of intense research interest. Among those antioxidants, dietary antioxidants are viewed as a promising therapeutic strategy to combat oxidative stress. As one of the main compounds present in the olive oil, tyrosol has been reported to have antioxidant activity. However, its role in renal I/R-induced oxidative stress damage remains speculative.

We hypothesized that (1) the reduced endogenous H_2S generation due to downregulation of CBS and CSE contributed to increased expression of pro-inflammatory cytokines in the kidney upon I/R injury; (2) proper restoration of endogenous H_2S production and/or the administration of exogenous H_2S donor had protective effect against renal I/R injury; (3) tyrosol treatment had antioxidant effect against I/R-induced kidney injury by the regulation of ROS production.

2.2 Objectives

The general objective of the research was to investigate the regulatory mechanisms of oxidative stress and inflammation in renal I/R-induced acute kidney injury.

The specific objectives were as follows:

(1) To investigate the regulation of H_2S generation and inflammation in I/R-induced acute kidney injury and to study the role of CBS and CSE-mediated H_2S production in pro-inflammatory cytokines (MCP-1, IL-6) expression *in vivo* and *in vitro*;

(2) To examine whether tyrosol has the protective effect against renal I/R-induced oxidative stress damage and to elucidate how tyrosol mediates the protective effect in SD rat I/R model.

Chapter 3. STUDY 1: Regulation of inflammatory response in renal I/R injury: Role of endogenous H_2S generation

(Manuscript 1: Downregulation of cystathionine *β*-synthase and cystathionine *γ*-lyase expression stimulates inflammation in kidney ischemia/reperfusion injury)

Physiological Reports, 2(12), no. e1225, 2014

3.1 Abstract

Inflammation plays a critical role in kidney ischemia/reperfusion (I/R) injury but the mechanisms of increased pro-inflammatory cytokine expression are not completely understood. Kidney has a high expression of cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) that can synthesize hydrogen sulfide (H_2S). CBE and CSE are also responsible for the synthesis of cysteine, an essential precursor for glutathione, an antioxidant. Reduced H_2S and glutathione production is associated with multiple organ injury. Although pro-and anti-inflammatory effects of H_2S have been reported, its role in I/R-induced inflammation in the kidney has not been well addressed. The aim of this study was to investigate the effect of CBS and CSE-mediated H_2S and glutathione production on kidney inflammatory response and the mechanism involved. The left kidney of Sprague-Dawley rat was subjected to ischemia (45 minutes) followed by reperfusion (24 hours). Ischemia reperfusion caused a significant decrease in CBS and CSE mRNA and protein levels with a concomitant reduction of glutathione and H_2S production in the kidney while the expression of pro-inflammatory cytokine expression (MCP-1, IL-6) was elevated. Hypoxia–reoxygenation of proximal tubular cells led to a decrease in CBS and CSE expression and an increase in pro-inflammatory cytokine expression. Supplementation of glutathione or H_2S donor (NaHS) effectively attenuated cytokine expression in tubular cells. These results suggested that I/R impaired CBS and CSE-mediated glutathione and H_2S production in the kidney, which augmented the expression of pro-inflammatory cytokines. Regulation of CBS and CSE expression may be therapeutically relevant in alleviating I/R-induced inflammation and improving kidney function.

3.2 Introduction

Kidney ischemia/reperfusion (I/R) is one of the common causes for acute kidney injury (AKI), which is associated with a high morbidity and mortality (Bellomo et al., 2012; Bonventre & Yang, 2011; Hoste & Kellum, 2007; Lameire et al., 2005). I/R injury occurs in many clinical conditions including renal transplantation and surgery. The pathogenesis of I/R-induced kidney injury is multifaceted. Depletion of oxygen and nutrients, oxidative stress, inflammatory response and induction of cell death contribute to kidney injury (Bellomo et al., 2012; Bonventre & Yang, 2011; Sharfuddin & Molitoris, 2011; A. P. Singh et al., 2012). I/R elicits inflammatory response characterized by increased expression of pro-inflammatory cytokines, which facilitates leukocyte recruitment into the kidney. The chain event of inflammatory response to I/R accelerates renal injury. Although inflammatory response is known to play a crucial role in kidney I/R injury, the mechanisms responsible for increased pro-inflammatory cytokine expression are not completely understood.

Inflammation is recognized as a major pathogenic process in kidney I/R injury (Bonventre & Zuk, 2004; Kinsey, Li, & Okusa, 2008; Sharfuddin & Molitoris, 2011; Thurman, 2007). I/R results in the generation of pro-inflammatory cytokines that play an important role in recruitment of leukocytes and exacerbates kidney injury (Bonventre & Yang, 2011; Bonventre & Zuk, 2004; Kinsey et al., 2008). Monocyte chemoattractant protein-1 (MCP-1) is a potent chemotactic cytokine that stimulates monocyte recruitment to the kidney (Munshi et al., 2011; Sung et al., 2002). We and others have observed an increased expression of MCP-1 and leukocyte accumulation in rat kidney subjected to I/R injury (Munshi et al., 2011; Sung et al., 2002). Attenuation of MCP-1 expression significantly improved kidney

function (Amann, Tinzmann, & Angelkort, 2003). Interleukin-6 (IL-6), one of the major inflammatory mediators, has been shown to have a strong correlation with the onset and severity of AKI (Kielar et al., 2005; Nechemia-Arbely et al., 2008). Despite recent advances in AKI research, the mechanisms of kidney I/R-induced inflammatory response are complex and not completely understood.

Hydrogen sulfide (H_2S) is a gasotransmitter that, at physiological levels, exerts beneficial effects through antioxidant and anti-inflammatory action, as well as serving as a neurotransmitter (Abe & Kimura, 1996; Elrod et al., 2007; Gadalla & Snyder, 2010; Wallace, Vong, McKnight, Dicay, & Martin, 2009; R. Wang, 2012). Kidney is one of the major organs regulating endogenous H_2S generation through cystathionine-β-synthase (CBS, EC 4.2.1.22) and cystathionine-γ-lyase (CSE, EC 4.4.1.1) (House, Brosnan, & Brosnan, 1997; Prathapasinghe, Siow, & O, 2007; Prathapasinghe et al., 2008; Xu et al., 2009). These two enzymes catalyze H_2S production through desulfhydration reations (M. Fu et al., 2012). Both CBS and CSE are also responsible for the synthesis of cysteine through the transsulfuration pathway (Paul & Snyder, 2012). Cysteine is an essential substrate for the biosynthesis of glutathione, a major endogenous antioxidant (Paul & Snyder, 2012).

In a recent study, we have observed that ischemia followed by a short period of reperfusion (6 hours) results in a significant decrease in hydrogen sulfide (H_2S) production in rat kidney (Xu et al., 2009). Supplementation of exogenous H_2S is shown to be renal protective in AKI and in chronic kidney disease (Lobb et al., 2012; Perna & Ingrosso, 2012; Xu et al., 2009). In mice with CSE depletion, renal H_2S production rate was markedly reduced (Bos et al., 2013). Generation of endogenous H_2S by CSE or the administration of H_2S donor attenuates kidney

I/R injury (Hunter et al., 2012; Tripatara et al., 2008). H_2S has been implicated as a mediator exerting anti- and pro-inflammatory effects (Chan & Wallace, 2013; Rivers, Badiei, & Bhatia, 2012; R. Wang, 2012; Whiteman & Winyard, 2011). The administration of exogenous H_2S is shown to be cytoprotective in various organs of I/R injury, including heart, brain, kidney and lung (Nicholson & Calvert, 2010). The treatment of hearts with a H_2S donor (NaHS) during reperfusion resulted in significant improvement in heart function, which was through the increased open probability of KATP channel in cardiac myocytes by H_2S (Z. Zhang, Huang, Liu, Tang, & Wang, 2007). In a global cerebral I/R model, the administration of NaHS after 24 h I/R had a potent protective effect against the severe cerebral injury, which was through the inhibition of oxidative stress, inflammation and apoptosis (Yin et al., 2012). Similarly, administration of H_2S or H_2S donor provided beneficial effect on lung and renal I/R injury (Z. Fu, Liu, Geng, Fang, & Tang, 2008; Tripatara et al., 2008). Although the effect of endogenous H_2S in I/R models, including renal I/R, has been reported, the mechanisms of such beneficial effect are not clearly understood. Since most of the experiments in renal I/R were still conducted only in animal model, the understanding the molecular mechanisms is very limited. Anti-inflammatory is one of the possible effects of H_2S. However the role of H_2S in I/R-induced inflammatory response in the kidney is not clear. To study the relationship between endogenous H_2S production and inflammation, proper I/R duration time is needed, since it takes time for the inflammatory reponse to be simulated and for the endogenous H_2S production to be regulated. The aim of this study was to investigate the effect of down-regulation of CBS and CSE expression on endogenous H_2S and glutathione production and its impact on kidney inflammatory response during ischemia followed by a longer period of reperfusion (24 hours) injury.

3.3 Materials and Methods

3.3.1 Animal model

Kidney ischemia was induced in male Sprague-Dawley rats (250-300 g) by clamping the left renal pedicle for 45 minutes followed by reperfusion for 24 hours (Prathapasinghe et al., 2007; Sung et al., 2002; P. Wang et al., 2013). In brief, rats were anesthetized by 3% isoflurane/oxygen gas prior to surgery. Surgery was performed when rats reach the stage 3 anesthesia. During the surgery, the 1-2% isoflurance/oxygen gas was maintained via inhalation. Rats were kept on a heat pad and the rectal temperature was maintained at 37 ℃ throughout the experimental procedure. To prevent a decrease in body temperature, rats were placed in a warm incubator for 12 hours after surgery. As a control, a sham-operated group of rats were subjected to the same surgical procedure but without inducing ischemia/reperfusion and were sacrificed at corresponding time points. A blood sample was collected and plasma was separated by centrifugation of blood at 3000 $\times g$ for 20 minutes at 4 ℃. Plasma creatinine level was measured by using a commercial assay kit (Genzyme diagnostics, Canada). Kidneys were harvested in ice-cold potassium phosphate buffer. 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. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

3.3.2 Simulated ischemia/reperfusion in cell culture

Human kidney cortex proximal tubular cells (HK-2; CRL-2190, American Type Culture Collection) were cultured in keratinocyte-serum free medium supplied with human

recombinant epidermal growth factor and bovine pituitary extract (GIBCO, Invitrogen) at 37 ℃ in a normal atmosphere of 95% air and 5% CO_2. Simulated ischemia was induced in cells by oxygen-glucose deprivation (N. Wu, Siow, & O, 2010). In brief, tubular cells were incubated for 2 hours in a modified Krebs buffer (137 mM NaCl, 3.8 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$, 4 mM HEPES) supplemented with 10 mM 2-deoxyglucose, 20 mM sodium lactate, 12 mM KCl and 1 mM sodium dithionite (pH 6.2) in a hypoxia chamber (Billups-Rothenberg, Inc., Del Mar, CA) containing 95% N_2 / 5% CO_2 at 37 ℃. Control cells were incubated in a modified Krebs buffer (pH 7.4) containing D-glucose at 37 ℃ in a normal atmosphere. After incubation for 2 hours, the Krebs buffer was replaced with keratinocyte-serum free medium and cells were cultured for another 24-48 hours.

3.3.3 Measurement of mRNA expression

Total RNAs were isolated from the kidney tissue with Trizol reagent (Invitrogen) according to the manufacturer's instruction. Total RNA (2 μg) was converted to cDNA by reverse transcription. The mRNA expression of CBS, CSE and cytokines was determined by a real-time PCR analysis. The real-time PCR reaction mixture contained 0.4 μM of 5′ and 3′ primers and 1 μl of cDNA product in iQ-SYBR green supermix reagent (Bio-Rad). The relative changes in mRNA expression were determined by the fold change analysis. The primers (Invitrogen) used in this study were listed in Table 3-1.

Table 3-1 Gene primer sequences used for RT-qPCR

Target gene	Forward Primer(5'-3')	Reverse Primer(5'-3')
Human		
CBS	GCAGATCCAGTACCACAGCA	CTCCGGACTTCACTTCTGGT
CSE	CAAGGTTTCCTGCCACACTT	GCTATATTCAAAACCCGAGTGC
IL-6	AGGAGACTTGCCTGGTGAAA	GTCAGGGGTGGTTATTGCAT
MCP-1	CCCAAAGAAGCTGTGATCTTCA	GTGTCTGGGGAAAGCTAGGG
GAPDH	GAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
Rat		
CBS	TCGTGATGCCTGAGAAGATG	TTGGGGATTTCGTTCTTCAG
CSE	GTATGGAGGCACCAACAGGT	GTTGGGTTTGTGGGTGTTTC
IL-6	CCGGAGAGGAGACTTCACAG	ACAGTGCATCATCGCTGTTC
MCP-1	CAGAAACCAGCCAACTCTCA	AGACAGCACGTGGATGCTAC
β-actin	ACAACCTTCTTGCAGCTCCTC	GACCCATACCCACCATCACA

3.3.4 Western immunoblotting analysis of CBS and CSE, measurement of cytokines

The protein levels of CBS and CSE in the kidney were measured by Western immunoblotting analysis. In brief, kidney proteins (20 μg) were separated by electrophoresis in 10% SDS polyacrylamide gels. Proteins in the gel were transferred to a nitrocellulose membrane. The membrane was probed with (1) mouse anti-CBS monoclonal (1:3000, Abnova Corporation) or rabbit anti-CSE monoclonal antibodies (1:3000, GeneTex) for rat proteins. HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (Cell Signaling Technology) were used as the secondary antibodies (1:5000). 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 re-probed with mouse anti-β-actin monoclonal antibodies (1:5000, Cell Signaling Technology). Pro-inflammatory factors (MCP-1, IL-6) were quantified by using the MesoScale Discovery electrochemiluminescence platform (Rockville, MD).

3.3.5 Measurement of H_2S production and glutathione levels in the kidney

H_2S production was measured based on a method described by Stipanuk and Beck (Stipanuk & Beck, 1982). Kidney tissue was homogenized in 50 mM potassium phosphate buffer (pH 6.9) followed by centrifugation at 15,000 *g* for 30 minutes at 4 ℃. The supernatant was collected and H_2S production was measured in a reaction mixture containing 0.3 ml supernatant, 10 mM L-cysteine, 10 mM DL-Hcy, 2 mM pyridoxal-5′-phosphate and 0.05 mM S-adenosylmethionine and prepared in 100 mM potassium phosphate buffer (pH 7.4). The reaction was carried out in an Erlenmeyer flask that was fitted with a septum stopper and

contained a plastic center well. A folded filter paper was soaked in 0.5 ml mixture of 1% zinc acetate and 12% NaOH. The tube was placed in the flask and the flask was blown with N_2. The flask was immediately covered and incubated in a water bath for 30 minutes at 37 ℃. The reaction was stopped with the injection of 30% trichloroacetic acid into the flask. The flask was incubated for an additional 60 minutes at 37 ℃. The filter paper was removed and transferred to a test tube containing 3.5 ml water to which 0.4 ml of 20 mM *N*,*N*,dimethyl-*p*-phenylenediamine sulfate dissolved in 7.2 M HCl and 0.4 ml of 30 mM $FeCl_3$ dissolved in 1.2 M HCl were added. The reaction was allowed to proceed for 10 minutes in the dark and the absorbance of the resulting solution was measured at 670 nm. Sodium hydrosulfide hydrate was used as standard (Hwang, Sarna, Siow, & O, 2013; Xu et al., 2009). Total glutathione was measured in kidney tissue and cell lysates as previously reported (I. Rahman, Kode, & Biswas, 2006).

3.3.6 Statistical analysis

Results were analyzed by using one-way ANOVA followed by Newman-Keuls test. Data were presented as the means ± SEM. The level of statistical significance was determined when a *p* value was less than 0.05.

3.4 Results

3.4.1 Effect of ischemia/reperfusion on kidney function, H_2S production, and glutathione level

The induction of kidney ischemia (45 minutes) followed by reperfusion (24 hours) resulted in a marked elevation of plasma creatinine (Figure 3-1), indicating that kidney function was impaired. Upon ischemia/reperfusion, the H_2S production in the kidney tissue was significantly decreased (Figure 3-2). Total glutathione level was significantly lower in ischemia-reperfused kidneys than that in the sham-operated group (Figure 3-3).

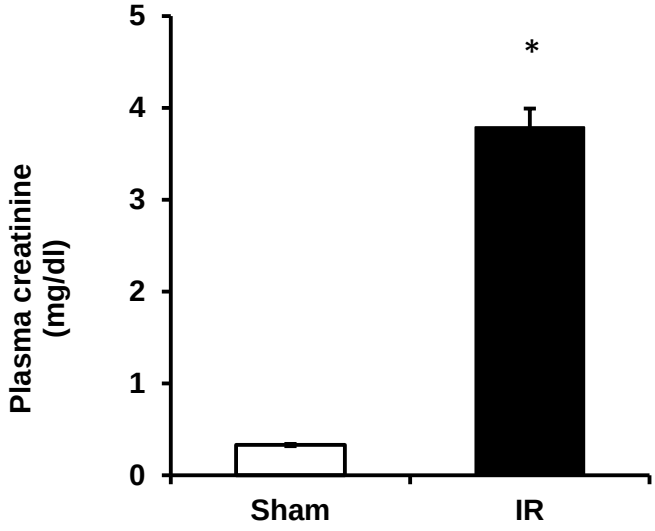

Figure 3-1 Effect of ischemia/reperfusion on plasma creatinine level

The left kidney of rats was subjected to ischemia (45 minutes) followed by reperfusion (24 hours) (I/R). As a control, rats were subjected to a sham operation but without inducing I/R. Plasma creatinine levels were determined. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from the sham-operated group.

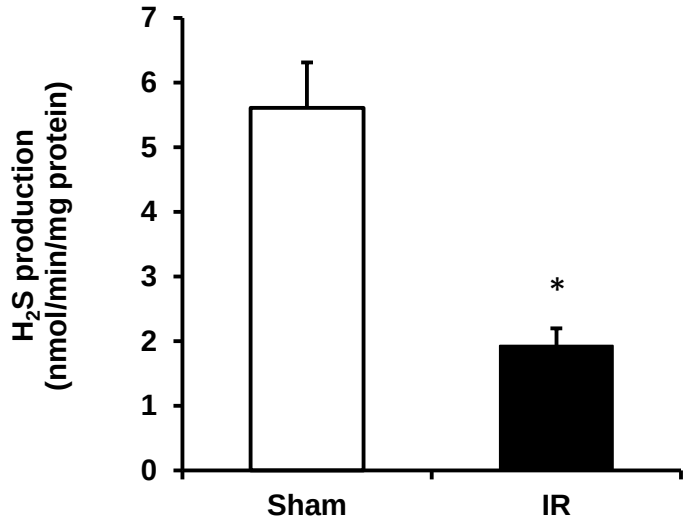

Figure 3-2 Effect of ischemia/reperfusion on kidney H_2S production level

The left kidney of rats was subjected to ischemia (45 minutes) followed by reperfusion (24 hours) (I/R). As a control, rats were subjected to a sham operation but without inducing I/R. H_2S production levels were determined. Results are expressed as mean ± SEM (n = 5 for each group). $*P < 0.05$ when compared with the value obtained from the sham-operated group.

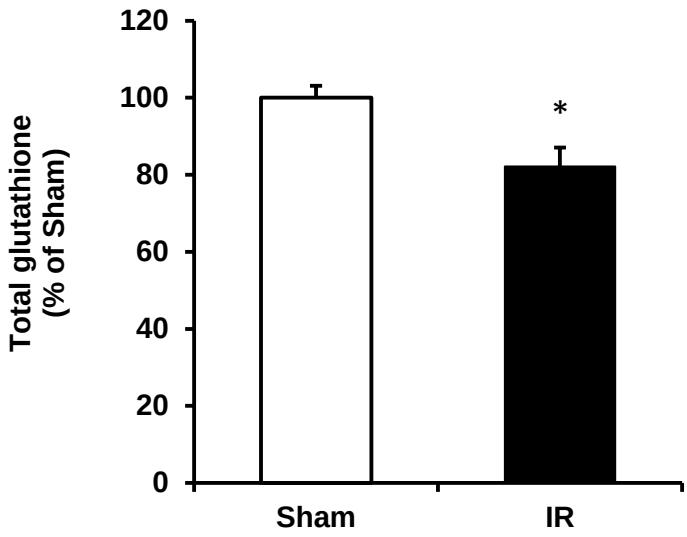

Figure 3-3 Effect of ischemia/reperfusion on kidney glutathione level

The left kidney of rats was subjected to ischemia (45 minutes) followed by reperfusion (24 hours) (I/R). As a control, rats were subjected to a sham operation but without inducing I/R. The glutathione level was measured in the kidney tissue. Results are expressed as mean ± SEM (n = 5 for each group). *$P < 0.05$ when compared with the value obtained from the sham-operated group.

3.4.2 Effect of ischemia/reperfusion on the expression of CBS, CSE, and pro-inflammatory cytokines in the kidney

To investigate whether a decrease in H_2S levels was due to downregulation of CBS and CSE expression in the kidney, CBS, and CSE mRNA was measured by a real-time PCR analysis. The levels of CBS (Figure 3-4) and CSE (Figure 3-5) mRNA were significantly lower in I/R kidneys than that in the sham-operated group. In accordance, the protein levels of CBS (Figure 3-6) and CSE (Figure 3-7) were significantly decreased in those kidneys. We then examined the effect of I/R on inflammatory response in the kidney. There was a significant increase in pro-inflammatory cytokines MCP-1 (Figure 3-8) and IL-6 (Figure 3-9) mRNA expression. The protein levels of MCP-1 (Figure 3-10) and IL-6 (Figure 3-11) were also elevated signifiantly in kidneys subjected to I/R. The level of MCP-1 was also significantly elevated in the plasma of rats subjected to kidney I/R as well (Figure 3-12). Neutrophil gelatinase-associated lipocalin (NGAL) is a small (25 kDa) protein that belongs to the lipocalin protein family. NGAL is produced by epithelial cells and neutrophils in response to tubular epithelial damage (Sharfuddin & Molitoris, 2011) . Plasma NGAL has been proposed as a marker of tubular damage in AKI (A. P. Singh et al., 2012; Sodha et al., 2009). The level of plasma NGAL was significantly elevated (Figure 3-13), indicating tubular damage in I/R-induced kidney injury.

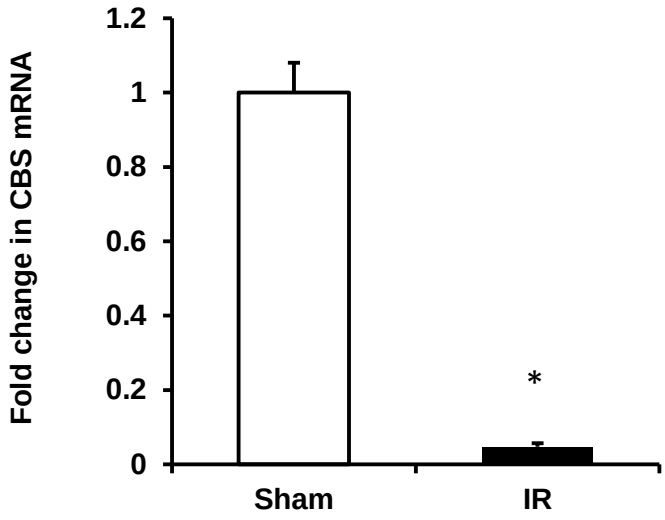

Figure 3-4 Effect of ischemia/reperfusion on kidney CBS gene expression level

The left kidney of rats was subjected to ischemia (45 minutes) followed by reperfusion (24 hours) (I/R). As a control, rats were subjected to a sham operation but without inducing I/R. The mRNA of CBS in the kidney tissue was determined by a real-time PCR analysis. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from the sham-operated group.

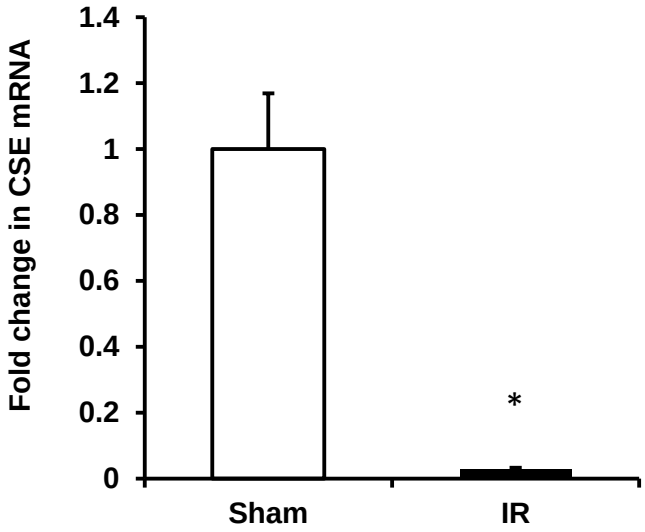

Figure 3-5 Effect of ischemia/reperfusion on kidney CSE gene expression level

The left kidney of rats was subjected to ischemia (45 minutes) followed by reperfusion (24 hours) (I/R). As a control, rats were subjected to a sham operation but without inducing I/R. The mRNA of CSE in the kidney tissue was determined by a real-time PCR analysis. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from the sham-operated group.

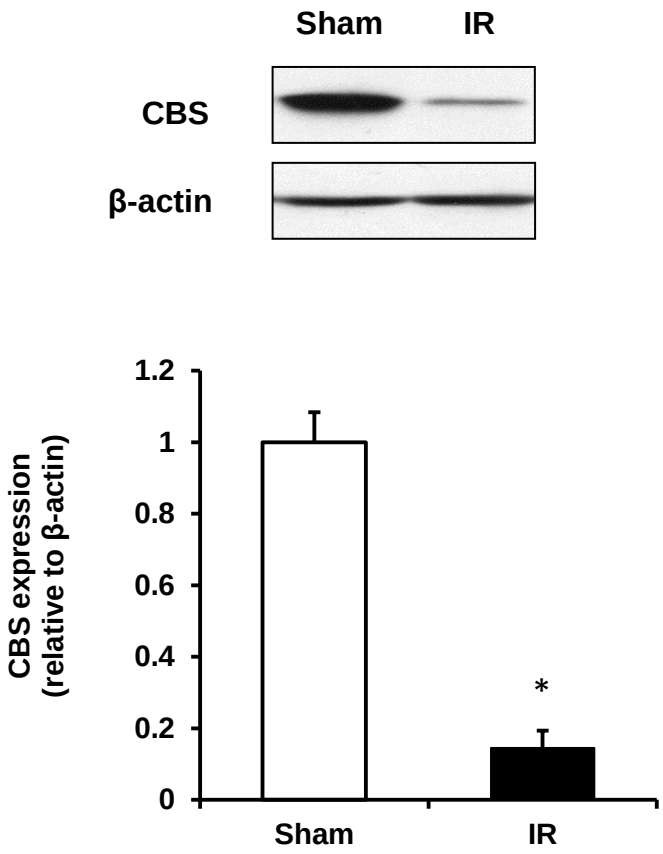

Figure 3-6 Effect of ischemia/reperfusion on kidney CBS protein level

The left kidney of rats was subjected to ischemia (45 minutes) followed by reperfusion (24 hours) (I/R). As a control, rats were subjected to a sham operation but without inducing I/R. The protein of CBS in the kidney tissue was determined by a Western immunoblotting analysis. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from the sham-operated group.

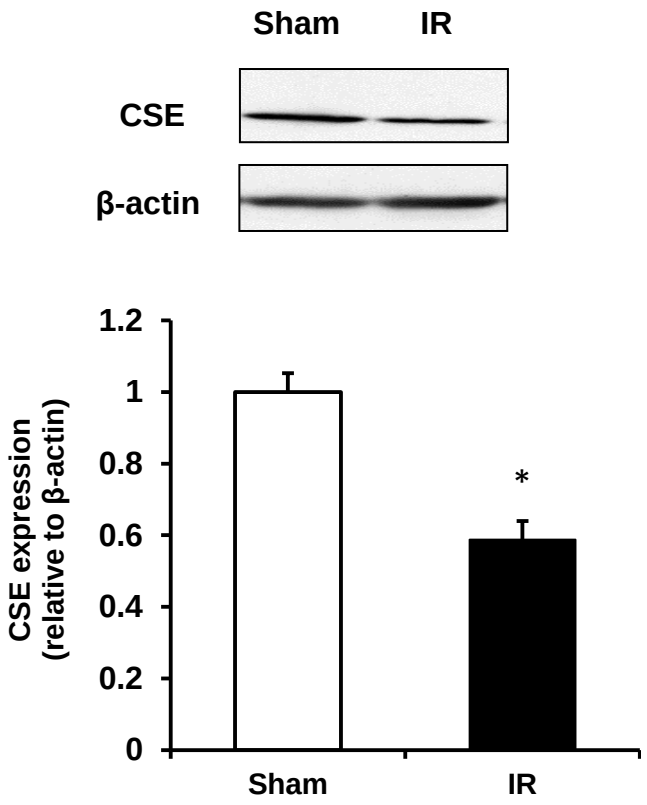

Figure 3-7 Effect of ischemia/reperfusion on kidney CSE protein level

The left kidney of rats was subjected to ischemia (45 minutes) followed by reperfusion (24 hours) (I/R). As a control, rats were subjected to a sham operation but without inducing I/R. The protein of CSE in the kidney tissue was determined by a Western immunoblotting analysis. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from the sham-operated group.

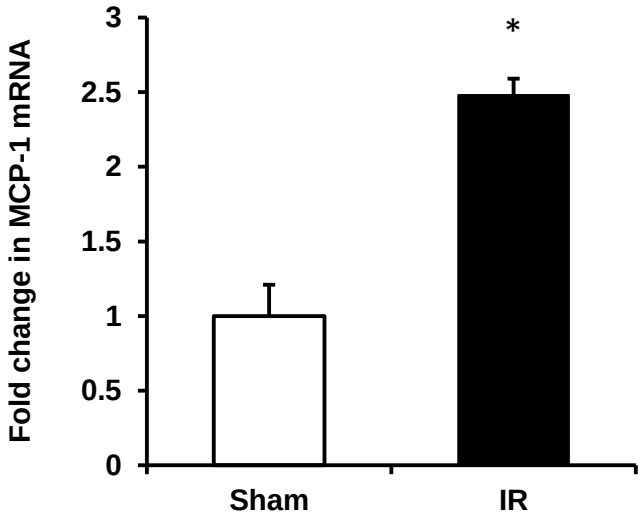

Figure 3-8 Effect of ischemia/reperfusion on MCP-1 gene expression in the kidney

The left kidney of rats was subjected to ischemia (45 minutes) followed by reperfusion (24 hours) (I/R). As a control, rats were subjected to a sham operation but without inducing I/R. The mRNA levels of cytokine MCP-1were measured in the kidney tissue. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from the sham-operated group.

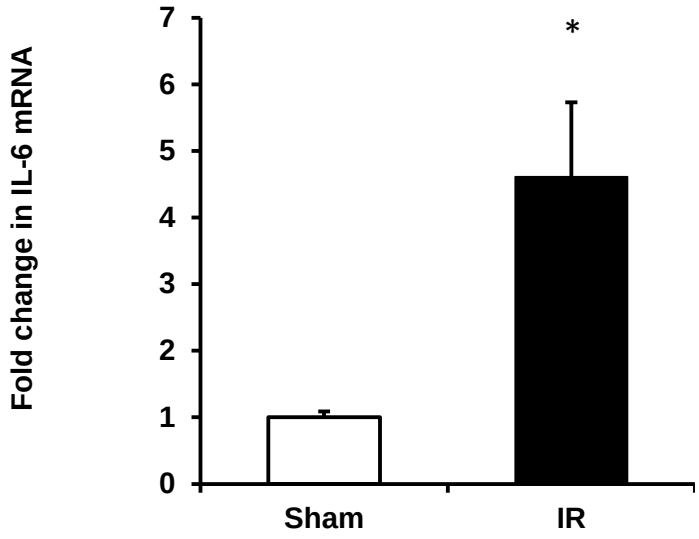

Figure 3-9 Effect of ischemia/reperfusion on IL-6 gene expression in the kidney

The left kidney of rats was subjected to ischemia (45 minutes) followed by reperfusion (24 hours) (I/R). As a control, rats were subjected to a sham operation but without inducing I/R. The mRNA levels of cytokine IL-6 were measured in the kidney tissue. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from the sham-operated group.

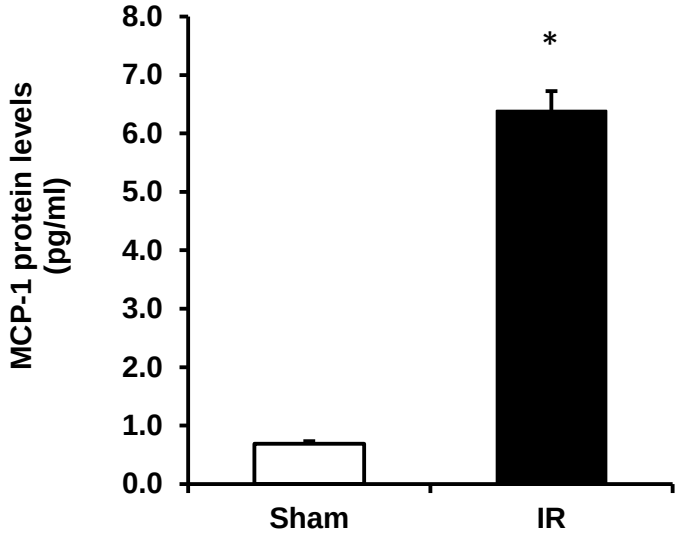

Figure 3-10 Effect of ischemia/reperfusion on MCP-1 protein levels in the kidney

The left kidney of rats was subjected to ischemia (45 minutes) followed by reperfusion (24 hours) (I/R). As a control, rats were subjected to a sham operation but without inducing I/R. The protein levels of cytokine MCP-1 were measured in the kidney tissue. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from the sham-operated group.

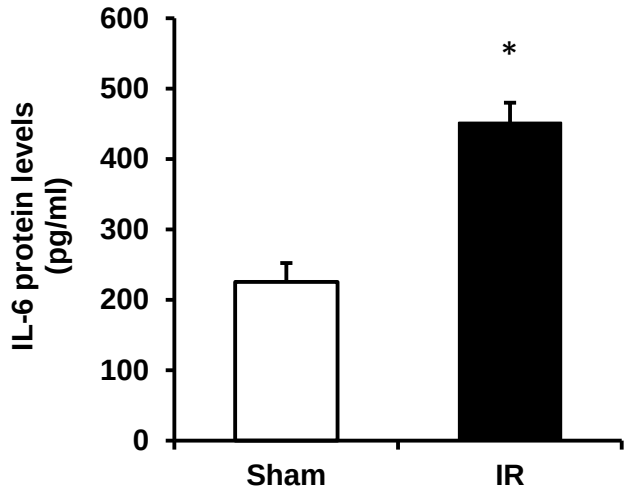

Figure 3-11 Effect of ischemia/reperfusion on IL-6 protein levels in the kidney

The left kidney of rats was subjected to ischemia (45 minutes) followed by reperfusion (24 hours) (I/R). As a control, rats were subjected to a sham operation but without inducing I/R. The protein levels of cytokine IL-6 were measured in the kidney tissue. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from the sham-operated group.

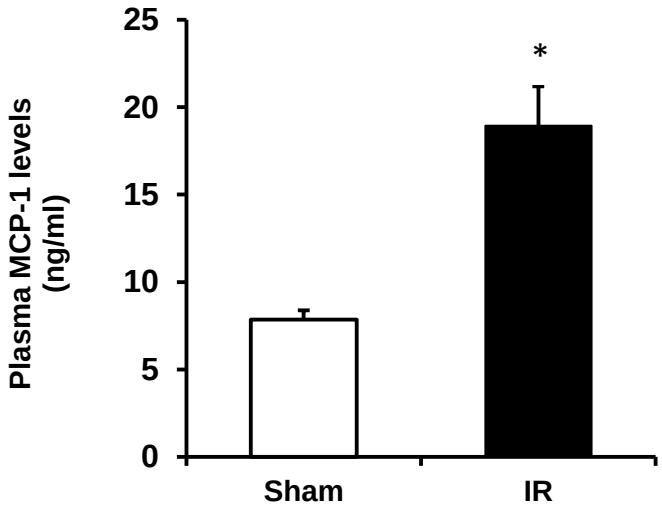

Figure 3-12 Effect of ischemia/reperfusion on plasma MCP-1 levels

The left kidney of rats was subjected to ischemia (45 minutes) followed by reperfusion (24 hours) (I/R). As a control, rats were subjected to a sham operation but without inducing I/R. The MCP-1 levels were measured in plasma. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from the sham-operated group.

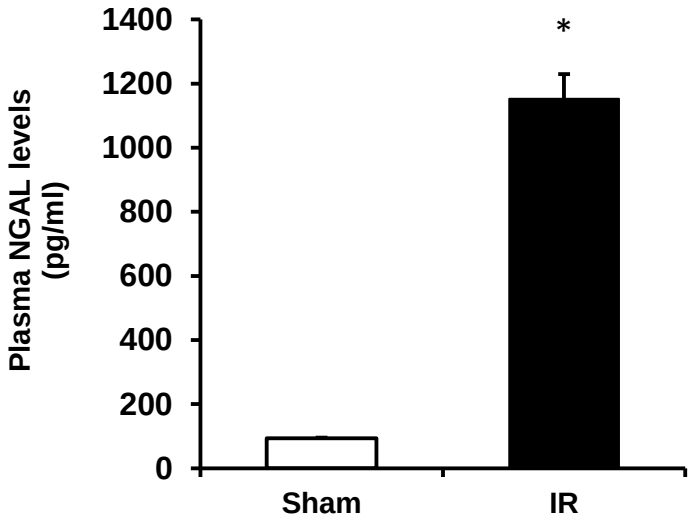

Figure 3-13 Effect of ischemia/reperfusion on plasma NGAL levels

The left kidney of rats was subjected to ischemia (45 minutes) followed by reperfusion (24 hours) (I/R). As a control, rats were subjected to a sham operation but without inducing I/R. The NGAL levels were measured in plasma. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from the sham-operated group.

3.4.3 Role of the transsulfuration pathway in ischemia/reperfusion-stimulated cytokine expression in the kidney and in tubular cells

To investigate whether downregulation of CBS and CSE expression contributed to increased cytokine expression, experiments were conducted in human proximal tubular cells, the type of cells in the kidney where CBS and CSE are highly expressed. Tubular cells were subjected to hypoxia followed by reoxygenation with regular culture medium (reperfusion). There was a significant reduction of CBS (Figure 3-14) and CSE (Figure 3-15) mRNA levels in cells subjected to 2 hours of hypoxia followed by reoxygenation with a regular culture medium for 24 hours while the levels of pro-inflammatory cytokines MCP-1 (Figure 3-16) and IL-6 (Figure 3-17) were significantly elevated.

Next, tubular cells were incubated with a CBS inhibitor aminooxyacetic acid (AOAA) or a CSE inhibitor DL-propargylglycine (PAG). Inhibition of CBS and CSE led to an elevation of MCP-1 (Figure 3-18) and IL-6 (Figure 3-19) mRNA in these cells. Inhibition of rate-limiting enzyme CBS in the transsulfuration pathway caused a significant reduction of intracellular glutathione levels in tubular cells (Figure 3-20).

To further investigate whether a reduction of CBS and CSE-mediated H_2S and glutathione production played a crucial role in pro-inflammatory cytokine expression, hydrogen sulfide donor sodium hydrosulfide (NaHS) and glutathione (GSH) were added to the culture medium prior to induction of hypoxia–reoxygenation. Addition of NaHS or GSH attenuated hypoxia–reoxygenation-induced MCP-1(Figure 3-21) and IL-6 (Figure 3-22) expression in tubular cells.

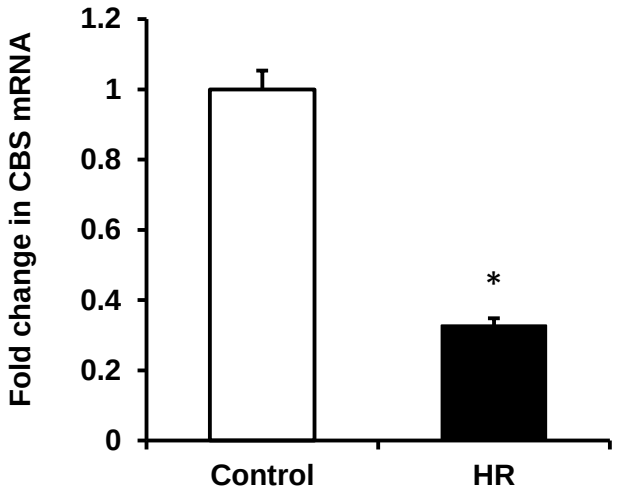

Figure 3-14 Effect of hypoxia–reoxygenation on the expression of CBS in renal tubular cells

Human proximal tubular cells were subjected to 2-h hypoxia followed by 24-h reoxygenation (HR). Cells cultured in the regular medium without being subjected to HR were used as a control. A real-time PCR analysis was used to measure the mRNA of CBS. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from control cells.

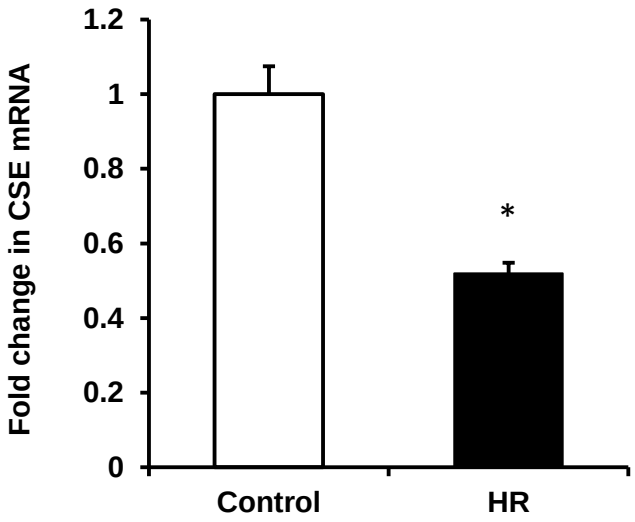

Figure 3-15 Effect of hypoxia–reoxygenation on the expression of CSE in renal tubular cells

Human proximal tubular cells were subjected to 2-h hypoxia followed by 24-h reoxygenation (HR). Cells cultured in the regular medium without being subjected to HR were used as a control. A real-time PCR analysis was used to measure the mRNA of CSE. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from control cells.

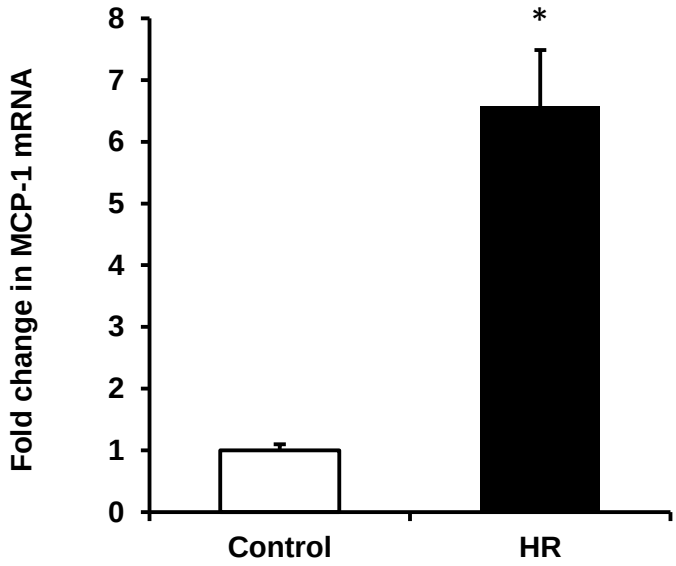

Figure 3-16 Effect of hypoxia–reoxygenation on the expression of MCP-1 in renal tubular cells

Human proximal tubular cells were subjected to 2-h hypoxia followed by 24-h reoxygenation (HR). Cells cultured in the regular medium without being subjected to HR were used as a control. A real-time PCR analysis was used to measure the mRNA of cytokine MCP-1. Results are expressed as mean ± SEM (n = 5 for each group). *P < 0.05 when compared with the value obtained from control cells.

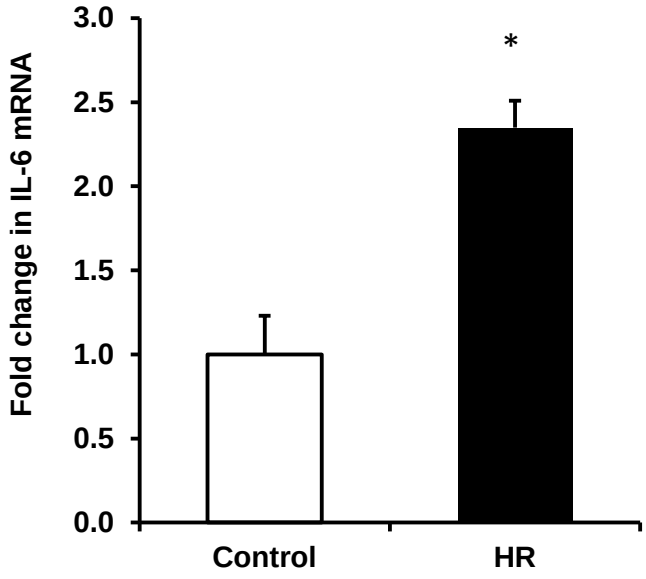

Figure 3-17 Effect of hypoxia–reoxygenation on the expression of IL-6 in renal tubular cells

Human proximal tubular cells were subjected to 2-h hypoxia followed by 24-h reoxygenation (HR). Cells cultured in the regular medium without being subjected to HR were used as a control. A real-time PCR analysis was used to measure the mRNA of cytokine IL-6. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from control cells.

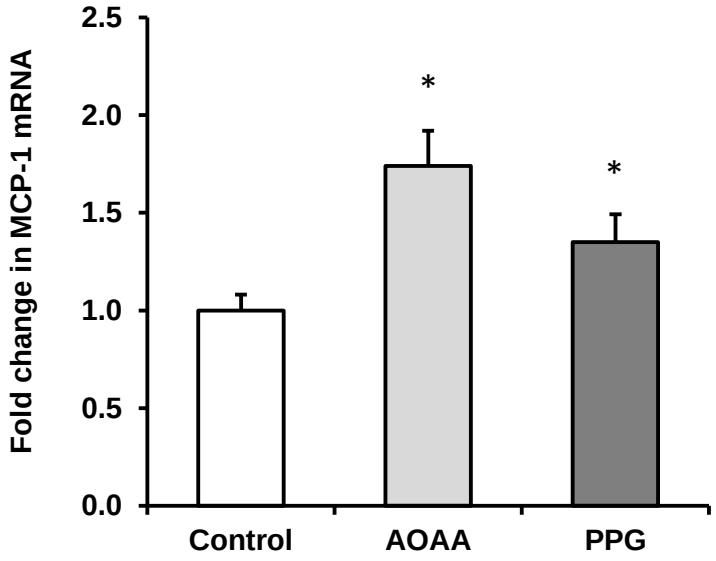

Figure 3-18 Effect of CBS and CSE inhibitors on pro-inflammatory cytokine MCP-1 expression in tubular cells

Human proximal tubular cells were incubated in the absence (control) or presence of a CBS inhibitor aminooxyacetic acid (AOAA, 0.2 mmol/L) or a CSE inhibitor DL-propargylglycine (PAG, 0.5 mmol/L). The mRNA of MCP-1 was measured by a real-time PCR analysis. Results are expressed as mean ± SEM (n = 5 for each group). *$P < 0.05$ when compared with the value obtained from control cells.

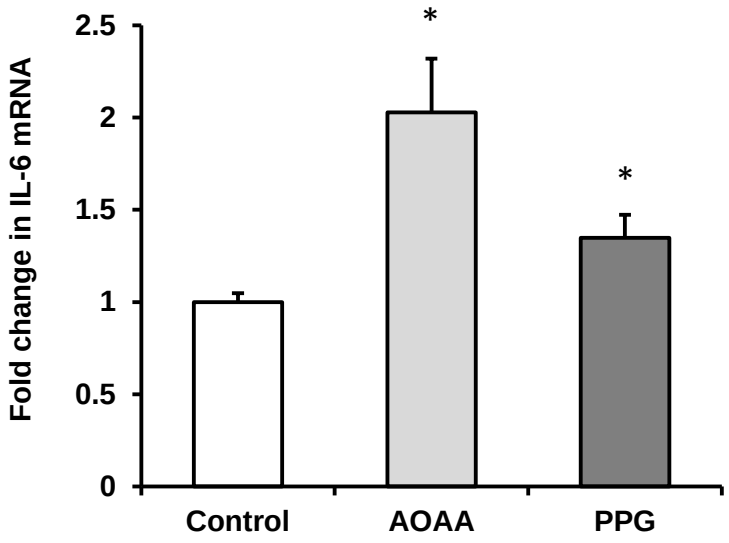

Figure 3-19 Effect of CBS and CSE inhibitors on pro-inflammatory cytokine IL-6 expression in tubular cells

Human proximal tubular cells were incubated in the absence (control) or presence of a CBS inhibitor aminooxyacetic acid (AOAA, 0.2 mmol/L) or a CSE inhibitor DL-propargylglycine (PAG, 0.5 mmol/L). The mRNA of IL-6 was measured by a real-time PCR analysis. Results are expressed as mean ± SEM (n = 5 for each group). *$P < 0.05$ when compared with the value obtained from control cells.

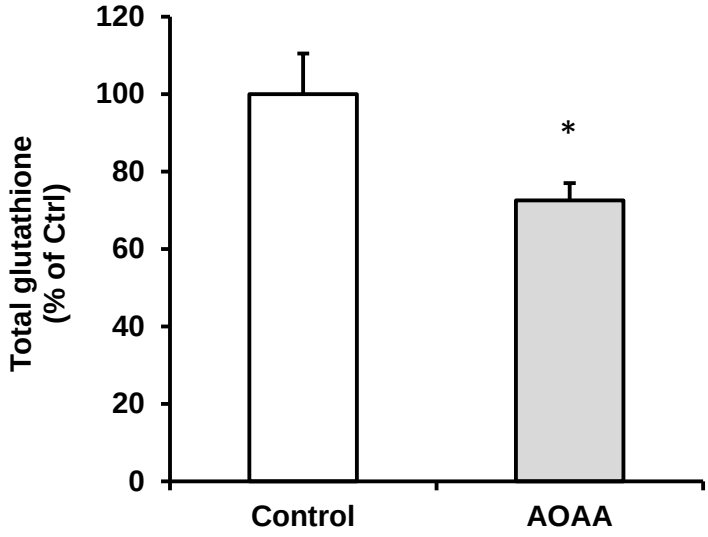

Figure 3-20 Effect of CBS inhibitors on glutathione levels in tubular cells in tubular cells

Human proximal tubular cells were incubated in the absence (control) or presence of a CBS inhibitor aminooxyacetic acid (AOAA, 0.2 mmol/L). Total intracellular glutathione levels were measured. Results are expressed as mean ± SEM (n = 5 for each group). *$P < 0.05$ when compared with the value obtained from control cells.

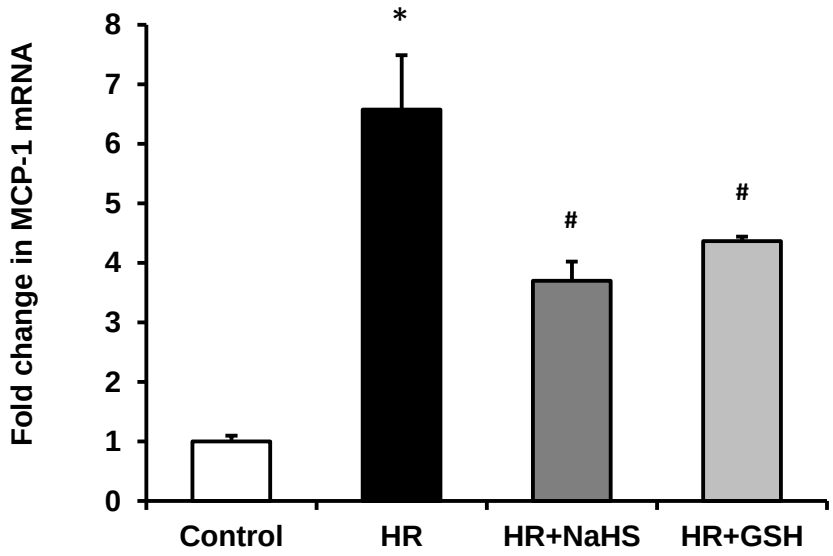

Figure 3-21 Effects of H_2S donor and glutathione on MCP-1 expression in tubular cells

Human proximal tubular cells were subjected to 2-h hypoxia followed by 48-h reoxygenation in the absence (HR) or presence of NaHS (10 μmol/L) or glutathione (GSH 0.01 mmol/L). Cells cultured in a regular medium without being subjected to HR were used as a control. The mRNA levels of MCP-1 were measured by a real-time PCR analysis. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from control cells. #$P < 0.05$ when compared with the value obtained from cells subjected to hypoxia–reoxygenation.

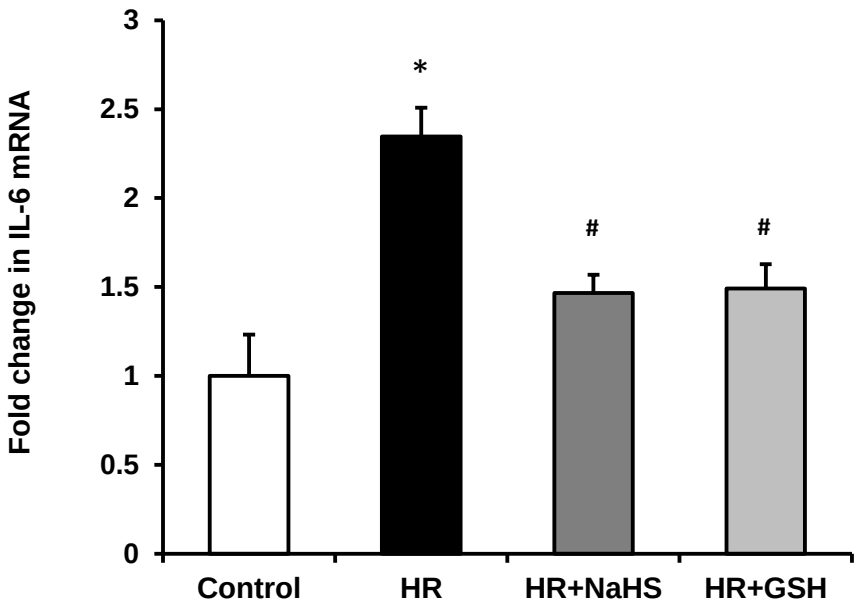

Figure 3-22 Effects of H_2S donor and glutathione on IL-6 expression in tubular cells

Human proximal tubular cells were subjected to 2-h hypoxia followed by 48-h reoxygenation in the absence (HR) or presence of NaHS (10 μmol/L) or glutathione (GSH 0.01 mmol/L). Cells cultured in a regular medium without being subjected to HR were used as a control. The mRNA levels of IL-6 were measured by a real-time PCR analysis. Results are expressed as mean ± SEM ($n = 5$ for each group). *$P < 0.05$ when compared with the value obtained from control cells. #$P < 0.05$ when compared with the value obtained from cells subjected to hypoxia–reoxygenation.

3.5 Discussion

Acute inflammatory response is a hallmark of I/R-induced kidney injury. Uncontrolled inflammatory response can exacerbate I/R injury. However, the mechanisms responsible for aberrant inflammatory response are not completely understood. This study for the first time demonstrates that reduced CBS and CSE expression contributes to inflammatory response in the kidney upon ischemia (45 minutes) followed by reperfusion (24 hours). Inhibition of CBS and CSE leads to reduced glutathione and H_2S production in the kidney and in proximal tubular cells, which correlates with increased expression of pro-inflammatory cytokines. Supplementation of glutathione or H_2S donor effectively attenuates the expression of pro-inflammatory cytokines in tubular cells.

Both CBS and CSE in the transsulfuration pathway are highly expressed in the kidney. These two enzymes are mainly located in the proximal tubule segments which are more susceptible to I/R injury (Beltowski, 2010). In this study, tubular damage persisted 24 hours after ischemia insult as indicated by a significantly elevated plasma NGAL level. The expression of CBS and CSE was significantly reduced in I/R kidney. Cysteine lies downstream of the transsulfuration pathway and serves as an essential substrate for glutathione biosynthesis. A decrease in CBS and CSE expression in the transsulfuration pathway could lead to a reduction of cysteine level and subsequently limit glutathione generation. In this study, I/R caused a significant reduction in glutathione levels in the kidney. Such an inhibitory effect on glutathione levels was also observed in tubular cells subjected to hypoxia–reoxygenation. Inhibition of the rate-limiting enzyme CBS also led to a decrease in intracellular glutathione levels in tubular cells. These results suggested that reduction of CBS and CSE expression in

the transsulfuration pathway was responsible for glutathione depletion in the kidney upon I/R injury. Glutathione is a major endogenous nonenzymatic antioxidant that has anti-inflammatory property through the regulation of redox-sensitive signaling pathways. Acute inflammatory response is recognized as one of the major factors of kidney I/R injury, which is characterized by increased expression of pro-inflammatory cytokines and recruitment of leukocytes. In this study, supplementation of glutathione to tubular cells effectively attenuated the expression of pro-inflammatory cytokines. Such beneficial effect of glutathione might be mediated via its antioxidant action.

Aside from their roles in the transsulfuration pathway, CBS and CSE are also a major source of H_2S production through desulfhydration reactions (Xu et al., 2009). In accordance with a reduction in CBS and CSE expression, there was a marked decrease in H_2S production in the kidney subjected to ischemia (45 minutes) and reperfusion (24 hours). H_2S has been shown to have protective effects against I/R injury in various organs including heart, brain, kidney, and liver (Calvert et al., 2009; Gheibi et al., 2014; Kang et al., 2009; Xu et al., 2009). Anti- and pro-inflammatory effects of endogenous H_2S or exogenous H_2S donor have been reported in various animal models (Whiteman & Winyard, 2011). The mechanisms by which H_2S exerts inflammatory response are complex including attenuation of nuclear factor-κB (NF-κB) activation, reduction of pro-inflammatory factor expression, and inhibition of leukocyte adhesion (Dongo, Hornyak, Benko, & Kiss, 2011; Whiteman & Winyard, 2011; H. Zhang, Moochhala, & Bhatia, 2008). For example, in a myocardial I/R injury porcine model, administration of H_2S donor (NaHS) prior to reperfusion limited inflammatory response and attenuated myocardial injury in Yorkshire pigs (Sodha et al., 2009). In a cerebral I/R injury rat model, administration of NaHS exerted a protective effect against severe cerebral injury

induced by a global I/R through attenuation of oxidative stress, inflammation, and apoptosis in the brain tissue (Yin et al., 2013). In mouse mesangial cells, increased endogenous H_2S generation or supplementation of exogenous H_2S partially inhibited Hcy-induced expression of pro-inflammatory cytokines (U. Sen, Givvimani, Abe, Lederer, & Tyagi, 2011). We previously reported that there was an acute inhibition of CBS-mediated H_2S production in I/R kidney 6 hours after the onset of reperfusion (Xu et al., 2009). Results obtained from this study indicated that the expression of both CBS and CSE was reduced in the kidney subjected to ischemia (45 minutes) followed by reperfusion (24 hours). Although H_2S has been implicated in the regulation of inflammatory response, its role in the expression of pro-inflammatory cytokines in AKI has not been well defined. Our recent study has demonstrated that administration of H_2S donor (NaHS) can restore I/R-impaired kidney function (Xu et al., 2009). This study provided several lines of evidence indicating that changes in CBS and CSE-mediated H_2S production had a profound effect on I/R-induced pro-inflammatory cytokine expression. First, low CBS and CSE expression along with decreased H_2S production was inversely associated with I/R-induced expression of pro-inflammatory cytokines (i.e., MCP-1, IL-6) in the kidney. Second, the expression of CBS and CSE was decreased in proximal tubular cells subjected to hypoxia–reoxygenation, while the expression of pro-inflammatory cytokines such as IL-6 and MCP-1 was elevated in these cells. Exogenous H_2S donor effectively attenuated I/R-induced cytokine expression in tubular cells. Third, inhibition of H_2S production by CBS and CSE inhibitors also led to an increase in pro-inflammatory cytokine expression in tubular cells. Taken together, these results suggested that reduced endogenous H_2S generation due to downregulation of CBS and CSE contributed to increased expression of pro-inflammatory cytokines in the kidney

upon I/R injury. Given the anti-inflammatory property of H_2S, its deficiency could potentially exacerbate I/R injury in the kidney. Our results indicated that CBS and CSE played an important role in regulating H_2S generation in the kidney. Reduction in CBS and CSE-mediated H_2S production during I/R contributed to inflammatory response in the kidney (Figure 3-23). Proper restoration of endogenous H_2S production and/or administration of exogenous H_2S donor may represent one of the therapeutic strategies in I/R-mediated tissue injury.

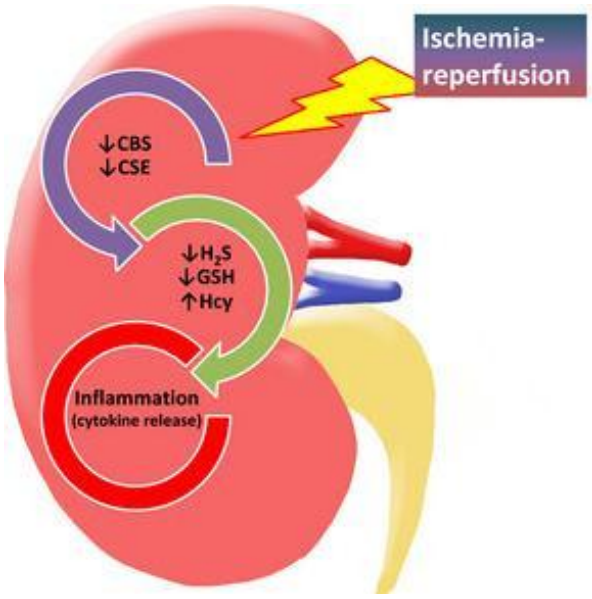

Figure 3-23 Proposed mechanism of CBS/CSE in AKI

Chapter 4. STUDY 2: Regulation of oxidative stress in renal I/R injury: Beneficial effect of tyrosol

(Manuscript 2: Tyrosol Attenuates Ischemia-reperfusion-Induced Kidney Injury via Inhibition of Inducible Nitric Oxide Synthase)

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4.1 Abstract

Tyrosol is a natural phenolic antioxidant compound. Oxidative stress represents one of the important mechanisms underlying ischemia/reperfusion (I/R) induced kidney injury. The aim of our study was to investigate the effect of tyrosol against acute kidney injury associated with I/R. The left kidney of Sprague-Dawley rat was subjected to ischemia (45 minutes) followed by reperfusion (6 hours). I/R caused an increase in peroxynitrite formation and lipid peroxidation. The level of nitric oxide (NO) metabolites and the mRNA of inducible nitric oxide synthase (iNOS) were elevated in ischemia-reperfused kidneys. The administration of tyrosol (100 mg/kg body weight) prior to the induction of ischemia significantly reduced peroxynitrite formation, lipid peroxidation and the level of NO metabolites. Tyrosol administration also attenuated I/R induced NF-κB activation and iNOS expression. Such a treatment improved kidney function. Results suggest that tyrosol may have a protective effect against acute kidney injury through inhibition of iNOS-mediated oxidative stress.

4.2 Introduction

Epidemiologic studies have indicated that moderate consumption of olive oil, one of the major components in the Mediterranean diet, is associated with the reduced risk of morbidity and mortality of diseases such as cancer, cardiovascular diseases and renal diseases (Buckland et al., 2012; de Lorgeril et al., 1999; Giugliano & Esposito, 2008; Ruiz-Canela & Martinez-Gonzalez, 2011; Vernaglione, 2009). The health benefits of olive oil are attributed to their high contents of polyphenol compounds (Briante, Febbraio, & Nucci, 2003; Carrasco-Pancorbo et al., 2005). Among the phenolic compounds found in olive oil, tyrosol (2-(4-hydroxyphenyl) ethanol) (Figure 4-1) is one of the major natural phenolic compounds present at high concentrations in olive oil (Covas et al., 2003; Di Carlo, Mascolo, Izzo, & Capasso, 1999) and has been studied extensively for its antioxidant potential. It has been reported that addition of tyrosol to the culture medium reduces ROS generation in PMA-activated rat peritoneal leukocytes (de la Puerta, Ruiz Gutierrez, & Hoult, 1999), in human endothelial cells (Bertelli et al., 2002) and in oxidized-LDL treated CaCo-2 intestinal mucosa cells (C. Giovannini et al., 1999). Moreover, it has been shown that its antioxidant property may also contribute to the neuroprotective effect of tyrosol against oxidative stress-induced injury (Shi et al., 2012; Vauzour et al., 2007).

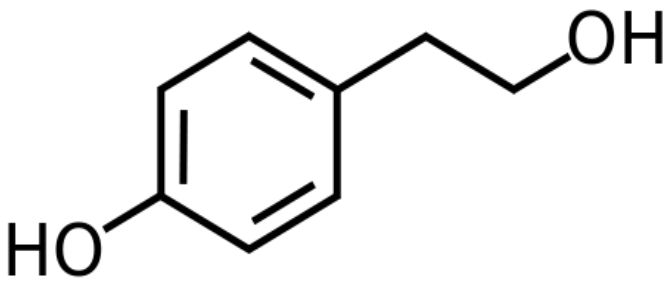

Figure 4-1 Chemical structure of tyrosol

Kidney ischemia/reperfusion (I/R) injury is a complication frequently experienced by patients in the perioperative period (El-Zoghby et al., 2009). Various clinical-surgical procedures such as renal angioplasty, bypass procedure and clamping of the renal pedicle or the aorta above the renal arteries lead to a temporary interruption or reduction of renal blood supply, resulting in an ischemic area (Brady & Singer, 1995; Gueler, Gwinner, Schwarz, & Haller, 2004). The reinstitution of blood to the ischemic area can subsequently result in I/R injury (Ploeg et al., 1992), which is associated with prolonged hospitalization, increased morbidity and mortality (Kelly, 2006; Star, 1998). Studies in humans and experimental animal models suggest that oxidative stress resulting from the production of reactive oxygen species (ROS) plays a key role in I/R injury (Nath & Norby, 2000; Noiri et al., 2001; Prathapasinghe et al., 2008; Sung et al., 2002; Weight, Bell, & Nicholson, 1996). Among ROS, superoxide anion can rapidly react with nitric oxide (NO) to form another potent free radical peroxynitrite (ONOO-) that, in turn, causes extensive protein tyrosine nitration (Pryor & Squadrito, 1995; Radi, Beckman, Bush, & Freeman, 1991). NO is an endogenous signal transduction gas molecule and plays an important role in kidney I/R injury (Weight & Nicholson, 1998). The NO is synthesized by nitric oxide synthase (NOS), which is found to have three isoforms of NOS, namely, inducible (iNOS), endothelial (eNOS) and neuronal (nNOS) in the kidney. The activation of iNOS often occurs in the kidney under pathological conditions. Excessive production of NO from iNOS exerts detrimental effects on the tissue (Chatterjee et al., 2002; Joles, Vos, Grone, & Rabelink, 2002). We have observed that I/R leads to increased iNOS-mediated NO production and peroxynitrite formation in rat kidney (Prathapasinghe et al., 2008). The expression of iNOS gene is mainly regulated by transcription factor, namely, nuclear factor-kappa B (NF-κB). Inhibition of iNOS expression

has been shown to attenuate oxidative stress and hence alleviate renal I/R injury (Chatterjee, Patel et al., 2003). Furthermore, inactivation of NF-κB by NF-κB decoy oligodeoxynucleotides treatment prevents I/R-induced iNOS expression in the kidney (Cao et al., 2004).

Although dialysis currently is a treatment of choice for acute ischemic renal failure, it is only a temporary replacement for the lost kidney function (Friedewald & Rabb, 2004; Rondon-Berrios & Palevsky, 2007). The identification of novel therapeutic interventions against kidney I/R injury has become a subject of intense research interest. Inhibition of iNOS-mediated NO production may antagonize oxidative stress induced by I/R and hence protect against renal injury. Dietary antioxidants are viewed as a promising therapeutic strategy to combat oxidative stress. As one of the main compounds present in the Mediterranean diet, tyrosol has been reported to have antioxidant activity (Gris et al., 2011; Manna, Napoli, Cacciapuoti, Porcelli, & Zappia, 2009; Thirunavukkarasu et al., 2008). The ready absorption of tyrosol leads to its effective bioavailability in humans (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). It has been reported that once absorbed, tyrosol concentrates mainly in the kidney (Loru et al., 2009). However, it remains unclear whether tyrosol can prevent or alleviate I/R-induced injury in the kidney. Therefore, in the present study, we aimed to investigate the protective effect of tyrosol on I/R-induced injury in the rat kidney and the mechanisms involved.

4.3 Materials and Methods

4.3.1 Renal ischemia/reperfusion

Male Sprague-Dawley rats weighing 250-300 g were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg). The rats were kept on a heat pad and the rectal temperature was maintained at 37 ℃ throughout the experimental procedure. Renal ischemia was induced by clamping the left renal vascular pedicle for 45 minutes with non-traumatic vascular clamp (Gris et al., 2011; Ko et al., 2010; N. Wu et al., 2010). At the end of ischemia period, the rats were subjected to reperfusion (1 hour or 6 hours) of by removal of the clamp, and right nephrectomy was performed (Prathapasinghe et al., 2008). A sham-operated group of rats was subjected to the same surgical procedure without inducing ischemia/reperfusion and were sacrificed at corresponding time points. Results obtained from this group were used as controls. In the tyrosol treatment experiment, rats were administered tyrosol (100 mg/kg) via intraperitoneal injection 30 minutes prior to the induction of ischemia followed by reperfusion. A blood sample was drawn from the abdominal aorta before rats were sacrificed. Plasma was separated by centrifugation of blood at 3,000 g for 20 minutes at 4 ℃. Plasma creatinine levels were determined by using Genzyme Diagnostics Creatinine Kit. Kidneys were collected and bisected in ice-cold potassium phosphate buffer. All of the 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. All of the chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

4.3.2 Determination of lipid peroxidation

The degree of lipid peroxidation in the kidney was determined by measurement of malondiadehyde (MDA) levels with thiobarbituric acid reactive substances (TBARS) assay as described previously (Hwang, Siow, Au-Yeung, House, & O, 2011). A portion of the kidney including both cortex and medulla was homogenized in 0.1M KCl solution containing 3 mM EDTA, followed by centrifugation at 600 g for 15 minutes at 4 ℃. An aliquot of supernatant was added to the reaction mixture containing 8% SDS, 20% acetic acid, 0.8% thiobarbituric acid and water. The reaction mixture was incubated at 95 ℃ for 1 hour. The amount of MDA formed in the reaction mixture was measured by using spectrophotometer at the absorbance of 532 nm. MDA was used as the external standard, and results were expressed as a percentage of sham-operated group. The amount of MDA levels correlates to the degree of lipid peroxidation produced in the kidney tissues.

4.3.3 Measurement of nitric oxide metabolites in the kidney

As stable end products of NO metabolites, nitrite and nitrate were measured to assess the NO levels in the kidney. The amount of nitrite and nitrate (nitrite-nitrate) was determined by Griess reaction assay based on azo coupling reaction as described previously (Prathapasinghe et al., 2007). In brief, the kidney was homogenized in a buffer (pH 7.4) containing 20 mM Tris and 2 mM EDTA followed by centrifugation at 600 g for 10 minutes at 4 ℃ and the supernatant was collected. After deproteinized with 0.3 N NaOH and 5% $ZnSO_4$, the supernatant was incubated with nitrate reductase to convert nitrate to nitrite. The Griess reaction was initiated by the addition of a reagent containing 12.5 mM sulfanilamide in 6 N HCl and 12.5 mM N-(1-naphthyl)-ethylenediamine. The absorbency of the reaction mixture

was read at 520 nm. Sodium nitrite at different concentrations was used as standards. The concentration of nitrite-nitrate in the kidney tissue was expressed as a percentage of the sham-operated group.

4.3.4 Western immunoblotting analysis

iNOS levels in kidney tissue were determined by a Western immunoblotting analysis. In brief, kidney proteins (100 μg) were separated by electrophoresis on a 8% SDS polyacrylamide gel. The proteins in the gel were transferred to a nitrocellulose membrane. The membrane was probed with rabbit polyclonal anti-iNOS antibodies (1:2000; Calbiochem, Ontario, Canada). For determination of renal nitrotyrosine protein levels, tissue proteins (40 μg) were separated by electrophoresis on a 15% SDS polyacrylamide gel, which was followed by electrophoretic transfer of proteins from the gel to a nitrocellulose membrane. The membrane was probed with mouse monoclonal anti-nitrotyrosine antibodies (1:1000, Cayman, Ann Arbor, MI). The bands were visualized by using enhanced chemiluminescence (ECL) reagents and exposed to Kodak BioMax Light film. Film was analyzed by using Bio-Rad Quantity-One image analysis software (Version 4.2.1). The same nitrocellulose membrane was probed with mouse monoclonal anti-β-actin antibodies (Cell Signaling Technology, Inc.) to confirm equal loading of proteins of individual samples.

4.3.5 Measurement of iNOS mRNA expression

Total RNAs were isolated from rat kidney tissues with TriZol reagent (Invitrogen). iNOS mRNA was determined by a real time PCR analysis using the iQ5 real time PCR detection system (Bio-Rad) (N. Wu et al., 2010). In brief, 2ug total RNA was reverse transcribed to cDNA in a total volume of 20 μl with the reverse transcription reagent mixer. The reaction

mixture of real time PCR contains 0.4 μM 5 ́and 3 ´primers, 2 μl of cDNA products and iQ-SYBR green supermix reagent (Bio-Rad) in a total volume of 25 μl. Crossing threshold values were normalized to β-actin. The nucleotide sequences of primers (Invitrogen) used for rat iNOS (178 bp) were (forward) 5 ´-GGAAGAAATGCAGGAGATGG-3 ´and (reverse) 5 ´-GCACATCGCCACAAACATAG-3 ´, and those used for rat β-actin were (forward) 5 ´-GTCGTACCACTGGCATTGTG-3 ´ and (reverse) 5 ´-TCTCAGCTGTGGTGGTGAAG-3 ´. All of the primers were purchased from Invitrogen. The result of iNOS mRNA expression was expressed as percentage change relative to the sham-operated group.

4.3.6 Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins were isolated from the kidney tissue and the NF-κB/DNA binding activity was determined by EMSA (F. Zhang et al., 2004). Briefly, 10 μg of nuclear protein was incubated with a reaction buffer for 15 minutes at room temperature, followed by the incubation with excess ^{32}P-end-labled oligonucleotides containing the NF-κB consensus sequence (Promega, Madison, WI). The sequence of the oligonucleotide probe was 5 ´-AGTTGAGGGGACTTCCCAGGC-3 ´. The radioactive mixture was separated in a 6% nondenaturing polyacrylamide gel and dried on a piece of filter paper followed by autoradiography. The cold competition experiment was performed by adding a 100-fold excess unlabeled NF-κB probe prior to the addition of the ^{32}P-end-labeled oligonucleotide in the reaction mixture to confirm that the binding of ^{32}P-end-labled oligonucleotides to NF-κB was sequence specific.

4.3.7 Histological staining

A portion of the kidney was immersion-fixed in 10% neutral-buffered formalin overnight followed by embedding in paraffin. Sequential 5-μm paraffin-embedded cross sections were prepared and stained with hematoxylin and eosin (H&E) to examine histological changes in the kidney (Hwang et al., 2011). Images were captured by using Olympus BX43 microscope with an Olympus Q-Color3 digital camera. The slides were analyzed at ×200 magnification using the Image-Pro Plus software (Version 7.0, Media Cybernetics, Bethesda, MD).

4.3.8 Statistical analysis

Results were analyzed by using one-way ANOVA followed by Newman-Keuls test. Data were presented as the means ± SEM. The level of statistical significance was determined when a *p* value was less than 0.05.

4.4 Results

4.4.1 Effect of tyrosol on the function and morphological changes in kidneys subjected to ischemia/reperfusion

As an indicator of kidney dysfunction, plasma creatinine levels were measured. The level of creatinine in rats subjected to ischemia (45 minutes) followed by reperfusion (6 hours) was significantly higher than that in the sham-operated rats (Table 4-1), indicating an impairment of kidney function upon I/R. Kidney tissue morphology was examined by using H&E staining. Tubular necrosis and interstitial congestion were observed in the kidney subjected to I/R (Figure 4-2). Administration of tyrosol (100 mg/kg, intraperitoneal injection) prior to the induction of ischemia caused a decrease in plasma creatinine levels in rats subjected to I/R (Table 4-1). In accordance with the improvement of kidney function, tyrosol treatment also protected the structural integrity of the kidney, which was characterized by reduced tubular cell necrosis and interstitial congestion (Figure 4-2). Administration of tyrosol preserved the gross appearance of kidney subjected to I/R (Figure 4-2).

Table 4-1 Plasma creatinine levels of the groups

Groups	n	Creatinine (mg/dL)
Sham	10	0.42 ± 0.04
I/R	10	1.93 ± 0.13[a]
I/R+Tyr	10	1.51 ± 0.13[a,b]

[a] $P < 0.05$ vs. sham-operated group [b] $P < 0.05$ vs. ischemia/reperfusion

The left kidney was subjected to sham operation (Sham), ischemia (45 minutes) followed by reperfusion (6 hours) (I/R), or ischemia (45 minutes) followed by reperfusion (6 hours) with intraperioneal injection of tyrosol (100 mg/kg) 30 minutes prior to the induction of ischemia (I/R+Tyr). Results are expressed as mean ± SEM.

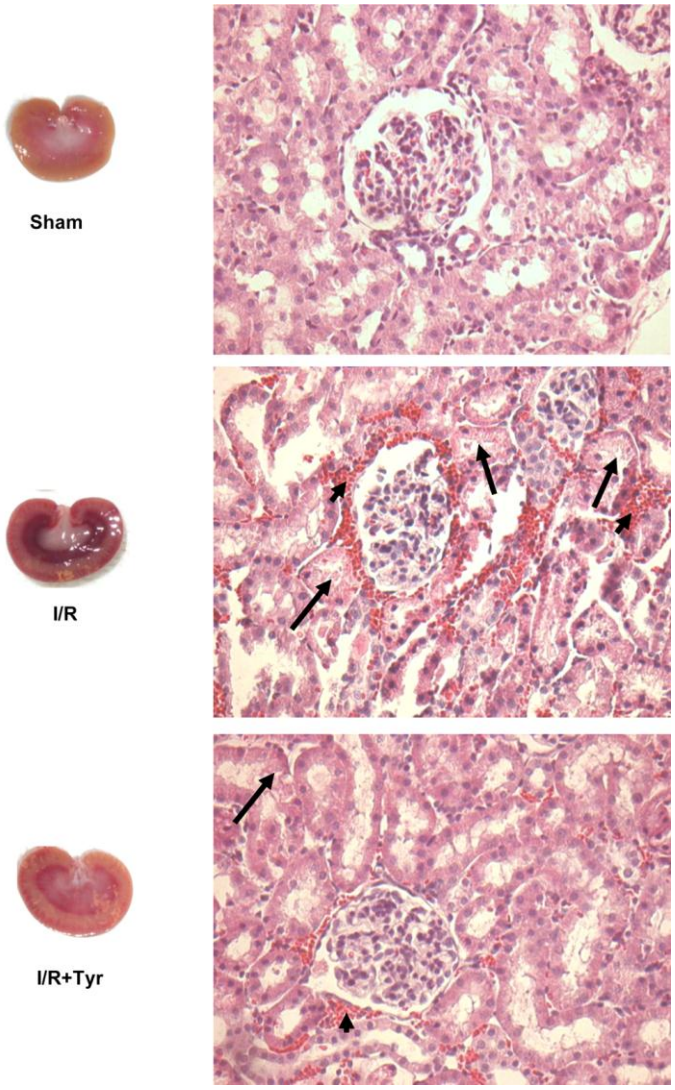

Figure 4-2 Effect of tyrosol on histological change of renal ischemia−reperfusion

The left kidney was subjected to sham operation (Sham), ischemia (45 minutes) followed by reperfusion (6 hours) (I/R), or ischemia (45 minutes) followed by reperfusion (6 hours) with tyrosol administration (100 mg/kg, intraperitoneal injection) 30 minutes prior to the induction of ischemia (I/R+Tyr). The gross appearance of a midtransverse plane of kidney was examined. Renal vascular integrity and cellular morphology were examined by hematoxylin and eosin (H&E) staining. A kidney section obtained from the I/R group showed tubular necrosis and interstitial congestion, compared with the sham-operated group. Long arrows point to the areas with tubular necrosis, and short arrowheads point to the areas with interstitial congestion.

4.4.2 Effect of tyrosol on ischemia/reperfusion induced oxidative stress in the kidney

The degree of lipid peroxidation in the kidney was examined by measuring the level of MDA, a stable by-product of lipid oxidation which is often used as an indicator of tissue oxidative stress. I/R resulted in a 2-fold increase in the MDA level in the kidney as compared to that in the sham-operated group, indicating that lipid peroxidation was increased (Figure 4-3). Administration of tyrosol significantly reduced the degree of lipid peroxidation in the kidneys subjected to I/R (Figure 4-3). To determine whether there was an increase in peroxynitrite formation in kidneys subjected to I/R, Western immunoblotting analysis was carried out to detect nitrotyrosine, a biomarker for peroxynitrite. A significant increase in the intensity of nitrotyrosine protein adduct was found in kidneys subjected to I/R (Figure 4-4), indicating an increase in peroxynitrite formation as compared to the sham-operated group. Administration of tyrosol significantly reduced the levels of nitrotyrosine in the kidneys subjected to I/R (Figure 4-4). These results suggested that administration of tyrosol to rats was able to alleviate I/R-induced oxidative stress in the kidney.

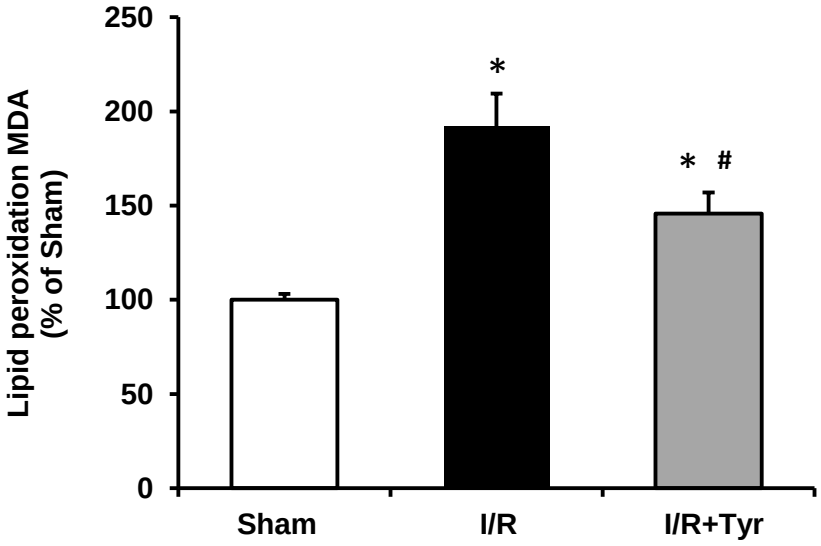

Figure 4-3 Measurement of lipid peroxidation in the kidney tissue

The left kidney was subjected to sham operation (Sham), ischemia (45 minutes) followed by reperfusion (6 hours) (I/R), or ischemia (45 minutes) followed by reperfusion (6 hours) with tyrosol administration (100 mg/kg, intraperitoneal injection) 30 minutes prior to the induction of ischemia (I/R+Tyr). Kidney lipid peroxides were determined by measuring the amount of MDA. Results are expressed as the mean ± SEM (n = 10). *P < 0.05 when compared with the value obtained from the sham-operated group; # P < 0.05 when compared with the value obtained from the I/R group.

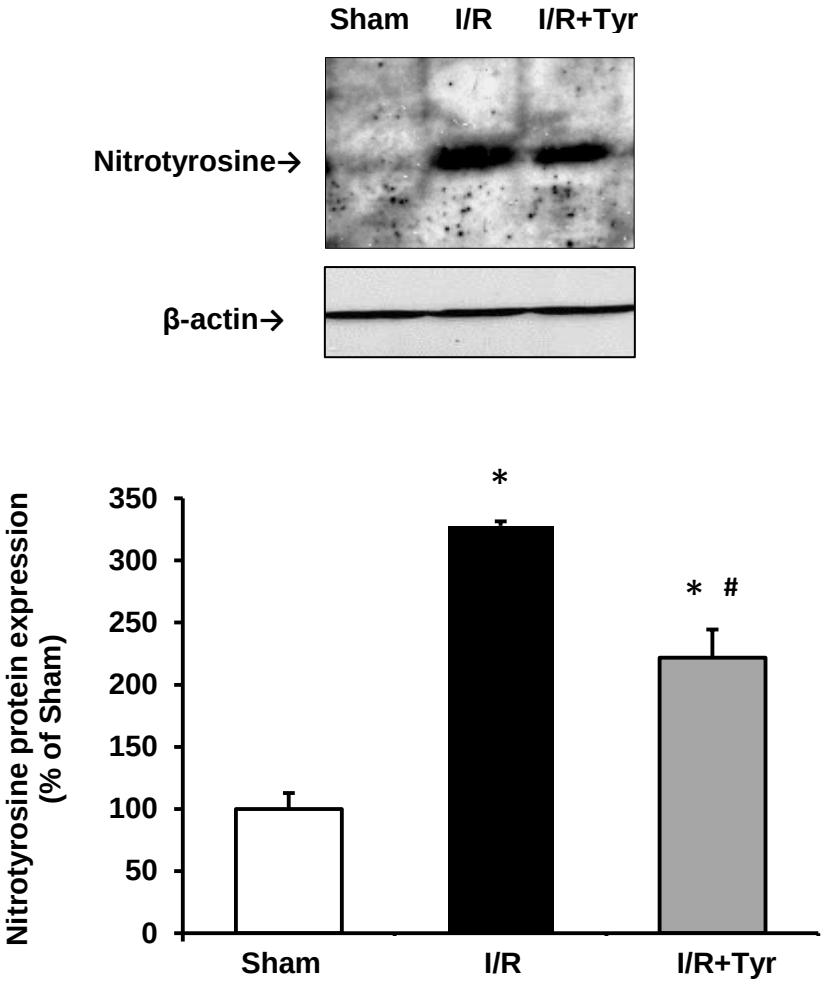

Figure 4-4 Effect of tyrosol treatment on nitrotyrosine-modified protein levels

The left kidney was subjected to sham operation (Sham), ischemia (45 minutes) followed by reperfusion (6 hours) (I/R), or ischemia (45 minutes) followed by reperfusion (6 hours) with tyrosol administration (100 mg/kg, intraperitoneal injection) 30 minutes prior to the induction of ischemia (I/R+Tyr). The histogram displays the relative density of nitrotyrosine-modified protein bands compared with β-actin. Results are expressed as the mean ± SEM (n = 5). *$P < 0.05$ when compared with the value obtained from the sham-operated group; #$P < 0.05$ when compared with the value obtained from the I/R group.

4.4.3 Effect of tyrosol on iNOS expression and NF-κB activation in the kidney

Induction of iNOS expression can lead to excessive NO production in the tissue. The NO is able to interact rapidly with superoxide to form peroxynitrite, a potent oxidant causing oxidative stress. The effect of I/R on iNOS expression in the kidney was examined. I/R caused a significant increase in iNOS mRNA (Figure 4-5) and protein (Figure 4-6) levels in the kidney tissue. Administration of tyrosol effectively attenuated I/R-induced elevation of iNOS mRNA and protein levels in the kidney tissue (Figure 4-5 and Figure 4-6). In accordance, I/R caused a significant elevation of NO metabolites levels (nitrite-nitrate) in the kidney (Figure 4-7). Administration of tyrosol attenuated I/R-induced iNOS expression (Figure 4-5 and Figure 4-6) and reduced the levels of NO metabolites (Figure 4-7) in the kidneys. Similar inhibitory effect of tyrosol was also observed in the kidneys subjected to ischemia (45 minutes) followed by reperfusion (1 hour) (data not shown). To investigate the mechanism by which tyrosol inhibited iNOS expression, nuclear proteins were prepared from the kidney tissue and EMSA was performed. I/R caused a significant increase in the NF-κB/DNA binding activity in the kidney tissue, which might lead to increased iNOS expression. Administration of tyrosol effectively inhibited I/R-induced NF-κB activation in the kidney tissue (Figure 4-8). These results suggested that tyrosol exerted an inhibitory effect on I/R-induced iNOS expression through its inhibition on NF-κB activation.

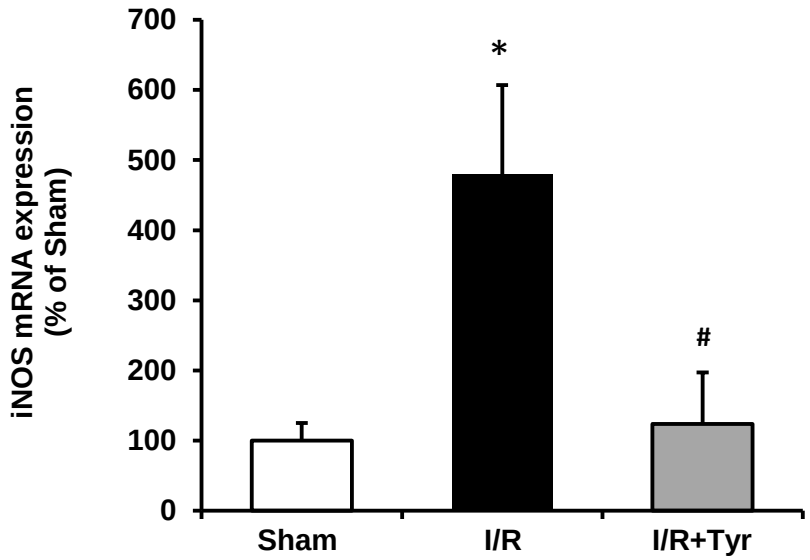

Figure 4-5 Effect of tyrosol treatment on iNOS gene expression in the kidney tissue

The left kidney was subjected to sham operation (Sham), ischemia (45 minutes) followed by reperfusion (6 hours) (I/R), or ischemia (45 minutes) followed by reperfusion (6 hours) with tyrosol administration (100 mg/kg, intraperitoneal injection) 30 minutes prior to the induction of ischemia (I/R+Tyr). iNOS mRNA in the kidney was determined by real-time PCR analysis. Results are expressed as the mean ± SEM (n = 8). *$P < 0.05$ when compared with the value obtained from the sham-operated group; #$P < 0.05$ when compared with the value obtained from the I/R group.

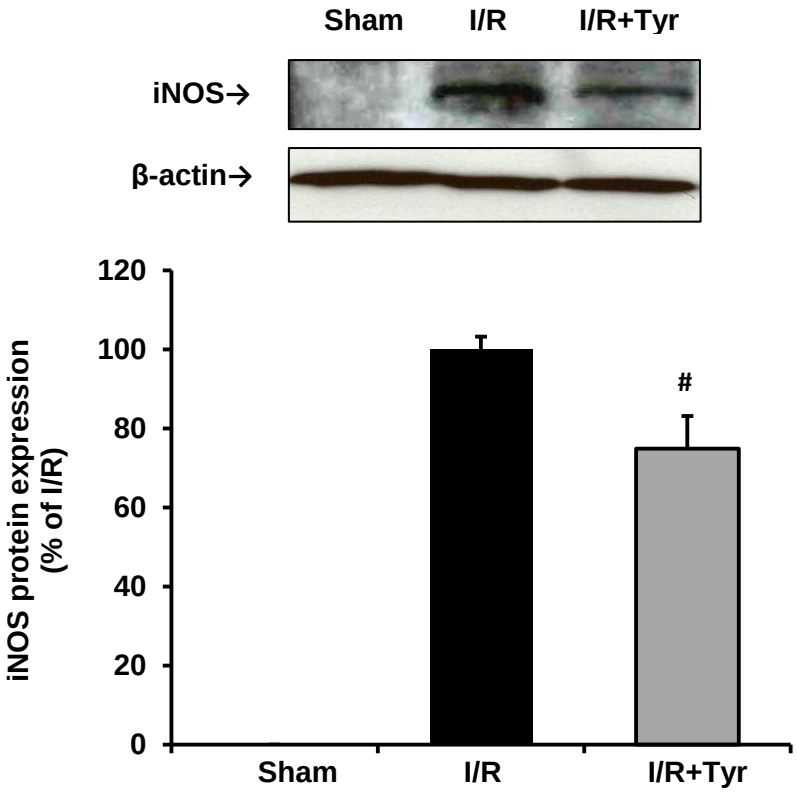

Figure 4-6 Effect of tyrosol treatment on iNOS protein expression in the kidney tissue

The left kidney was subjected to sham operation (Sham), ischemia (45 minutes) followed by reperfusion (6 hours) (I/R), or ischemia (45 minutes) followed by reperfusion (6 hours) with tyrosol administration (100 mg/kg, intraperitoneal injection) 30 minutes prior to the induction of ischemia (I/R+Tyr). iNOS protein was determined by Western immunoblotting analysis. Results are expressed as the mean ± SEM (n = 8). #$P < 0.05$ when compared with the value obtained from the I/R group.

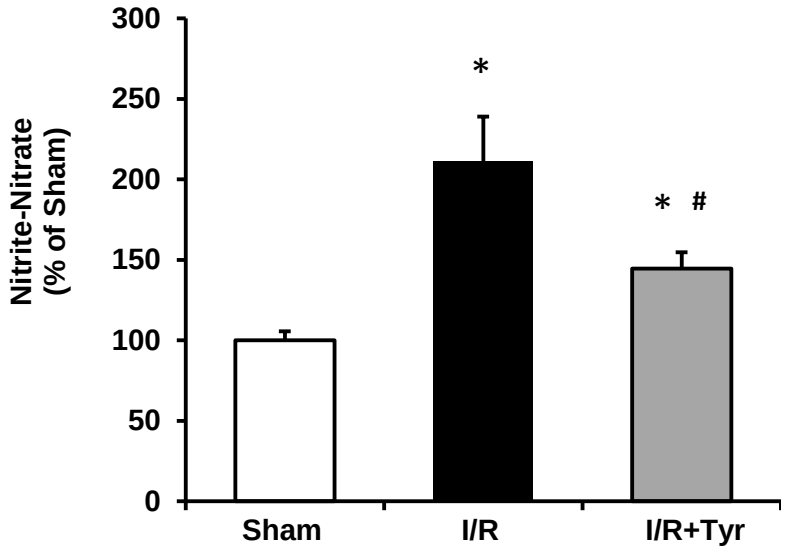

Figure 4-7 Effect of tyrosol treatment on NO metabolites levels in the kidney tissue

The left kidney was subjected to sham operation (Sham), ischemia (45 minutes) followed by reperfusion (6 hours) (I/R), or ischemia (45 minutes) followed by reperfusion (6 hours) with tyrosol administration (100 mg/kg, intraperitoneal injection) 30 minutes prior to the induction of ischemia (I/R+Tyr). Kidney NO metabolites (nitrite-nitrate) were determined. Results are expressed as the mean ± SEM (n = 8). * $P < 0.05$ when compared with the value obtained from the sham-operated group; # $P < 0.05$ when compared with the value obtained from the I/R group.

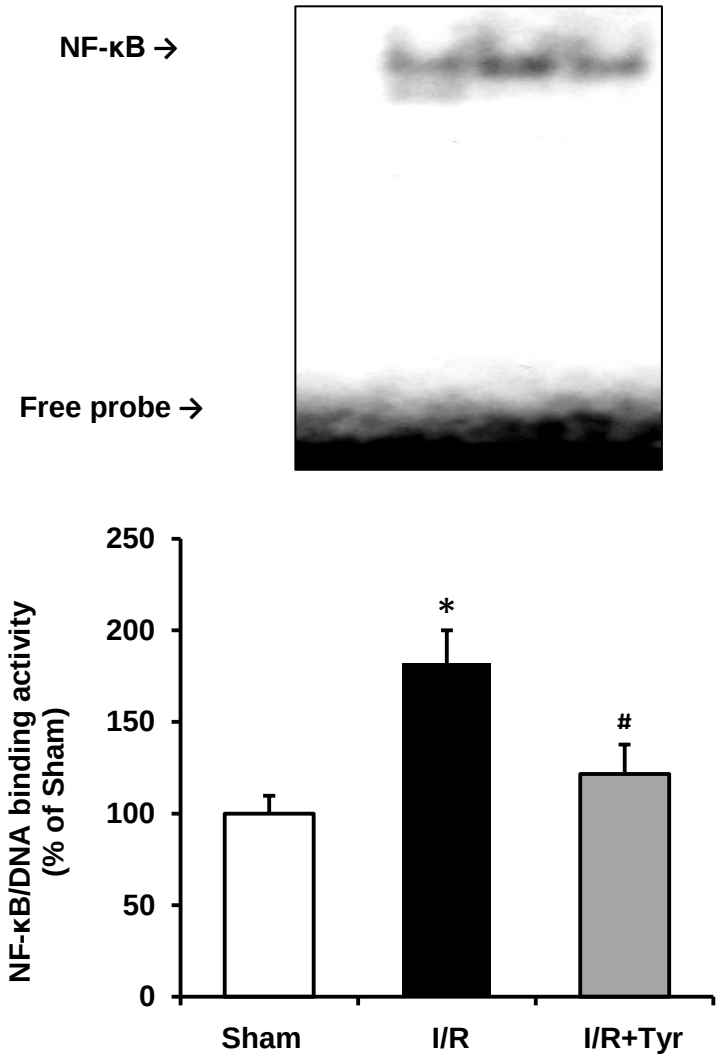

Figure 4-8 Effect of tyrosol treatment on the activation of NF-κB in kidney

The left kidney was subjected to sham operation (Sham), ischemia (45 minutes) followed by reperfusion (6 hours) (I/R), or ischemia (45 minutes) followed by reperfusion (6 hours) with tyrosol administration (100 mg/kg, intraperitoneal injection) 30 minutes prior to the induction of ischemia (I/R+Tyr). The DNA binding activity of NF-κB in the kidney was determined by EMSA. Results are expressed as the mean ± SEM (n = 5). *P < 0.05 when compared with the value obtained from the sham-operated group; #P < 0.05 when compared with the value obtained from the I/R group.

4.5 Discussion

The results obtained from the present study revealed that ischemia (45 min) followed by reperfusion (6 hours) led to impaired kidney function in rats. Kidney dysfunction was accompanied by increased iNOS-mediated NO production, increased protein nitration and lipid peroxidation. The novel findings of the present study are that 1) administration of tyrosol prior to the induction of ischemia effectively reduced oxidative stress and improved the function of kidney subjected to I/R; 2) such a beneficial effect of tyrosol was mediated, in part, through its inhibition on NF-κB activation and iNOS-mediated NO production.

NO plays an important role in physiological processes in the body. At physiological concentrations, NO regulates vasodilation and participates in cell signal transduction. However, activation of the iNOS isoform can lead to excessive production of NO, which is detrimental to tissues. The contribution of excessive NO to cell damage may be attributable to the effect mediated by the NO molecule itself or an indirect effect by peroxynitrite, the product of an interaction of NO with superoxide (Pacher et al., 2007). Studies in experimental animals have demonstrated that scavenging excessive NO or selective inhibition of iNOS protects kidney from I/R injury (Chatterjee, Patel et al., 2003; Mark, Robinson, & Schulak, 2005; Prathapasinghe et al., 2008). In the present study, I/R caused a significant increase in iNOS expression in the kidney. This was accompanied by an elevation of NO metabolites in the same kidney tissue. We previously reported that I/R resulted in an activation of NF-κB (Sung et al., 2002). This transcription factor regulates the expression of many inflammatory genes including iNOS (Sung et al., 2002). Indeed, we observed a significant increase in NF-κB/DNA binding activity in the kidneys subjected to I/R.

Administration of tyrosol not only attenuated I/R-induced NF-κB activation but also reduced I/R-induced iNOS expression in the kidney. In accordance, the levels of NO metabolites in these kidneys were markedly reduced, which might account for a reduction of peroxynitrite formation in the kidney tissue.

Oxidative stress is characterized by an excessive accumulation of reactive radicals causing tissue damage (Nath & Norby, 2000; Sung et al., 2002). Peroxynitrite is a well known reactive oxidant and even modest increase in peroxynitrite formation can result in extensive oxidation of macromolecules (Pacher et al., 2007). In the present study, upregulation of iNOS expression led to excessive NO production in the kidney upon I/R, which might contribute to an elevation of peroxynitrite formation and subsequently protein tyrosine nitration. This was accompanied by impaired kidney function. Histological examination revealed tubular cell swelling and massive blood cell infiltration in the medulla region in the kidney subjected to I/R. Administration of tyrosol prior to the induction of ischemia significantly reduced nitrotyrosine levels and lipid peroxidation in the kidney. Such treatment also improved renal function and alleviated histological damage in the kidney subjected to I/R. These results suggested that reduction of peroxynitrite formation by tyrosol treatment might contribute to its beneficial effect against I/R injury in the kidney.

The beneficial effects of tyrosol as a natural phenolic antioxidant have also been observed in other experimental models. Polyphenol extract from olive pomace with tyrosol as one of the main constituents has been shown to reduce NO levels in human endothelial cells subjected to anoxia (Palmieri et al., 2012). Tyrosol was also shown to reduce phorbol 12-myristate 13-acetate (PMA) induced NO release from macrophage (Moreno, 2003). A recent study suggests that tyrosol has a strong antioxidant capacity to exert protective effect against I/R-

induced apoptosis (Sun et al., 2012). Another mechanism by which tyrosol exerts the antioxidant effect is mediated by scavenging peroxynitrite and superoxide (Bertelli et al., 2002; de la Puerta, Martinez Dominguez, Ruiz-Gutierrez, Flavill, & Hoult, 2001). Results from the present study provided evidence on the renal protective effect of tyrosol during I/R (Figure 4-9). Our results suggested that regulation of NF-κB activation might be one of the mechanisms by which tyrosol attenuated I/R-induced iNOS expression in the kidney.

In summary, the present study has demonstrated, for the first time, that tyrosol treatment attenuated iNOS-mediated NO production which in turn reduced oxidative stress and minimized the extent of kidney injury induced by I/R. Our results suggest that tyrosol may be considered as a potential natural health product for the prevention and treatment of I/R-induced tissue injury such as acute kidney injury.

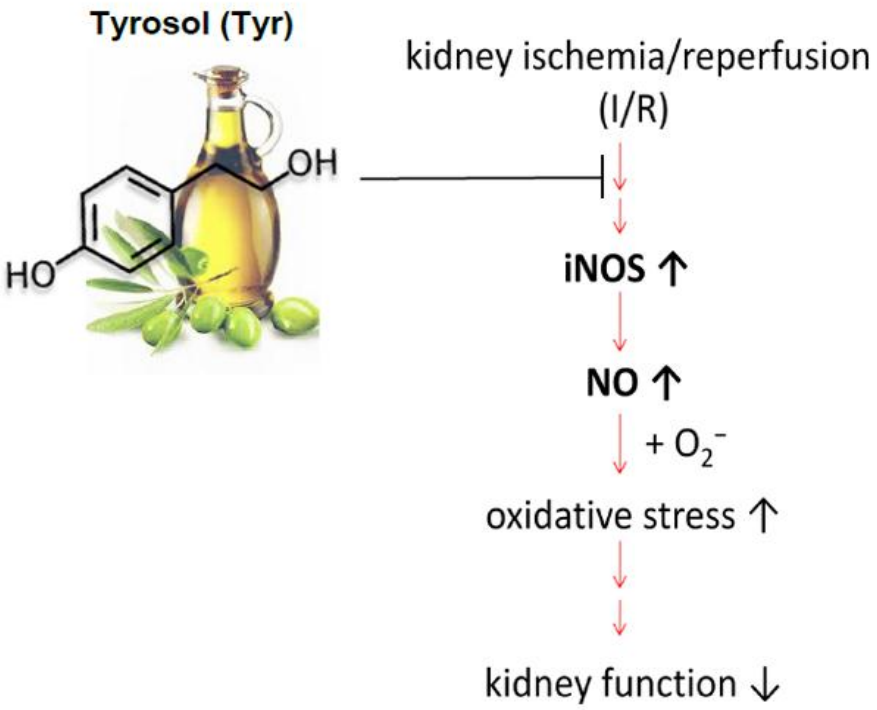

Figure 4-9 Proposed mechanisms of the protetive effect of tyrosol on I/R-induced kidney injury

Chapter 5. GENERAL DISCUSSION, LIMITATIONS AND FUTURE PERSPECTIVES

5.1 General discussion

The present study mainly focused on the investigation of the regulatory mechanisms of renal inflammatory response and oxidative stress in I/R-induced AKI models, *in vivo* and *in vitro*. The inflammation and oxidative stress injury were induced by I/R in the kidney. The findings have clearly demonstrated that (1) there is reduced endogenous H_2S generation due to downregulation of CBS and CSE, which contributes to increased expression of pro-inflammatory cytokines, e.g. MCP-1, IL-6, in the kidney upon I/R injury. (2) Proper restoration of endogenous H_2S production and/or administration of exogenous H_2S donor have a protective effect against renal I/R injury. (3) The increased iNOS-mediated NO production and peroxynitrite formation play an important role in causing the oxidative stress damage in renal I/R kidneys. (4) Tyrosol administration offers a renal protective effect by reducing I/R-induced oxidative stress injury via the inhibition of NF-κB activation and iNOS expression.

5.1.1 Renal I/R-induced AKI causes renal injury by increasing oxidative stress and inflammatory response

Acute kidney injury is a common and serious problem that affects millions of patients from around the world, which leads to decreased survival and promotes the progression of chronic kidney disease (CKD). Research into the mechanisms and pathophysiology of AKI has yielded potential targets in the prevention and treatment of AKI. Increasing evidence has indicated that AKI is associated with inflammation and oxidative stress. An increase in oxidative stress favors endothelial dysfunction by injuring endothelial and smooth muscle cell membranes and by reducing NO availability. Endothelial dysfunction plays a major role

in renal I/R-induced inflammatory responses. This results in the expression of leukocyte adhesion molecules and the subsequent binding of circulating inflammatory cells and their migration into the subendothelial space. During the inflammatory process, the inflammatory cells can cause direct injury to renal tubular epithelial cells through direct cell-cell contact, or indirectly by upregulating the expression of cytokine and chemokine. Oxidative stress is also associated with renal tissue damage and dysfunction via attacking, denaturing, and modifying the structural and functional molecules and by regulating signal transduction pathways. There are a number of transcription factors which can be activated under oxidative stress, including NF-κB, AP-1, p53, HIF-1α, PPAR-γ, β-catenin/Wnt, and Nrf2 (Reuter, Gupta, Chaturvedi, & Aggarwal, 2010). Activation of these transcription factors results in the expression of hundreds of genes, the gene products of which include inflammatory cytokines, chemokines and anti-inflammatory molecules (Wojdasiewicz, Poniatowski, & Szukiewicz, 2014). Experimental AKI models are pivotal for understanding the mechanism of AKI and for developing effective therapies.

In this study, *in vitro*, AKI was induced by hypoxia/reoxygenation in renal tubular epithelial cell. *In vivo* experimental AKI was successfully induced by renal pedicle clamping for 45 minutes, followed by specified reperfusion time period (1 hour, 6 hours or 24 hours). The plasma creatinine level, as a marker of renal function, was significantly higher in the I/R group than in the sham-operation group. I/R caused an increase in peroxynitrite formation and lipid peroxidation, showing the increased oxidative stress damage. There was a significant increase in pro-inflammatory cytokines (MCP-1, IL-6) mRNA and protein levels in kidneys subjected to I/R. In addition, plasma neutrophil gelatinase-associated lipocalin (NGAL) levels were examined. NGAL is a biomarker of tubular damage, and the significant

increase in the I/R group suggested that there was acute tubular damage induced by renal I/R. Histological analysis of H&E-stained kidney sections revealed cell swelling in renal tubular cells and massive blood cell infiltration in the medulla region in the kidney subjected to I/R. I/R has been shown to rapidly induce structural and functional alterations in kidneys by increasing oxidative stress and inflammatory response.

5.1.2 Downregulation of CBS and CSE mediated H_2S production contributes to the increased pro-inflammatory cytokine expression in the kidney upon I/R injury

Inflammation plays a detrimental role in the pathophysiology of AKI following renal I/R. I/R stimulates the synthesis of pro-inflammatory cytokines, such as TNF-α, IL-6, MCP-1 in the kidney. The activation of multiple pro-inflammatory cytokines and the infiltration of inflammatory cells in the kidney may activate several pathways and lead to inflammation-mediated tissue injury. It has been demonstrated that MCP-1 plays an important role in the recruitment of monocytes and/or macrophages to inflamed tissue. Our previous study clearly demonstrated that there was enhanced MCP-1 expression in rat kidneys during I/R injury which was mediated by NF-κB activation (Sung et al., 2002). Apart from MCP-1, another cytokine, IL-6, was a major contributor of renal I/R injury and inflammation (Patel et al., 2005). Recently, hydrogen sulfide (H_2S) has emerged as an important endogenous gasotransmitter involved in numerous physiological and pathological processes. However, the role of H_2S in inflammation remains poorly elucidated with both pro- and anti-inflammatory actions of this gas being described. By using the same model, our previous study has revealed that CBS-mediated renal H_2S production is decreased during renal I/R, suggesting that maintenance of tissue H_2S levels may offer a renal protective effect against

kidney I/R injury (Xu et al., 2009). Therefore, the first part of my study was aimed at investigating the role of H_2S in renal I/R-induced inflammation and the mechanisms by which H_2S might exert anti-inflammatory and renal protective effects. Our results demonatrated that I/R caused a significant reduction of H_2S-producing enzymes, CBS and CSE, in mRNA as well as in protein levels in the kidney. There was a marked decrease in H_2S production in the same kidney tissue. In accordance with our findings in the rat (*in vivo*) model, reduction of CBS and CSE expression was also observed in proximal tubular cells in the I/R model (*in vitro*). Several lines of evidence from my study suggested that changes in CBS and CSE-mediated H_2S production had a profound effect on I/R-induced cytokine expression. First, low CBS and CSE expression and decreased H_2S production were inversely associated with I/R-induced expression of MCP-1 and IL-6 in the kidney. Secondly, the expression of CBS and CSE was decreased in proximal tubular cells subjected to simulated I/R while the expression of cytokines was elevated in these cells. The H_2S donor effectively attenuated I/R-induced cytokine expression in tubular cells. Third, inhibition of H_2S production by CBS and CSE inhibitors also led to an increase in cytokine expression in tubular cells. Taken together, the first part of my study identified, for the first time, that reduced endogenous H_2S generation was responsible for an inflammatory response and renal injury in the kidney upon I/R insult. It was confirmed in our renal I/R model that H_2S, as a novel gaseous transmitter, exerted anti-inflammatory effect in pathophysiological conditions. Thus, H_2S-based therapeutics (e.g. H_2S-releasing compounds) may represent a potential pharmacological approach in the treatment of inflammation of AKI. It is known that I/R decreases renal blood flow with renal vasoconstriction (Sutton, Fisher, & Molitoris, 2002). H_2S has been known to act as a vasodilator mediated by the opening of KATP channels in

vascular smooth muscle cells (Zhao, Zhang, Lu, & Wang, 2001). It has been reported that H_2S treatment attenuates kidney injury in angiotensin II-induced renal disease in rats (Snijder et al., 2014). Thus, it is possible that the vasodilator action of H_2S may be involved in the accelerated renal recovery from I/R-induced AKI. By targeting biosynthesis and metabolism pathways, the therapeutic effects of H_2S can be established. A lot of research is ongoing to develop novel H_2S donors, and more research will aid in determining suitable routes of administration and dosages of H_2S in treating AKI-induced inflammation.

5.1.3 Tyrosol treatment protects against I/R-induced AKI through inhibition of iNOS-mediated oxidative stress

Oxidative stress, like inflammation, plays an important role in different types of renal disorders, including I/R-induced AKI. There are several kinds of evidence implicating ROS production during renal I/R. Our previous study suggested, by measuring the end product MDA, that there was a significant increase in ROS-mediated lipid peroxidation production (Prathapasinghe et al., 2008). Nitric oxide (NO), produced via inducible nitric oxide synthase (iNOS) from L-arginine, is implicated in the pathophysiology of renal I/R (Chatterjee et al., 2002). Once formed, NO will combine with superoxide spontaneously to form a strong oxidant, peroxynitrite, and lead to further oxidative stress damage. Oxidative stress can lead to direct tissue damage and stimulate inflammatory reactions, which, in turn, contribute to tissue injury. Therefore, oxidative stress is thought to be one of important mechanisms for I/R-induced AKI. Tyrosol has been shown to be able to exert antioxidant activity in *in vitro* studies. In the second part of my study, our aim was to investigate whether tyrosol administration had a protective effect on I/R-induced oxidative stress injury in kidneys, and also to better understand the mechanisms underlying this protective effect. The results obtained from the second study revealed that ischemia (45 minutes)

followed by reperfusion (6 hours) led to impaired kidney function in rats. Kidney dysfunction was accompanied by increased iNOS-mediated NO production, increased protein nitration and lipid peroxidation. We previously reported that I/R resulted in an activation of NF-κB (Sung et al., 2002). This transcription factor regulates the expression of many inflammatory genes including iNOS (Sung et al., 2002). Indeed, we observed a significant increase in NF-κB/DNA binding activity in the kidneys subjected to I/R. Administration of tyrosol not only attenuated I/R-induced NF-κB activation but also reduced iNOS expression in the kidney. Collectively, our results from the present study demonstrate that tyrosol exerts a renalprotective effect through attenuation of oxidative stress in an I/R-induced AKI model in rats, and it may be considered as an antioxidant therapy for AKI. Our findings suggest that tyrosol intake or administration of a dietary supplement may provide a protective effect against I/R-induced AKI. These results also provide an insight into the renal protective activity associated with the consumption of food such as olive oil, which contains tyrosol as a major constituent. The findings from my present study underscore the importance of continuing research into the effect and underlying mechanisms of olive oil phenolics and into other natural health products sharing a similar mechanism of action. It will link the diet with a healthy life in humans.

In conclusion, the present study has illustrated that there are oxidative stress and inflammatory responses in the kidney during I/R-induced AKI. I/R-induced oxidative stress and inflammatory responses represent two important mechanisms for AKI. H_2S exerts an anti-inflammatory effect on the inflammation after I/R insult in kidneys. Tyrosol administration is able to protect the kidney from oxidative stress-mediated structural and

functional impairment. Understanding the molecular mechanisms of renal injury during I/R may help identify more therapeutic targets and potential approaches for AKI.

5.2 Limitations and future perspectives

5.2.1 Study 1

5.2.1.1 Mechanisms by which H_2S acts anti-inflammatory effect against renal I/R-induced AKI

In the present study, inflammatory response was observed in I/R rat kidneys. H_2S generation was inversely correlated with the level of pro-inflammatory cytokines, IL-6 and MCP-1. Inhibitors of H_2S-producing enzymes and H_2S donors were used to further confirm the association between H_2S and pro-inflammatory cytokines. The inhibition of H_2S-producing enzymes resulted in the increased production of pro-inflammatory cytokines, and the administration of H_2S donors appears to lower the levels of inflammation. The present study provides supporting evidence that H_2S displays anti-inflammatory activity against I/R-induced renal injury. Consistently, other recent studies have also demonstrated that administration of sulfide prior to ischemia or prior to reperfusion reduced pro-inflammatory cytokine levels. However, large gaps remain in the mechanistic understanding of these protective effects occur. The detailed mechanisms or signaling pathways involved are worthy of further investigation. It has been shown that H_2S generated by CSE stimulated DNA binding and gene activation of NF-κB in CSE-deleted mice (N. Sen et al., 2012). H_2S has been shown to mediate cardioprotection through Nrf2 signaling in the setting of ischemic-induced heart failure (Calvert et al., 2009). The anti-inflammatory effect of H_2S was reported

to be related to BK channel signaling (Zuidema et al., 2010). Therefore, in the renal I/R-induced AKI model, the regulatory mechanisms of H_2S on inflammation remain to be elucidated.

5.2.1.2 Effect of H_2S on distant organ injury induced by renal I/R

Experimental evidence has clarified that there are distant organ dysfunctions induced by I/R-induced AKI, such as liver injury. However, the mechanisms by which AKI causes distant organ injury remain poorly understood. We and other investigators have observed an increased expression of inflammatory cytokines in the liver of rats subjected to renal I/R (Golab et al., 2009). In my present study, we have identified the relationship between H_2S and pro-inflammatory cytokines in the kidney during I/R. The involvement of H_2S and inflammatory response in liver injury during renal I/R remains to be investigated in future studies. In addition, accurate identification of the distant organ effect regulation will be critical in developing targeted therapies to improve outcomes in AKI.

5.2.1.3 Effect of combination of H_2S donor and tyrosol as antioxidants on renal I/R-induced damage

Recent studies suggest that H_2S exhibits potent antioxidant capacity (Kimura et al., 2009; Peake et al., 2013). Our study demonstrated that tyrosol treatment was able to suppress oxidative stress damage induced by renal I/R. However, whether the combination of H_2S donor and tyrosol as antoxidants has a protective effect against renal I/R injury has not been studied yet. Therefore, the effect of the combination of H_2S donor and tyrosol on renal I/R oxidative stressdamage warrants further investigation.

5.2.2 Study 2

5.2.2.1 Effect of tyrosol treatment on inflammatory response and apoptosis in the kidney during renal I/R

In the present study, we investigated the antioxidant effect of tyrosol. As a dietary polyphenol, in addition to its antioxidant effect, tyrosol has also been reported to possess anti-apoptotic and anti-inflammatory effects (Dewapriya et al., 2013; Je et al., 2015). Recently, virgin olive oil, rich in polyphenols (e.g. tyrosol, hydroxytyrosol), was shown to be effective in lowering the inflammatory markers in stable coronary heart disease patients (Fito et al., 2008). Olive oil phenolic extract exerted anti-apoptotic effects in H_2O_2-induced apoptotic cell death in HeLa cells (Erol-Dayi, Arda, & Erdem, 2012). Since oxidative stress, inflammation and apoptosis are all involved in the mechanisms underlying I/R injury, the role of tyrosol in renal I/R may be more complex than the antioxidant effect. The complexity is worthy of further investigation. However, there are only limited available studies using tyrosol itself instead of in the form of olive oil. It may be possible that tyrosol exhibits anti-inflammatory and anti-apoptocic activities during I/R process in kidneys. Future studies evaluating tyrosol's capacity to manage renal inflammation and apoptosis are therefore warranted.

5.2.2.2 Effect of chronic administration of tyrosol on kidney disease

Although our study has demonstrated the beneficial effect of a one-time administration of tyrosol on the AKI animal model, its long-term effects on chronic kidney disease (CKD) are not clear. Renal I/R induces tubular epithelial cell structural disruption and renal function

impairment. The injured epithelial cells can be restored through proliferation within several days after renal blood flow recovery (Duffield et al., 2005). However, if the damage caused by AKI persists or the recovery is inadequate, it can eventually progress to CKD (Venkatachalam et al., 2010). Some accelerating factors, such as ROS, are associated with the progression of AKI to CKD. The one-time administration of tyrosol in my study has been shown to combat the ROS production during I/R-induced AKI. However, the effect of tyrosol during the recovery phase of AKI has not been studied yet. The major therapeutic goals are to prevent further damage, improve post-injury survival, and prevent or delay the progression of CKD. Therefore, chronic tyrosol administration may exert a long term protective effect against AKI-induced chronic renal diseases.

Chapter 6. REFERENCES

Abe, K., & Kimura, H. (1996). The possible role of hydrogen sulfide as an endogenous neuromodulator. *J Neurosci, 16*(3), 1066-1071.

Agarwal, I., Kirubakaran, C., & Markandeyulu, V. (2004). Clinical profile and outcome of acute renal failure in South Indian children. *J Indian Med Assoc, 102*(7), 353-354, 356.

Akcay, A., Nguyen, Q., & Edelstein, C. L. (2009). Mediators of inflammation in acute kidney injury. *Mediators Inflamm, 2009*, 137072.

Amann, B., Tinzmann, R., & Angelkort, B. (2003). ACE inhibitors improve diabetic nephropathy through suppression of renal MCP-1. *Diabetes Care, 26*(8), 2421-2425.

Anders, H. J., Vielhauer, V., & Schlondorff, D. (2003). Chemokines and chemokine receptors are involved in the resolution or progression of renal disease. *Kidney Int, 63*(2), 401-415.

Aragno, M., Cutrin, J. C., Mastrocola, R., Perrelli, M. G., Restivo, F., Poli, G., Danni, O., & Boccuzzi, G. (2003). Oxidative stress and kidney dysfunction due to ischemia/reperfusion in rat: attenuation by dehydroepiandrosterone. *Kidney Int, 64*(3), 836-843.

Awad, A. S., Ye, H., Huang, L., Li, L., Foss, F. W., Jr., Macdonald, T. L., Lynch, K. R., & Okusa, M. D. (2006). Selective sphingosine 1-phosphate 1 receptor activation reduces ischemia-reperfusion injury in mouse kidney. *Am J Physiol Renal Physiol, 290*(6), F1516-1524.

Badrichani, A. Z., Stroka, D. M., Bilbao, G., Curiel, D. T., Bach, F. H., & Ferran, C. (1999). Bcl-2 and Bcl-XL serve an anti-inflammatory function in endothelial cells through inhibition of NF-kappaB. *J Clin Invest, 103*(4), 543-553.

Basile, D. P., Anderson, M. D., & Sutton, T. A. (2012). Pathophysiology of acute kidney injury. *Compr Physiol, 2*(2), 1303-1353.

Beauchamp, R. O., Jr., Bus, J. S., Popp, J. A., Boreiko, C. J., & Andjelkovich, D. A. (1984). A critical review of the literature on hydrogen sulfide toxicity. *Crit Rev Toxicol, 13*(1), 25-97.

Beckman, J. S., Chen, J., Ischiropoulos, H., & Crow, J. P. (1994). Oxidative chemistry of peroxynitrite. *Methods Enzymol, 233*, 229-240.

Bellomo, R., Kellum, J. A., & Ronco, C. (2012). Acute kidney injury. *Lancet, 380*(9843), 756-766.

Beltowski, J. (2010). Hypoxia in the renal medulla: implications for hydrogen sulfide signaling. *J Pharmacol Exp Ther, 334*(2), 358-363.

Berlett, B. S., & Stadtman, E. R. (1997). Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem, 272*(33), 20313-20316.

Bertelli, A. A., Migliori, M., Panichi, V., Longoni, B., Origlia, N., Ferretti, A., Cuttano, M. G., & Giovannini, L. (2002). Oxidative stress and inflammatory reaction modulation by white wine. *Ann N Y Acad Sci, 957*, 295-301.

Betteridge, D. J. (2000). What is oxidative stress? *Metabolism, 49*(2 Suppl 1), 3-8.

Bhatia, M., Sidhapuriwala, J. N., Ng, S. W., Tamizhselvi, R., & Moochhala, S. M. (2008). Pro-inflammatory effects of hydrogen sulphide on substance P in caerulein-induced acute pancreatitis. *J Cell Mol Med, 12*(2), 580-590.

Bogdan, C., & Nathan, C. (1993). Modulation of macrophage function by transforming growth factor beta, interleukin-4, and interleukin-10. *Ann N Y Acad Sci, 685*, 713-739.

Boniface, K., Bak-Jensen, K. S., Li, Y., Blumenschein, W. M., McGeachy, M. J., McClanahan, T. K., McKenzie, B. S., Kastelein, R. A., Cua, D. J., & de Waal Malefyt, R. (2009). Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. *J Exp Med, 206*(3), 535-548.

Bonventre, J. V., & Yang, L. (2011). Cellular pathophysiology of ischemic acute kidney injury. *J Clin Invest, 121*(11), 4210-4221.

Bonventre, J. V., & Zuk, A. (2004). Ischemic acute renal failure: an inflammatory disease? *Kidney Int, 66*(2), 480-485.

Bos, E. M., Wang, R., Snijder, P. M., Boersema, M., Damman, J., Fu, M., Moser, J., Hillebrands, J. L., Ploeg, R. J., Yang, G., Leuvenink, H. G., & van Goor, H. (2013). Cystathionine gamma-lyase protects against renal ischemia/reperfusion by modulating oxidative stress. *J Am Soc Nephrol, 24*(5), 759-770.

Bouayed, J., & Bohn, T. (2010). Exogenous antioxidants - Double-edged swords in cellular redox state: Health beneficial effects at physiologic doses versus deleterious effects at high doses. *Oxid Med Cell Longev, 3*(4), 228-237.

Brady, H. R., & Singer, G. G. (1995). Acute renal failure. *Lancet, 346*(8989), 1533-1540.

Breggia, A. C., & Himmelfarb, J. (2008). Primary mouse renal tubular epithelial cells have variable injury tolerance to ischemic and chemical mediators of oxidative stress. *Oxid Med Cell Longev, 1*(1), 33-38.

Briante, R., Febbraio, F., & Nucci, R. (2003). Antioxidant properties of low molecular weight phenols present in the mediterranean diet. *J Agric Food Chem, 51*(24), 6975-6981.

Buckland, G., Mayen, A. L., Agudo, A., Travier, N., Navarro, C., Huerta, J. M., Chirlaque, M. D., Barricarte, A., Ardanaz, E., Moreno-Iribas, C., Marin, P., Quiros, J. R., Redondo, M. L., Amiano, P., Dorronsoro, M., Arriola, L., Molina, E., Sanchez, M. J., & Gonzalez, C. A. (2012). Olive oil intake and mortality within the Spanish population (EPIC-Spain). *Am J Clin Nutr, 96*(1), 142-149.

Burney, S., Niles, J. C., Dedon, P. C., & Tannenbaum, S. R. (1999). DNA damage in deoxynucleosides and oligonucleotides treated with peroxynitrite. *Chem Res Toxicol, 12*(6), 513-520.

Calvert, J. W., Jha, S., Gundewar, S., Elrod, J. W., Ramachandran, A., Pattillo, C. B., Kevil, C. G., & Lefer, D. J. (2009). Hydrogen sulfide mediates cardioprotection through Nrf2 signaling. *Circ Res, 105*(4), 365-374.

Cao, C. C., Ding, X. Q., Ou, Z. L., Liu, C. F., Li, P., Wang, L., & Zhu, C. F. (2004). In vivo transfection of NF-kappaB decoy oligodeoxynucleotides attenuate renal ischemia/reperfusion injury in rats. *Kidney Int, 65*(3), 834-845.

Carballal, S., Trujillo, M., Cuevasanta, E., Bartesaghi, S., Moller, M. N., Folkes, L. K., Garcia-Bereguiain, M. A., Gutierrez-Merino, C., Wardman, P., Denicola, A., Radi, R., & Alvarez, B.

(2010). Reactivity of hydrogen sulfide with peroxynitrite and other oxidants of biological interest. *Free Radic Biol Med, 50*(1), 196-205.

Carrasco-Pancorbo, A., Cerretani, L., Bendini, A., Segura-Carretero, A., Del Carlo, M., Gallina-Toschi, T., Lercker, G., Compagnone, D., & Fernandez-Gutierrez, A. (2005). Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil. *J Agric Food Chem, 53*(23), 8918-8925.

Caruso, D., Visioli, F., Patelli, R., Galli, C., & Galli, G. (2001). Urinary excretion of olive oil phenols and their metabolites in humans. *Metabolism, 50*(12), 1426-1428.

Chan, M. V., & Wallace, J. L. (2013). Hydrogen sulfide-based therapeutics and gastrointestinal diseases: translating physiology to treatments. *Am J Physiol Gastrointest Liver Physiol, 305*(7), G467-473.

Charo, I. F., & Taubman, M. B. (2004). Chemokines in the pathogenesis of vascular disease. *Circ Res, 95*(9), 858-866.

Chatterjee, P. K. (2007). Novel pharmacological approaches to the treatment of renal ischemia-reperfusion injury: a comprehensive review. *Naunyn Schmiedebergs Arch Pharmacol, 376*(1-2), 1-43.

Chatterjee, P. K., Cuzzocrea, S., Brown, P. A., Zacharowski, K., Stewart, K. N., Mota-Filipe, H., & Thiemermann, C. (2000). Tempol, a membrane-permeable radical scavenger, reduces oxidant stress-mediated renal dysfunction and injury in the rat. *Kidney Int, 58*(2), 658-673.

Chatterjee, P. K., di Villa Bianca, R. D., Sivarajah, A., McDonald, M. C., Cuzzocrea, S., & Thiemermann, C. (2003). Pyrrolidine dithiocarbamate reduces renal dysfunction and injury caused by ischemia/reperfusion of the rat kidney. *Eur J Pharmacol, 482*(1-3), 271-280.

Chatterjee, P. K., Patel, N. S., Kvale, E. O., Cuzzocrea, S., Brown, P. A., Stewart, K. N., Mota-Filipe, H., & Thiemermann, C. (2002). Inhibition of inducible nitric oxide synthase reduces renal ischemia/reperfusion injury. *Kidney Int, 61*(3), 862-871.

Chatterjee, P. K., Patel, N. S., Sivarajah, A., Kvale, E. O., Dugo, L., Cuzzocrea, S., Brown, P. A., Stewart, K. N., Mota-Filipe, H., Britti, D., Yaqoob, M. M., & Thiemermann, C. (2003). GW274150, a potent and highly selective inhibitor of iNOS, reduces experimental renal ischemia/reperfusion injury. *Kidney Int, 63*(3), 853-865.

Chawla, L. S., & Kimmel, P. L. (2012). Acute kidney injury and chronic kidney disease: an integrated clinical syndrome. *Kidney Int, 82*(5), 516-524.

Chen, Q., Jin, M., Yang, F., Zhu, J., Xiao, Q., & Zhang, L. (2013). Matrix metalloproteinases: inflammatory regulators of cell behaviors in vascular formation and remodeling. *Mediators Inflamm, 2013*, 928315.

Chen, Q., Vazquez, E. J., Moghaddas, S., Hoppel, C. L., & Lesnefsky, E. J. (2003). Production of reactive oxygen species by mitochondria: central role of complex III. *J Biol Chem, 278*(38), 36027-36031.

Chernyak, B. V., Izyumov, D. S., Lyamzaev, K. G., Pashkovskaya, A. A., Pletjushkina, O. Y., Antonenko, Y. N., Sakharov, D. V., Wirtz, K. W., & Skulachev, V. P. (2006). Production of reactive oxygen species in mitochondria of HeLa cells under oxidative stress. *Biochim Biophys Acta, 1757*(5-6), 525-534.

Choi, J. Y., Nam, S. A., Jin, D. C., Kim, J., & Cha, J. H. (2012). Expression and cellular localization of inducible nitric oxide synthase in lipopolysaccharide-treated rat kidneys. *J Histochem Cytochem, 60*(4), 301-315.

Chung, H. Y., Cesari, M., Anton, S., Marzetti, E., Giovannini, S., Seo, A. Y., Carter, C., Yu, B. P., & Leeuwenburgh, C. (2009). Molecular inflammation: underpinnings of aging and age-related diseases. *Ageing Res Rev, 8*(1), 18-30.

Coca, S. G., Singanamala, S., & Parikh, C. R. (2012). Chronic kidney disease after acute kidney injury: a systematic review and meta-analysis. *Kidney Int, 81*(5), 442-448.

Coca, S. G., Yalavarthy, R., Concato, J., & Parikh, C. R. (2008). Biomarkers for the diagnosis and risk stratification of acute kidney injury: a systematic review. *Kidney Int, 73*(9), 1008-1016.

Cooke, M. S., Evans, M. D., Dizdaroglu, M., & Lunec, J. (2003). Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J, 17*(10), 1195-1214.

Covas, M. I., Miro-Casas, E., Fito, M., Farre-Albadalejo, M., Gimeno, E., Marrugat, J., & De La Torre, R. (2003). Bioavailability of tyrosol, an antioxidant phenolic compound present in wine and olive oil, in humans. *Drugs Exp Clin Res, 29*(5-6), 203-206.

Cray, C., Zaias, J., & Altman, N. H. (2009). Acute phase response in animals: a review. *Comp Med, 59*(6), 517-526.

de Haij, S., Woltman, A. M., Bakker, A. C., Daha, M. R., & van Kooten, C. (2002). Production of inflammatory mediators by renal epithelial cells is insensitive to glucocorticoids. *Br J Pharmacol, 137*(2), 197-204.

de la Puerta, R., Martinez Dominguez, M. E., Ruiz-Gutierrez, V., Flavill, J. A., & Hoult, J. R. (2001). Effects of virgin olive oil phenolics on scavenging of reactive nitrogen species and upon nitrergic neurotransmission. *Life Sci, 69*(10), 1213-1222.

de la Puerta, R., Ruiz Gutierrez, V., & Hoult, J. R. (1999). Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. *Biochem Pharmacol, 57*(4), 445-449.

de la Torre, R. (2008). Bioavailability of olive oil phenolic compounds in humans. *Inflammopharmacology, 16*(5), 245-247.

de Lorgeril, M., Salen, P., Martin, J. L., Monjaud, I., Delaye, J., & Mamelle, N. (1999). Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. *Circulation, 99*(6), 779-785.

De Vriese, A. S., Colardyn, F. A., Philippe, J. J., Vanholder, R. C., De Sutter, J. H., & Lameire, N. H. (1999). Cytokine removal during continuous hemofiltration in septic patients. *J Am Soc Nephrol, 10*(4), 846-853.

Devarajan, P. (2006). Update on mechanisms of ischemic acute kidney injury. *J Am Soc Nephrol, 17*(6), 1503-1520.

Dewapriya, P., Himaya, S. W., Li, Y. X., & Kim, S. K. (2013). Tyrosol exerts a protective effect against dopaminergic neuronal cell death in in vitro model of Parkinson's disease. *Food Chem, 141*(2), 1147-1157.

Di Carlo, G., Mascolo, N., Izzo, A. A., & Capasso, F. (1999). Flavonoids: old and new aspects of a class of natural therapeutic drugs. *Life Sci, 65*(4), 337-353.

Di Giorno, C., Pinheiro, H. S., Heinke, T., Franco, M. F., Galante, N. Z., Pacheco-Silva, A., & Camara, N. O. (2006). Beneficial effect of N-acetyl-cysteine on renal injury triggered by ischemia and reperfusion. *Transplant Proc, 38*(9), 2774-2776.

Dinarello, C. A. (1997). Induction of interleukin-1 and interleukin-1 receptor antagonist. *Semin Oncol, 24*(3 Suppl 9), S9-81-S89-93.

Dinarello, C. A. (2000). Proinflammatory cytokines. *Chest, 118*(2), 503-508.

Distrutti, E., Sediari, L., Mencarelli, A., Renga, B., Orlandi, S., Antonelli, E., Roviezzo, F., Morelli, A., Cirino, G., Wallace, J. L., & Fiorucci, S. (2006). Evidence that hydrogen sulfide exerts antinociceptive effects in the gastrointestinal tract by activating KATP channels. *J Pharmacol Exp Ther, 316*(1), 325-335.

Docimo, S. G. (2006). *The Kelalis-King-Belman Textbook of Clinical Pediatric Urology*: CRC Press.

Doi, K., Suzuki, Y., Nakao, A., Fujita, T., & Noiri, E. (2004). Radical scavenger edaravone developed for clinical use ameliorates ischemia/reperfusion injury in rat kidney. *Kidney Int, 65*(5), 1714-1723.

Dongo, E., Hornyak, I., Benko, Z., & Kiss, L. (2011). The cardioprotective potential of hydrogen sulfide in myocardial ischemia/reperfusion injury (review). *Acta Physiol Hung, 98*(4), 369-381.

Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiol Rev, 82*(1), 47-95.

Duffield, J. S., Park, K. M., Hsiao, L. L., Kelley, V. R., Scadden, D. T., Ichimura, T., & Bonventre, J. V. (2005). Restoration of tubular epithelial cells during repair of the postischemic kidney occurs independently of bone marrow-derived stem cells. *J Clin Invest, 115*(7), 1743-1755.

El-Zoghby, Z. M., Stegall, M. D., Lager, D. J., Kremers, W. K., Amer, H., Gloor, J. M., & Cosio, F. G. (2009). Identifying specific causes of kidney allograft loss. *Am J Transplant, 9*(3), 527-535.

Ellenberger, C., Schweizer, A., Diaper, J., Kalangos, A., Murith, N., Katchatourian, G., Panos, A., & Licker, M. (2006). Incidence, risk factors and prognosis of changes in serum creatinine early after aortic abdominal surgery. *Intensive Care Med, 32*(11), 1808-1816.

Elrod, J. W., Calvert, J. W., Morrison, J., Doeller, J. E., Kraus, D. W., Tao, L., Jiao, X., Scalia, R., Kiss, L., Szabo, C., Kimura, H., Chow, C. W., & Lefer, D. J. (2007). Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. *Proc Natl Acad Sci U S A, 104*(39), 15560-15565.

England, K., & Cotter, T. G. (2005). Direct oxidative modifications of signalling proteins in mammalian cells and their effects on apoptosis. *Redox Rep, 10*(5), 237-245.

Enokido, Y., Suzuki, E., Iwasawa, K., Namekata, K., Okazawa, H., & Kimura, H. (2005). Cystathionine beta-synthase, a key enzyme for homocysteine metabolism, is preferentially expressed in the radial glia/astrocyte lineage of developing mouse CNS. *FASEB J, 19*(13), 1854-1856.

Epstein, F. H. (1997). Oxygen and renal metabolism. *Kidney Int, 51*(2), 381-385.

Erol-Dayi, O., Arda, N., & Erdem, G. (2012). Protective effects of olive oil phenolics and gallic acid on hydrogen peroxide-induced apoptosis. *Eur J Nutr, 51*(8), 955-960.

Ferran, C., Stroka, D. M., Badrichani, A. Z., Cooper, J. T., Wrighton, C. J., Soares, M., Grey, S. T., & Bach, F. H. (1998). A20 inhibits NF-kappaB activation in endothelial cells without sensitizing to tumor necrosis factor-mediated apoptosis. *Blood, 91*(7), 2249-2258.

Fiaccadori, E., Parenti, E., & Maggiore, U. (2008). Nutritional support in acute kidney injury. *J Nephrol, 21*(5), 645-656.

Fito, M., Cladellas, M., de la Torre, R., Marti, J., Munoz, D., Schroder, H., Alcantara, M., Pujadas-Bastardes, M., Marrugat, J., Lopez-Sabater, M. C., Bruguera, J., & Covas, M. I. (2008). Anti-inflammatory effect of virgin olive oil in stable coronary disease patients: a randomized, crossover, controlled trial. *Eur J Clin Nutr, 62*(4), 570-574.

Forstermann, U., Boissel, J. P., & Kleinert, H. (1998). Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). *FASEB J, 12*(10), 773-790.

Frankel, E. N. (2010). Chemistry of extra virgin olive oil: adulteration, oxidative stability, and antioxidants. *J Agric Food Chem, 58*(10), 5991-6006.

Frankel, E. N., Kanner, J., German, J. B., Parks, E., & Kinsella, J. E. (1993). Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet, 341*(8843), 454-457.

Friedewald, J. J., & Rabb, H. (2004). Inflammatory cells in ischemic acute renal failure. *Kidney Int, 66*(2), 486-491.

Fry, A. C., & Farrington, K. (2006). Management of acute renal failure. *Postgrad Med J, 82*(964), 106-116.

Fu, M., Zhang, W., Wu, L., Yang, G., Li, H., & Wang, R. (2012). Hydrogen sulfide (H2S) metabolism in mitochondria and its regulatory role in energy production. *Proc Natl Acad Sci U S A, 109*(8), 2943-2948.

Fu, Z., Liu, X., Geng, B., Fang, L., & Tang, C. (2008). Hydrogen sulfide protects rat lung from ischemia-reperfusion injury. *Life Sci, 82*(23-24), 1196-1202.

Fubini, B., & Hubbard, A. (2003). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis. *Free Radic Biol Med, 34*(12), 1507-1516.

Funk, C. D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science, 294*(5548), 1871-1875.

Furuichi, K., Wada, T., Iwata, Y., Sakai, N., Yoshimoto, K., Kobayashi Ki, K., Mukaida, N., Matsushima, K., & Yokoyama, H. (2002). Administration of FR167653, a new anti-inflammatory compound, prevents renal ischaemia/reperfusion injury in mice. *Nephrol Dial Transplant, 17*(3), 399-407.

Furusu, A., Miyazaki, M., Abe, K., Tsukasaki, S., Shioshita, K., Sasaki, O., Miyazaki, K., Ozono, Y., Koji, T., Harada, T., Sakai, H., & Kohno, S. (1998). Expression of endothelial and inducible nitric oxide synthase in human glomerulonephritis. *Kidney Int, 53*(6), 1760-1768.

Gadalla, M. M., & Snyder, S. H. (2010). Hydrogen sulfide as a gasotransmitter. *J Neurochem, 113*(1), 14-26.

Gheibi, S., Aboutaleb, N., Khaksari, M., Kalalian-Moghaddam, H., Vakili, A., Asadi, Y., Mehrjerdi, F. Z., & Gheibi, A. (2014). Hydrogen sulfide protects the brain against ischemic reperfusion injury in a transient model of focal cerebral ischemia. *J Mol Neurosci, 54*(2), 264-270.

Giovannini, C., Straface, E., Modesti, D., Coni, E., Cantafora, A., De Vincenzi, M., Malorni, W., & Masella, R. (1999). Tyrosol, the major olive oil biophenol, protects against oxidized-LDL-induced injury in Caco-2 cells. *J Nutr, 129*(7), 1269-1277.

Giovannini, L., Migliori, M., Longoni, B. M., Das, D. K., Bertelli, A. A., Panichi, V., Filippi, C., & Bertelli, A. (2001). Resveratrol, a polyphenol found in wine, reduces ischemia reperfusion injury in rat kidneys. *J Cardiovasc Pharmacol, 37*(3), 262-270.

Giugliano, D., & Esposito, K. (2008). Mediterranean diet and metabolic diseases. *Curr Opin Lipidol, 19*(1), 63-68.

Goicoechea, M., de Vinuesa, S. G., Gomez-Campdera, F., Aragoncillo, I., Verdalles, U., Mosse, A., & Luno, J. (2008). Serum fibrinogen levels are an independent predictor of mortality in patients with chronic kidney disease (CKD) stages 3 and 4. *Kidney Int Suppl*(111), S67-70.

Golab, F., Kadkhodaee, M., Zahmatkesh, M., Hedayati, M., Arab, H., Schuster, R., Zahedi, K., Lentsch, A. B., & Soleimani, M. (2009). Ischemic and non-ischemic acute kidney injury cause hepatic damage. *Kidney Int, 75*(8), 783-792.

Goligorsky, M. S., Brodsky, S. V., & Noiri, E. (2002). Nitric oxide in acute renal failure: NOS versus NOS. *Kidney Int, 61*(3), 855-861.

Gottmann, U., Mueller-Falcke, A., Schnuelle, P., Birck, R., Nickeleit, V., van der Woude, F. J., Yard, B. A., & Braun, C. (2007). Influence of hypersulfated and low molecular weight heparins on ischemia/reperfusion: injury and allograft rejection in rat kidneys. *Transpl Int, 20*(6), 542-549.

Gouvea Junior, V. T., Caporossi, C., Salomao, A. B., Cortes, E., Munhoz, M. F., & Nascimento, J. E. (2011). Effect of glutamine on the total antioxidant system of rats subjected to renal ischemia and reperfusion. *Acta Cir Bras, 26*(6), 445-450.

Gris, E. F., Mattivi, F., Ferreira, E. A., Vrhovsek, U., Filho, D. W., Pedrosa, R. C., & Bordignon-Luiz, M. T. (2011). Stilbenes and tyrosol as target compounds in the assessment of antioxidant and hypolipidemic activity of Vitis vinifera red wines from southern Brazil. *J Agric Food Chem, 59*(14), 7954-7961.

Gueler, F., Gwinner, W., Schwarz, A., & Haller, H. (2004). Long-term effects of acute ischemia and reperfusion injury. *Kidney Int, 66*(2), 523-527.

Gueler, F., Park, J. K., Rong, S., Kirsch, T., Lindschau, C., Zheng, W., Elger, M., Fiebeler, A., Fliser, D., Luft, F. C., & Haller, H. (2007). Statins attenuate ischemia-reperfusion injury by inducing heme oxygenase-1 in infiltrating macrophages. *Am J Pathol, 170*(4), 1192-1199.

Guijarro, C., & Egido, J. (2001). Transcription factor-kappa B (NF-kappa B) and renal disease. *Kidney Int, 59*(2), 415-424.

Guo, R., Wu, K., Chen, J., Mo, L., Hua, X., Zheng, D., Chen, P., Chen, G., Xu, W., & Feng, J. (2013). Exogenous hydrogen sulfide protects against doxorubicin-induced inflammation and

cytotoxicity by inhibiting p38MAPK/NFkappaB pathway in H9c2 cardiac cells. *Cell Physiol Biochem, 32*(6), 1668-1680.

Gutteridge, J. M., & Halliwell, B. (1990). The measurement and mechanism of lipid peroxidation in biological systems. *Trends Biochem Sci, 15*(4), 129-135.

Gyory, A. Z., & Kinne, R. (1971). Energy source for transepithelial sodium transport in rat renal proximal tubules. *Pflugers Arch, 327*(3), 234-260.

Hagemann, J. H., Thomasova, D., Mulay, S. R., & Anders, H. J. (2013). Nrf2 signalling promotes ex vivo tubular epithelial cell survival and regeneration via murine double minute (MDM)-2. *Nephrol Dial Transplant, 28*(8), 2028-2037.

Han, W. K., Bailly, V., Abichandani, R., Thadhani, R., & Bonventre, J. V. (2002). Kidney Injury Molecule-1 (KIM-1): a novel biomarker for human renal proximal tubule injury. *Kidney Int, 62*(1), 237-244.

Heemskerk, S., Masereeuw, R., Russel, F. G., & Pickkers, P. (2009). Selective iNOS inhibition for the treatment of sepsis-induced acute kidney injury. *Nat Rev Nephrol, 5*(11), 629-640.

Herget-Rosenthal, S., Marggraf, G., Husing, J., Goring, F., Pietruck, F., Janssen, O., Philipp, T., & Kribben, A. (2004). Early detection of acute renal failure by serum cystatin C. *Kidney Int, 66*(3), 1115-1122.

Hiromura, K., Kurosawa, M., Yano, S., & Naruse, T. (1998). Tubulointerstitial mast cell infiltration in glomerulonephritis. *Am J Kidney Dis, 32*(4), 593-599.

Hoste, E. A., & Kellum, J. A. (2007). Incidence, classification, and outcomes of acute kidney injury. *Contrib Nephrol, 156*, 32-38.

House, J. D., Brosnan, M. E., & Brosnan, J. T. (1997). Characterization of homocysteine metabolism in the rat kidney. *Biochem J, 328 (Pt 1)*, 287-292.

Hsu, C. Y., McCulloch, C. E., Fan, D., Ordonez, J. D., Chertow, G. M., & Go, A. S. (2007). Community-based incidence of acute renal failure. *Kidney Int, 72*(2), 208-212.

Hsu, R. K., McCulloch, C. E., Dudley, R. A., Lo, L. J., & Hsu, C. Y. (2012). Temporal changes in incidence of dialysis-requiring AKI. *J Am Soc Nephrol, 24*(1), 37-42.

Hughes, M. N., Centelles, M. N., & Moore, K. P. (2009). Making and working with hydrogen sulfide: The chemistry and generation of hydrogen sulfide in vitro and its measurement in vivo: a review. *Free Radic Biol Med, 47*(10), 1346-1353.

Hunter, J. P., Hosgood, S. A., Patel, M., Rose, R., Read, K., & Nicholson, M. L. (2012). Effects of hydrogen sulphide in an experimental model of renal ischaemia-reperfusion injury. *Br J Surg, 99*(12), 1665-1671.

Hwang, S. Y., Sarna, L. K., Siow, Y. L., & O, K. (2013). High-fat diet stimulates hepatic cystathionine beta-synthase and cystathionine gamma-lyase expression. *Can J Physiol Pharmacol, 91*(11), 913-919.

Hwang, S. Y., Siow, Y. L., Au-Yeung, K. K., House, J., & O, K. (2011). Folic acid supplementation inhibits NADPH oxidase-mediated superoxide anion production in the kidney. *Am J Physiol Renal Physiol, 300*(1), F189-198.

Imig, J. D., & Ryan, M. J. (2013). Immune and inflammatory role in renal disease. *Compr Physiol, 3*(2), 957-976.

Inthorn, D., & Hoffmann, J. N. (1996). Elimination of inflammatory mediators by hemofiltration. *Int J Artif Organs, 19*(2), 124-126.

Ishigami, M., Hiraki, K., Umemura, K., Ogasawara, Y., Ishii, K., & Kimura, H. (2009). A source of hydrogen sulfide and a mechanism of its release in the brain. *Antioxid Redox Signal, 11*(2), 205-214.

Ishii, I., Akahoshi, N., Yu, X. N., Kobayashi, Y., Namekata, K., Komaki, G., & Kimura, H. (2004). Murine cystathionine gamma-lyase: complete cDNA and genomic sequences, promoter activity, tissue distribution and developmental expression. *Biochem J, 381*(Pt 1), 113-123.

Jaeschke, H. (2000). Reactive oxygen and mechanisms of inflammatory liver injury. *J Gastroenterol Hepatol, 15*(7), 718-724.

Je, I. G., Kim, D. S., Kim, S. W., Lee, S., Lee, H. S., Park, E. K., Khang, D., & Kim, S. H. (2015). Tyrosol Suppresses Allergic Inflammation by Inhibiting the Activation of Phosphoinositide 3-Kinase in Mast Cells. *PLoS One, 10*(6), e0129829.

Joles, J. A., Vos, I. H., Grone, H. J., & Rabelink, T. J. (2002). Inducible nitric oxide synthase in renal transplantation. *Kidney Int, 61*(3), 872-875.

Jourd'heuil, D., Jourd'heuil, F. L., Kutchukian, P. S., Musah, R. A., Wink, D. A., & Grisham, M. B. (2001). Reaction of superoxide and nitric oxide with peroxynitrite. Implications for peroxynitrite-mediated oxidation reactions in vivo. *J Biol Chem, 276*(31), 28799-28805.

Kalogeris, T., Baines, C. P., Krenz, M., & Korthuis, R. J. (2012). Cell biology of ischemia/reperfusion injury. *Int Rev Cell Mol Biol, 298*, 229-317.

Kang, K., Zhao, M., Jiang, H., Tan, G., Pan, S., & Sun, X. (2009). Role of hydrogen sulfide in hepatic ischemia-reperfusion-induced injury in rats. *Liver Transpl, 15*(10), 1306-1314.

Kasama, T., Strieter, R. M., Lukacs, N. W., Burdick, M. D., & Kunkel, S. L. (1994). Regulation of neutrophil-derived chemokine expression by IL-10. *J Immunol, 152*(7), 3559-3569.

KDIGO clinical practice guidelines for acute kidney injury (2012). *The Nephron journals, 120*(4).

Kellum, J. A., & Lameire, N. (2013). Diagnosis, evaluation, and management of acute kidney injury: a KDIGO summary (Part 1). *Crit Care, 17*(1), 204.

Kelly, K. J. (2006). Acute renal failure: much more than a kidney disease. *Semin Nephrol, 26*(2), 105-113.

Khallouki, F., Younos, C., Soulimani, R., Oster, T., Charrouf, Z., Spiegelhalder, B., Bartsch, H., & Owen, R. W. (2003). Consumption of argan oil (Morocco) with its unique profile of fatty acids, tocopherols, squalene, sterols and phenolic compounds should confer valuable cancer chemopreventive effects. *Eur J Cancer Prev, 12*(1), 67-75.

Kielar, M. L., John, R., Bennett, M., Richardson, J. A., Shelton, J. M., Chen, L., Jeyarajah, D. R., Zhou, X. J., Zhou, H., Chiquett, B., Nagami, G. T., & Lu, C. Y. (2005). Maladaptive role of IL-6 in ischemic acute renal failure. *J Am Soc Nephrol, 16*(11), 3315-3325.

Kimura, Y., Goto, Y., & Kimura, H. (2009). Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. *Antioxid Redox Signal, 12*(1), 1-13.

Kinsey, G. R., Li, L., & Okusa, M. D. (2008). Inflammation in acute kidney injury. *Nephron Exp Nephrol, 109*(4), e102-107.

Kiss, L., Deitch, E. A., & Szabo, C. (2008). Hydrogen sulfide decreases adenosine triphosphate levels in aortic rings and leads to vasorelaxation via metabolic inhibition. *Life Sci, 83*(17-18), 589-594.

Knoops, K. T., de Groot, L. C., Kromhout, D., Perrin, A. E., Moreiras-Varela, O., Menotti, A., & van Staveren, W. A. (2004). Mediterranean diet, lifestyle factors, and 10-year mortality in elderly European men and women: the HALE project. *JAMA, 292*(12), 1433-1439.

Ko, G. J., Grigoryev, D. N., Linfert, D., Jang, H. R., Watkins, T., Cheadle, C., Racusen, L., & Rabb, H. (2010). Transcriptional analysis of kidneys during repair from AKI reveals possible roles for NGAL and KIM-1 as biomarkers of AKI-to-CKD transition. *Am J Physiol Renal Physiol, 298*(6), F1472-1483.

Korkmaz, A., & Kolankaya, D. (2009). The protective effects of ascorbic acid against renal ischemia-reperfusion injury in male rats. *Ren Fail, 31*(1), 36-43.

Kostadinova, R., Wahli, W., & Michalik, L. (2005). PPARs in diseases: control mechanisms of inflammation. *Curr Med Chem, 12*(25), 2995-3009.

Kroncke, K. D., Fehsel, K., & Kolb-Bachofen, V. (1998). Inducible nitric oxide synthase in human diseases. *Clin Exp Immunol, 113*(2), 147-156.

Kushner, I. (1982). The phenomenon of the acute phase response. *Ann N Y Acad Sci, 389*, 39-48.

Lai, L. W., Yong, K. C., Igarashi, S., & Lien, Y. H. (2007). A sphingosine-1-phosphate type 1 receptor agonist inhibits the early T-cell transient following renal ischemia-reperfusion injury. *Kidney Int, 71*(12), 1223-1231.

Lameire, N., Van Biesen, W., & Vanholder, R. (2005). Acute renal failure. *Lancet, 365*(9457), 417-430.

Langer, H. F., & Chavakis, T. (2009). Leukocyte-endothelial interactions in inflammation. *J Cell Mol Med, 13*(7), 1211-1220.

Lattanzio, M. R., & Kopyt, N. P. (2009). Acute kidney injury: new concepts in definition, diagnosis, pathophysiology, and treatment. *J Am Osteopath Assoc, 109*(1), 13-19.

Lefer, A. M., Tsao, P., Aoki, N., & Palladino, M. A., Jr. (1990). Mediation of cardioprotection by transforming growth factor-beta. *Science, 249*(4964), 61-64.

Legrand, M., Mik, E. G., Johannes, T., Payen, D., & Ince, C. (2008). Renal hypoxia and dysoxia after reperfusion of the ischemic kidney. *Mol Med, 14*(7-8), 502-516.

Lewington, A., & Kanagasundaram, S. (2011). Renal Association Clinical Practice Guidelines on acute kidney injury. *Nephron Clin Pract, 118 Suppl 1*, c349-390.

Li, L., & Moore, P. K. (2008). Putative biological roles of hydrogen sulfide in health and disease: a breath of not so fresh air? *Trends Pharmacol Sci, 29*(2), 84-90.

Li, L., Rossoni, G., Sparatore, A., Lee, L. C., Del Soldato, P., & Moore, P. K. (2007). Anti-inflammatory and gastrointestinal effects of a novel diclofenac derivative. *Free Radic Biol Med, 42*(5), 706-719.

Li, L., Salto-Tellez, M., Tan, C. H., Whiteman, M., & Moore, P. K. (2009). GYY4137, a novel hydrogen sulfide-releasing molecule, protects against endotoxic shock in the rat. *Free Radic Biol Med, 47*(1), 103-113.

Liang, J., Jiang, D., Griffith, J., Yu, S., Fan, J., Zhao, X., Bucala, R., & Noble, P. W. (2007). CD44 is a negative regulator of acute pulmonary inflammation and lipopolysaccharide-TLR signaling in mouse macrophages. *J Immunol, 178*(4), 2469-2475.

Lien, Y. H., Yong, K. C., Cho, C., Igarashi, S., & Lai, L. W. (2006). S1P(1)-selective agonist, SEW2871, ameliorates ischemic acute renal failure. *Kidney Int, 69*(9), 1601-1608.

Linas, S. L., Whittenburg, D., & Repine, J. E. (1990). Role of xanthine oxidase in ischemia/reperfusion injury. *Am J Physiol, 258*(3 Pt 2), F711-716.

Ling, H., Edelstein, C., Gengaro, P., Meng, X., Lucia, S., Knotek, M., Wangsiripaisan, A., Shi, Y., & Schrier, R. (1999). Attenuation of renal ischemia-reperfusion injury in inducible nitric oxide synthase knockout mice. *Am J Physiol, 277*(3 Pt 2), F383-390.

Lobb, I., Mok, A., Lan, Z., Liu, W., Garcia, B., & Sener, A. (2012). Supplemental hydrogen sulphide protects transplant kidney function and prolongs recipient survival after prolonged cold ischaemia-reperfusion injury by mitigating renal graft apoptosis and inflammation. *BJU Int, 110*(11 Pt C), E1187-1195.

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev, 4*(8), 118-126.

Loru, D., Incani, A., Deiana, M., Corona, G., Atzeri, A., Melis, M. P., Rosa, A., & Dessi, M. A. (2009). Protective effect of hydroxytyrosol and tyrosol against oxidative stress in kidney cells. *Toxicol Ind Health, 25*(4-5), 301-310.

Luther, S. A., & Cyster, J. G. (2001). Chemokines as regulators of T cell differentiation. *Nat Immunol, 2*(2), 102-107.

Luyckx, V. A., & Bonventre, J. V. (2008). *Chapter 42: Models of Ischemic Renal Injury*: Saunders/Elsevier.

Madamanchi, N. R., Vendrov, A., & Runge, M. S. (2005). Oxidative stress and vascular disease. *Arterioscler Thromb Vasc Biol, 25*(1), 29-38.

Manach, C., Williamson, G., Morand, C., Scalbert, A., & Remesy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr, 81*(1 Suppl), 230S-242S.

Mann, D. L., & Young, J. B. (1994). Basic mechanisms in congestive heart failure. Recognizing the role of proinflammatory cytokines. *Chest, 105*(3), 897-904.

Manna, C., Napoli, D., Cacciapuoti, G., Porcelli, M., & Zappia, V. (2009). Olive oil phenolic compounds inhibit homocysteine-induced endothelial cell adhesion regardless of their different antioxidant activity. *J Agric Food Chem, 57*(9), 3478-3482.

Mark, L. A., Robinson, A. V., & Schulak, J. A. (2005). Inhibition of nitric oxide synthase reduces renal ischemia/reperfusion injury. *J Surg Res, 129*(2), 236-241.

Mazumder, B., Li, X., & Barik, S. (2010). Translation control: a multifaceted regulator of inflammatory response. *J Immunol, 184*(7), 3311-3319.

Meagher, E. A., & FitzGerald, G. A. (2000). Indices of lipid peroxidation in vivo: strengths and limitations. *Free Radic Biol Med, 28*(12), 1745-1750.

Medzhitov, R. (2008). Origin and physiological roles of inflammation. *Nature, 454*(7203), 428-435.

Mehta, R. L., & Chertow, G. M. (2003). Acute renal failure definitions and classification: time for change? *J Am Soc Nephrol, 14*(8), 2178-2187.

Mehta, R. L., Kellum, J. A., Shah, S. V., Molitoris, B. A., Ronco, C., Warnock, D. G., & Levin, A. (2007). Acute Kidney Injury Network: report of an initiative to improve outcomes in acute kidney injury. *Crit Care, 11*(2), R31.

Meisinger, C., Baumert, J., Khuseyinova, N., Loewel, H., & Koenig, W. (2005). Plasma oxidized low-density lipoprotein, a strong predictor for acute coronary heart disease events in apparently healthy, middle-aged men from the general population. *Circulation, 112*(5), 651-657.

Miro Casas, E., Farre Albadalejo, M., Covas Planells, M. I., Fito Colomer, M., Lamuela Raventos, R. M., & de la Torre Fornell, R. (2001). Tyrosol bioavailability in humans after ingestion of virgin olive oil. *Clin Chem, 47*(2), 341-343.

Mishra, J., Ma, Q., Prada, A., Mitsnefes, M., Zahedi, K., Yang, J., Barasch, J., & Devarajan, P. (2003). Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol, 14*(10), 2534-2543.

Mok, Y. Y., & Moore, P. K. (2008). Hydrogen sulphide is pro-inflammatory in haemorrhagic shock. *Inflamm Res, 57*(11), 512-518.

Molitoris, B. A., & Sutton, T. A. (2004). Endothelial injury and dysfunction: role in the extension phase of acute renal failure. *Kidney Int, 66*(2), 496-499.

Moreno, J. J. (2003). Effect of olive oil minor components on oxidative stress and arachidonic acid mobilization and metabolism by macrophages RAW 264.7. *Free Radic Biol Med, 35*(9), 1073-1081.

Munshi, R., Johnson, A., Siew, E. D., Ikizler, T. A., Ware, L. B., Wurfel, M. M., Himmelfarb, J., & Zager, R. A. (2011). MCP-1 gene activation marks acute kidney injury. *J Am Soc Nephrol, 22*(1), 165-175.

Murugan, R., & Kellum, J. A. (2011). Acute kidney injury: what's the prognosis? *Nat Rev Nephrol, 7*(4), 209-217.

Nath, K. A., & Norby, S. M. (2000). Reactive oxygen species and acute renal failure. *Am J Med, 109*(8), 665-678.

Nathan, C. (2002). Points of control in inflammation. *Nature, 420*(6917), 846-852.

Nechemia-Arbely, Y., Barkan, D., Pizov, G., Shriki, A., Rose-John, S., Galun, E., & Axelrod, J. H. (2008). IL-6/IL-6R axis plays a critical role in acute kidney injury. *J Am Soc Nephrol, 19*(6), 1106-1115.

NICE (2013). Acute kidney injury: Prevention, detection and management of acute kidney injury up to the point of renal replacement therapy. *NICE clinical guideline 169.*

Nicholson, C. K., & Calvert, J. W. (2010). Hydrogen sulfide and ischemia-reperfusion injury. *Pharmacol Res, 62*(4), 289-297.

Noiri, E., Nakao, A., Uchida, K., Tsukahara, H., Ohno, M., Fujita, T., Brodsky, S., & Goligorsky, M. S. (2001). Oxidative and nitrosative stress in acute renal ischemia. *Am J Physiol Renal Physiol, 281*(5), F948-957.

Noronha, I. L., Fujihara, C. K., & Zatz, R. (2002). The inflammatory component in progressive renal disease--are interventions possible? *Nephrol Dial Transplant, 17*(3), 363-368.

Ogasawara, Y., Isoda, S., & Tanabe, S. (1994). Tissue and subcellular distribution of bound and acid-labile sulfur, and the enzymic capacity for sulfide production in the rat. *Biol Pharm Bull, 17*(12), 1535-1542.

Oh, G. S., Pae, H. O., Lee, B. S., Kim, B. N., Kim, J. M., Kim, H. R., Jeon, S. B., Jeon, W. K., Chae, H. J., & Chung, H. T. (2006). Hydrogen sulfide inhibits nitric oxide production and nuclear factor-kappaB via heme oxygenase-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide. *Free Radic Biol Med, 41*(1), 106-119.

Owen, R. W., Haubner, R., Wurtele, G., Hull, E., Spiegelhalder, B., & Bartsch, H. (2004). Olives and olive oil in cancer prevention. *Eur J Cancer Prev, 13*(4), 319-326.

Ozbek, E. (2012). Induction of oxidative stress in kidney. *Int J Nephrol, 2012*, 465897.

Pacher, P., Beckman, J. S., & Liaudet, L. (2007). Nitric oxide and peroxynitrite in health and disease. *Physiol Rev, 87*(1), 315-424.

Pacifici, R. E., & Davies, K. J. (1991). Protein, lipid and DNA repair systems in oxidative stress: the free-radical theory of aging revisited. *Gerontology, 37*(1-3), 166-180.

Palmieri, D., Aliakbarian, B., Casazza, A. A., Ferrari, N., Spinella, G., Pane, B., Cafueri, G., Perego, P., & Palombo, D. (2012). Effects of polyphenol extract from olive pomace on anoxia-induced endothelial dysfunction. *Microvasc Res, 83*(3), 281-289.

Papapetropoulos, A., Pyriochou, A., Altaany, Z., Yang, G. D., Marazioti, A., Zhou, Z. M., Jeschke, M. G., Branski, L. K., Herndon, D. N., Wang, R., & Szabo, C. (2009). Hydrogen sulfide is an endogenous stimulator of angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America, 106*(51), 21972-21977.

Parikh, C. R., Abraham, E., Ancukiewicz, M., & Edelstein, C. L. (2005). Urine IL-18 is an early diagnostic marker for acute kidney injury and predicts mortality in the intensive care unit. *J Am Soc Nephrol, 16*(10), 3046-3052.

Park, S. K., Yang, W. S., Lee, S. K., Ahn, H., Park, J. S., Hwang, O., & Lee, J. D. (2000). TGF-beta(1) down-regulates inflammatory cytokine-induced VCAM-1 expression in cultured human glomerular endothelial cells. *Nephrol Dial Transplant, 15*(5), 596-604.

Park, S. W., Chen, S. W., Kim, M., Brown, K. M., Kolls, J. K., D'Agati, V. D., & Lee, H. T. (2011). Cytokines induce small intestine and liver injury after renal ischemia or nephrectomy. *Lab Invest, 91*(1), 63-84.

Patel, N. S., Chatterjee, P. K., Di Paola, R., Mazzon, E., Britti, D., De Sarro, A., Cuzzocrea, S., & Thiemermann, C. (2005). Endogenous interleukin-6 enhances the renal injury, dysfunction, and inflammation caused by ischemia/reperfusion. *J Pharmacol Exp Ther, 312*(3), 1170-1178.

Paul, B. D., & Snyder, S. H. (2012). H(2)S signalling through protein sulfhydration and beyond. *Nat Rev Mol Cell Biol, 13*(8), 499-507.

Peake, B. F., Nicholson, C. K., Lambert, J. P., Hood, R. L., Amin, H., Amin, S., & Calvert, J. W. (2013). Hydrogen sulfide preconditions the db/db diabetic mouse heart against ischemia-reperfusion injury by activating Nrf2 signaling in an Erk-dependent manner. *Am J Physiol Heart Circ Physiol, 304*(9), H1215-1224.

Perna, A. F., & Ingrosso, D. (2012). Low hydrogen sulphide and chronic kidney disease: a dangerous liaison. *Nephrol Dial Transplant, 27*(2), 486-493.

Phadke, K. D., & Dinakar, C. (2001). The challenges of treating children with renal failure in a developing country. *Perit Dial Int, 21 Suppl 3*, S326-329.

Ploeg, R. J., van Bockel, J. H., Langendijk, P. T., Groenewegen, M., van der Woude, F. J., Persijn, G. G., Thorogood, J., & Hermans, J. (1992). Effect of preservation solution on results of cadaveric kidney transplantation. The European Multicentre Study Group. *Lancet, 340*(8812), 129-137.

Pooler, C. (2009). *Porth pathophysiology: concepts of altered health states*: Lippincott Williams & Wilkins.

Portilla, D., Dent, C., Sugaya, T., Nagothu, K. K., Kundi, I., Moore, P., Noiri, E., & Devarajan, P. (2008). Liver fatty acid-binding protein as a biomarker of acute kidney injury after cardiac surgery. *Kidney Int, 73*(4), 465-472.

Prathapasinghe, G. A., Siow, Y. L., & O, K. (2007). Detrimental role of homocysteine in renal ischemia-reperfusion injury. *Am J Physiol Renal Physiol, 292*(5), F1354-1363.

Prathapasinghe, G. A., Siow, Y. L., Xu, Z., & O, K. (2008). Inhibition of cystathionine-beta-synthase activity during renal ischemia-reperfusion: role of pH and nitric oxide. *Am J Physiol Renal Physiol, 295*(4), F912-922.

Pryor, W. A., & Squadrito, G. L. (1995). The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *Am J Physiol, 268*(5 Pt 1), L699-722.

Racusen, L. C., Fivush, B. A., Li, Y. L., Slatnik, I., & Solez, K. (1991). Dissociation of tubular cell detachment and tubular cell death in clinical and experimental "acute tubular necrosis". *Lab Invest, 64*(4), 546-556.

Radi, R., Beckman, J. S., Bush, K. M., & Freeman, B. A. (1991). Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys, 288*(2), 481-487.

Rahman, I., Kode, A., & Biswas, S. K. (2006). Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat Protoc, 1*(6), 3159-3165.

Rahman, M., Shad, F., & Smith, M. C. (2012). Acute kidney injury: a guide to diagnosis and management. *Am Fam Physician, 86*(7), 631-639.

Ramesh, G., & Reeves, W. B. (2004). Inflammatory cytokines in acute renal failure. *Kidney Int Suppl*(91), S56-61.

Rao, R. M., Yang, L., Garcia-Cardena, G., & Luscinskas, F. W. (2007). Endothelial-dependent mechanisms of leukocyte recruitment to the vascular wall. *Circ Res, 101*(3), 234-247.

Reiffenstein, R. J., Hulbert, W. C., & Roth, S. H. (1992). Toxicology of hydrogen sulfide. *Annu Rev Pharmacol Toxicol, 32*, 109-134.

Reuter, S., Gupta, S. C., Chaturvedi, M. M., & Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med, 49*(11), 1603-1616.

Rietjens, S. J., Bast, A., de Vente, J., & Haenen, G. R. (2007). The olive oil antioxidant hydroxytyrosol efficiently protects against the oxidative stress-induced impairment of the NObullet response of isolated rat aorta. *Am J Physiol Heart Circ Physiol, 292*(4), H1931-1936.

Riley, S., Diro, E., Batchelor, P., Abebe, A., Amsalu, A., Tadesse, Y., Williams, J., & Phillips, A. O. (2013). Renal impairment among acute hospital admissions in a rural Ethiopian hospital. *Nephrology (Carlton), 18*(2), 92-96.

Rivers, J. R., Badiei, A., & Bhatia, M. (2012). Hydrogen sulfide as a therapeutic target for inflammation. *Expert Opin Ther Targets, 16*(5), 439-449.

Roczniak, A., Fryer, J. N., Levine, D. Z., & Burns, K. D. (1999). Downregulation of neuronal nitric oxide synthase in the rat remnant kidney. *J Am Soc Nephrol, 10*(4), 704-713.

Romero, C., & Brenes, M. (2012). Analysis of total contents of hydroxytyrosol and tyrosol in olive oils. *J Agric Food Chem, 60*(36), 9017-9022.

Rondon-Berrios, H., & Palevsky, P. M. (2007). Treatment of acute kidney injury: an update on the management of renal replacement therapy. *Curr Opin Nephrol Hypertens, 16*(2), 64-70.

Ruiz-Canela, M., & Martinez-Gonzalez, M. A. (2011). Olive oil in the primary prevention of cardiovascular disease. *Maturitas, 68*(3), 245-250.

Samimagham, H. R., Kheirkhah, S., Haghighi, A., & Najmi, Z. (2011). Acute kidney injury in intensive care unit: incidence, risk factors and mortality rate. *Saudi J Kidney Dis Transpl, 22*(3), 464-470.

Samuelsson, B. (1983). Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science, 220*(4597), 568-575.

Sancaktutar, A. A., Bodakci, M. N., Hatipoglu, N. K., Soylemez, H., Basarili, K., & Turkcu, G. (2014). The protective effects of pomegranate extracts against renal ischemia-reperfusion injury in male rats. *Urol Ann, 6*(1), 46-50.

Savas, M., Yeni, E., Ciftci, H., Yildiz, F., Gulum, M., Keser, B. S., Verit, A., Utangac, M., Kocyigit, A., Celik, H., & Bitiren, M. (2010). The antioxidant role of oral administration of garlic oil on renal ischemia-reperfusion injury. *Ren Fail, 32*(3), 362-367.

Schieber, M., & Chandel, N. S. (2014). ROS function in redox signaling and oxidative stress. *Curr Biol, 24*(10), R453-462.

Schottelius, A. J., Mayo, M. W., Sartor, R. B., & Baldwin, A. S., Jr. (1999). Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. *J Biol Chem, 274*(45), 31868-31874.

Seedat, Y. K., & Nathoo, B. C. (1993). Acute renal failure in blacks and Indians in South Africa--comparison after 10 years. *Nephron, 64*(2), 198-201.

Sen, N., Paul, B. D., Gadalla, M. M., Mustafa, A. K., Sen, T., Xu, R., Kim, S., & Snyder, S. H. (2012). Hydrogen sulfide-linked sulfhydration of NF-kappaB mediates its antiapoptotic actions. *Mol Cell, 45*(1), 13-24.

Sen, U., Givvimani, S., Abe, O. A., Lederer, E. D., & Tyagi, S. C. (2011). Cystathionine beta-synthase and cystathionine gamma-lyase double gene transfer ameliorate homocysteine-mediated mesangial inflammation through hydrogen sulfide generation. *Am J Physiol Cell Physiol, 300*(1), C155-163.

Sener, G., Sener, E., Sehirli, O., Ogunc, A. V., Cetinel, S., Gedik, N., & Sakarcan, A. (2005). Ginkgo biloba extract ameliorates ischemia reperfusion-induced renal injury in rats. *Pharmacol Res, 52*(3), 216-222.

Serhan, C. N., Chiang, N., & Van Dyke, T. E. (2008). Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol, 8*(5), 349-361.

Shankar, A., Sun, L., Klein, B. E., Lee, K. E., Muntner, P., Nieto, F. J., Tsai, M. Y., Cruickshanks, K. J., Schubert, C. R., Brazy, P. C., Coresh, J., & Klein, R. (2012). Markers of inflammation predict the long-term risk of developing chronic kidney disease: a population-based cohort study. *Kidney Int, 80*(11), 1231-1238.

Sharfuddin, A. A., & Molitoris, B. A. (2011). Pathophysiology of ischemic acute kidney injury. *Nat Rev Nephrol, 7*(4), 189-200.

Shi, T. Y., Feng, S. F., Xing, J. H., Wu, Y. M., Li, X. Q., Zhang, N., Tian, Z., Liu, S. B., & Zhao, M. G. (2012). Neuroprotective effects of Salidroside and its analogue tyrosol galactoside against focal cerebral ischemia in vivo and H2O2-induced neurotoxicity in vitro. *Neurotox Res, 21*(4), 358-367.

Shibuya, N., Tanaka, M., Yoshida, M., Ogasawara, Y., Togawa, T., Ishii, K., & Kimura, H. (2009). 3-Mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain. *Antioxid Redox Signal, 11*(4), 703-714.

Sidhapuriwala, J., Li, L., Sparatore, A., Bhatia, M., & Moore, P. K. (2007). Effect of S-diclofenac, a novel hydrogen sulfide releasing derivative, on carrageenan-induced hindpaw oedema formation in the rat. *Eur J Pharmacol, 569*(1-2), 149-154.

Sies, H. (1997). Oxidative stress: oxidants and antioxidants. *Exp Physiol, 82*(2), 291-295.

Simmons, E. M., Himmelfarb, J., Sezer, M. T., Chertow, G. M., Mehta, R. L., Paganini, E. P., Soroko, S., Freedman, S., Becker, K., Spratt, D., Shyr, Y., & Ikizler, T. A. (2004). Plasma cytokine levels predict mortality in patients with acute renal failure. *Kidney Int, 65*(4), 1357-1365.

Singh, A. P., Junemann, A., Muthuraman, A., Jaggi, A. S., Singh, N., Grover, K., & Dhawan, R. (2012). Animal models of acute renal failure. *Pharmacol Rep, 64*(1), 31-44.

Singh, D., & Chopra, K. (2004). The effect of naringin, a bioflavonoid on ischemia-reperfusion induced renal injury in rats. *Pharmacol Res, 50*(2), 187-193.

Singh, S., & Banerjee, R. (2011). PLP-dependent H(2)S biogenesis. *Biochim Biophys Acta, 1814*(11), 1518-1527.

Sivarajah, A., Collino, M., Yasin, M., Benetti, E., Gallicchio, M., Mazzon, E., Cuzzocrea, S., Fantozzi, R., & Thiemermann, C. (2009). Anti-apoptotic and anti-inflammatory effects of hydrogen sulfide in a rat model of regional myocardial I/R. *Shock, 31*(3), 267-274.

Snijder, P. M., Frenay, A. R., Koning, A. M., Bachtler, M., Pasch, A., Kwakernaak, A. J., van den Berg, E., Bos, E. M., Hillebrands, J. L., Navis, G., Leuvenink, H. G., & van Goor, H. (2014). Sodium thiosulfate attenuates angiotensin II-induced hypertension, proteinuria and renal damage. *Nitric Oxide, 42*, 87-98.

Sodha, N. R., Clements, R. T., Feng, J., Liu, Y., Bianchi, C., Horvath, E. M., Szabo, C., Stahl, G. L., & Sellke, F. W. (2009). Hydrogen sulfide therapy attenuates the inflammatory response in a porcine model of myocardial ischemia/reperfusion injury. *J Thorac Cardiovasc Surg, 138*(4), 977-984.

Sofi, F., Cesari, F., Abbate, R., Gensini, G. F., & Casini, A. (2008). Adherence to Mediterranean diet and health status: meta-analysis. *BMJ, 337*, a1344.

St-Laurent-Thibault, C., Arseneault, M., Longpre, F., & Ramassamy, C. (2011). Tyrosol and hydroxytyrosol, two main components of olive oil, protect N2a cells against amyloid-beta-induced toxicity. Involvement of the NF-kappaB signaling. *Curr Alzheimer Res, 8*(5), 543-551.

Star, R. A. (1998). Treatment of acute renal failure. *Kidney Int, 54*(6), 1817-1831.

Stipanuk, M. H., & Beck, P. W. (1982). Characterization of the enzymic capacity for cysteine desulphhydration in liver and kidney of the rat. *Biochem J, 206*(2), 267-277.

Stipanuk, M. H., & Ueki, I. (2010). Dealing with methionine/homocysteine sulfur: cysteine metabolism to taurine and inorganic sulfur. *J Inherit Metab Dis, 34*(1), 17-32.

Sun, L., Fan, H., Yang, L., Shi, L., & Liu, Y. (2015). Tyrosol prevents ischemia/reperfusion-induced cardiac injury in H9c2 cells: involvement of ROS, Hsp70, JNK and ERK, and apoptosis. *Molecules, 20*(3), 3758-3775.

Sun, L., Isaak, C. K., Zhou, Y., Petkau, J. C., O, K., Liu, Y., & Siow, Y. L. (2012). Salidroside and tyrosol from Rhodiola protect H9c2 cells from ischemia/reperfusion-induced apoptosis. *Life Sci, 91*(5-6), 151-158.

Sung, F. L., Zhu, T. Y., Au-Yeung, K. K., Siow, Y. L., & O, K. (2002). Enhanced MCP-1 expression during ischemia/reperfusion injury is mediated by oxidative stress and NF-kappaB. *Kidney Int, 62*(4), 1160-1170.

Susantitaphong, P., Cruz, D. N., Cerda, J., Abulfaraj, M., Alqahtani, F., Koulouridis, I., & Jaber, B. L. (2013). World incidence of AKI: a meta-analysis. *Clin J Am Soc Nephrol, 8*(9), 1482-1493.

Sutton, T. A., Fisher, C. J., & Molitoris, B. A. (2002). Microvascular endothelial injury and dysfunction during ischemic acute renal failure. *Kidney Int, 62*(5), 1539-1549.

Sutton, T. A., Mang, H. E., Campos, S. B., Sandoval, R. M., Yoder, M. C., & Molitoris, B. A. (2003). Injury of the renal microvascular endothelium alters barrier function after ischemia. *Am J Physiol Renal Physiol, 285*(2), F191-198.

Szabo, C. (2003). Multiple pathways of peroxynitrite cytotoxicity. *Toxicol Lett, 140-141*, 105-112.

Takeda, R., Nishimatsu, H., Suzuki, E., Satonaka, H., Nagata, D., Oba, S., Sata, M., Takahashi, M., Yamamoto, Y., Terauchi, Y., Kadowaki, T., Kangawa, K., Kitamura, T., Nagai, R., & Hirata, Y. (2006). Ghrelin improves renal function in mice with ischemic acute renal failure. *J Am Soc Nephrol, 17*(1), 113-121.

Takeuchi, O., & Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell, 140*(6), 805-820.

Tang, M. J., Suresh, K. R., & Tannen, R. L. (1989). Carbohydrate metabolism by primary cultures of rabbit proximal tubules. *Am J Physiol, 256*(3 Pt 1), C532-539.

Tedgui, A., & Mallat, Z. (2001). Anti-inflammatory mechanisms in the vascular wall. *Circ Res, 88*(9), 877-887.

Thirunavukkarasu, M., Penumathsa, S. V., Samuel, S. M., Akita, Y., Zhan, L., Bertelli, A. A., Maulik, G., & Maulik, N. (2008). White wine induced cardioprotection against ischemia-reperfusion injury is mediated by life extending Akt/FOXO3a/NFkappaB survival pathway. *J Agric Food Chem, 56*(15), 6733-6739.

Thomas, M. E., Blaine, C., Dawnay, A., Devonald, M. A., Ftouh, S., Laing, C., Latchem, S., Lewington, A., Milford, D. V., & Ostermann, M. (2014). The definition of acute kidney injury and its use in practice. *Kidney Int, 87*(1), 62-73.

Thurman, J. M. (2007). Triggers of inflammation after renal ischemia/reperfusion. *Clin Immunol, 123*(1), 7-13.

Tracey, K. J. (2002). The inflammatory reflex. *Nature, 420*(6917), 853-859.

Trachootham, D., Lu, W., Ogasawara, M. A., Nilsa, R. D., & Huang, P. (2008). Redox regulation of cell survival. *Antioxid Redox Signal, 10*(8), 1343-1374.

Trichopoulou, A., Lagiou, P., Kuper, H., & Trichopoulos, D. (2000). Cancer and Mediterranean dietary traditions. *Cancer Epidemiol Biomarkers Prev, 9*(9), 869-873.

Tripatara, P., Patel, N. S., Collino, M., Gallicchio, M., Kieswich, J., Castiglia, S., Benetti, E., Stewart, K. N., Brown, P. A., Yaqoob, M. M., Fantozzi, R., & Thiemermann, C. (2008). Generation of endogenous hydrogen sulfide by cystathionine gamma-lyase limits renal ischemia/reperfusion injury and dysfunction. *Lab Invest, 88*(10), 1038-1048.

Tuck, K. L., & Hayball, P. J. (2002). Major phenolic compounds in olive oil: metabolism and health effects. *J Nutr Biochem, 13*(11), 636-644.

Uehlinger, D. E., Jakob, S. M., Ferrari, P., Eichelberger, M., Huynh-Do, U., Marti, H. P., Mohaupt, M. G., Vogt, B., Rothen, H. U., Regli, B., Takala, J., & Frey, F. J. (2005). Comparison of continuous and intermittent renal replacement therapy for acute renal failure. *Nephrol Dial Transplant, 20*(8), 1630-1637.

Ujiie, K., Yuen, J., Hogarth, L., Danziger, R., & Star, R. A. (1994). Localization and regulation of endothelial NO synthase mRNA expression in rat kidney. *Am J Physiol, 267*(2 Pt 2), F296-302.

Uttara, B., Singh, A. V., Zamboni, P., & Mahajan, R. T. (2009). Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr Neuropharmacol, 7*(1), 65-74.

Uysal, F., Girgin, F. K., Tuzun, S., Aldemir, S., & Sozmen, E. Y. (1998). Effect of vitamin E on antioxidant enzymes and nitric oxide in ischemia-reperfused kidney injury. *Biochem Mol Biol Int, 44*(6), 1255-1263.

Vauzour, D., Vafeiadou, K., Corona, G., Pollard, S. E., Tzounis, X., & Spencer, J. P. (2007). Champagne wine polyphenols protect primary cortical neurons against peroxynitrite-induced injury. *J Agric Food Chem, 55*(8), 2854-2860.

Venkatachalam, M. A., Griffin, K. A., Lan, R., Geng, H., Saikumar, P., & Bidani, A. K. (2010). Acute kidney injury: a springboard for progression in chronic kidney disease. *Am J Physiol Renal Physiol, 298*(5), F1078-1094.

Vernaglione, L. (2009). The Mediterranean diet: a matter of history, tradition, culture and health. *J Nephrol, 22 Suppl 14*, 149-158.

Vinsonneau, C., Camus, C., Combes, A., Costa de Beauregard, M. A., Klouche, K., Boulain, T., Pallot, J. L., Chiche, J. D., Taupin, P., Landais, P., & Dhainaut, J. F. (2006). Continuous venovenous haemodiafiltration versus intermittent haemodialysis for acute renal failure in patients with multiple-organ dysfunction syndrome: a multicentre randomised trial. *Lancet, 368*(9533), 379-385.

Vivancos, M., & Moreno, J. J. (2008). Effect of resveratrol, tyrosol and beta-sitosterol on oxidised low-density lipoprotein-stimulated oxidative stress, arachidonic acid release and prostaglandin E2 synthesis by RAW 264.7 macrophages. *Br J Nutr, 99*(6), 1199-1207.

Waikar, S. S., Curhan, G. C., Wald, R., McCarthy, E. P., & Chertow, G. M. (2006). Declining mortality in patients with acute renal failure, 1988 to 2002. *J Am Soc Nephrol, 17*(4), 1143-1150.

Walker, L. M., Walker, P. D., Imam, S. Z., Ali, S. F., & Mayeux, P. R. (2000). Evidence for peroxynitrite formation in renal ischemia-reperfusion injury: studies with the inducible nitric oxide synthase inhibitor L-N(6)-(1-Iminoethyl)lysine. *J Pharmacol Exp Ther, 295*(1), 417-422.

Wallace, J. L., Vong, L., McKnight, W., Dicay, M., & Martin, G. R. (2009). Endogenous and exogenous hydrogen sulfide promotes resolution of colitis in rats. *Gastroenterology, 137*(2), 569-578, 578 e561.

Wallace, J. L., & Wang, R. (2015). Hydrogen sulfide-based therapeutics: exploiting a unique but ubiquitous gasotransmitter. *Nat Rev Drug Discov, 14*(5), 329-345.

Wang, P., Zhu, Q., Wu, N., Siow, Y. L., Aukema, H., & O, K. (2013). Tyrosol attenuates ischemia-reperfusion-induced kidney injury via inhibition of inducible nitric oxide synthase. *J Agric Food Chem, 61*(15), 3669-3675.

Wang, R. (2002). Two's company, three's a crowd: can H2S be the third endogenous gaseous transmitter? *FASEB J, 16*(13), 1792-1798.

Wang, R. (2012). Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. *Physiol Rev, 92*(2), 791-896.

Weber, D., Kneschke, N., Grimm, S., Bergheim, I., Breusing, N., & Grune, T. (2012). Rapid and sensitive determination of protein-nitrotyrosine by ELISA: Application to human plasma. *Free Radic Res, 46*(3), 276-285.

Weight, S. C., Bell, P. R., & Nicholson, M. L. (1996). Renal ischaemia--reperfusion injury. *Br J Surg, 83*(2), 162-170.

Weight, S. C., & Nicholson, M. L. (1998). Nitric oxide and renal reperfusion injury: a review. *Eur J Vasc Endovasc Surg, 16*(2), 98-103.

Weinbrenner, T., Fito, M., Farre Albaladejo, M., Saez, G. T., Rijken, P., Tormos, C., Coolen, S., De La Torre, R., & Covas, M. I. (2004). Bioavailability of phenolic compounds from olive oil and oxidative/antioxidant status at postprandial state in healthy humans. *Drugs Exp Clin Res, 30*(5-6), 207-212.

Whiteman, M., & Moore, P. K. (2009). Hydrogen sulfide and the vasculature: a novel vasculoprotective entity and regulator of nitric oxide bioavailability? *J Cell Mol Med, 13*(3), 488-507.

Whiteman, M., & Winyard, P. G. (2011). Hydrogen sulfide and inflammation: the good, the bad, the ugly and the promising. *Expert Rev Clin Pharmacol, 4*(1), 13-32.

Wojdasiewicz, P., Poniatowski, L. A., & Szukiewicz, D. (2014). The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. *Mediators Inflamm, 2014*, 561459.

Wu, K., Lei, W., Tian, J., & Li, H. (2014). Atorvastatin treatment attenuates renal injury in an experimental model of ischemia-reperfusion in rats. *BMC Nephrol, 15*, 14.

Wu, N., Siow, Y. L., & O, K. (2010). Ischemia/reperfusion reduces transcription factor Sp1-mediated cystathionine beta-synthase expression in the kidney. *J Biol Chem, 285*(24), 18225-18233.

Xu, Z., Prathapasinghe, G., Wu, N., Hwang, S. Y., Siow, Y. L., & O, K. (2009). Ischemia-reperfusion reduces cystathionine-beta-synthase-mediated hydrogen sulfide generation in the kidney. *Am J Physiol Renal Physiol, 297*(1), F27-35.

Yadav, R., Larbi, K. Y., Young, R. E., & Nourshargh, S. (2003). Migration of leukocytes through the vessel wall and beyond. *Thromb Haemost, 90*(4), 598-606.

Yamamoto, Y., & Gaynor, R. B. (2001). Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. *J Clin Invest, 107*(2), 135-142.

Yang, G., Zhao, K., Ju, Y., Mani, S., Cao, Q., Puukila, S., Khaper, N., Wu, L., & Wang, R. (2012). Hydrogen sulfide protects against cellular senescence via S-sulfhydration of Keap1 and activation of Nrf2. *Antioxid Redox Signal, 18*(15), 1906-1919.

Yellon, D. M., & Hausenloy, D. J. (2007). Myocardial reperfusion injury. *N Engl J Med, 357*(11), 1121-1135.

Yin, J., Tu, C., Zhao, J., Ou, D., Chen, G., Liu, Y., & Xiao, X. (2012). Exogenous hydrogen sulfide protects against global cerebral ischemia/reperfusion injury via its anti-oxidative, anti-inflammatory and anti-apoptotic effects in rats. *Brain Res, 1491*, 188-196.

Yin, J., Tu, C., Zhao, J., Ou, D., Chen, G., Liu, Y., & Xiao, X. (2013). Exogenous hydrogen sulfide protects against global cerebral ischemia/reperfusion injury via its anti-oxidative, anti-inflammatory and anti-apoptotic effects in rats. *Brain Res, 1491*, 188-196.

Zanardo, R. C., Brancaleone, V., Distrutti, E., Fiorucci, S., Cirino, G., & Wallace, J. L. (2006). Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. *FASEB J, 20*(12), 2118-2120.

Zhang, F., Siow, Y. L., & O, K. (2004). Hyperhomocysteinemia activates NF-kappaB and inducible nitric oxide synthase in the kidney. *Kidney Int, 65*(4), 1327-1338.

Zhang, H., Moochhala, S. M., & Bhatia, M. (2008). Endogenous hydrogen sulfide regulates inflammatory response by activating the ERK pathway in polymicrobial sepsis. *J Immunol, 181*(6), 4320-4331.

Zhang, H., Zhi, L., Moochhala, S., Moore, P. K., & Bhatia, M. (2007). Hydrogen sulfide acts as an inflammatory mediator in cecal ligation and puncture-induced sepsis in mice by upregulating the production of cytokines and chemokines via NF-kappaB. *Am J Physiol Lung Cell Mol Physiol, 292*(4), L960-971.

Zhang, Z., Huang, H., Liu, P., Tang, C., & Wang, J. (2007). Hydrogen sulfide contributes to cardioprotection during ischemia-reperfusion injury by opening K ATP channels. *Can J Physiol Pharmacol, 85*(12), 1248-1253.

Zhao, W., Zhang, J., Lu, Y., & Wang, R. (2001). The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. *EMBO J, 20*(21), 6008-6016.

Zuidema, M. Y., Yang, Y., Wang, M., Kalogeris, T., Liu, Y., Meininger, C. J., Hill, M. A., Davis, M. J., & Korthuis, R. J. (2010). Antecedent hydrogen sulfide elicits an anti-inflammatory phenotype in postischemic murine small intestine: role of BK channels. *Am J Physiol Heart Circ Physiol, 299*(5), H1554-1567.

Appendix I

Reagents/Chemicals	*Company*
2-(4-Hydroxyphenyl) ethanol, Tyrosol	Sigma-Aldrich
Acetic acid, Glacial	Fisher Scientific
Acrylamide/bis 30% solution 37.5:1	Bio-Rad
Adenosine triphosphate-^{32}P, [^{32}P]-ATP	PerkinElmer
anti-CBS antibody	Abnova Corporation
anti-CSE antibody	GeneTex
anti-iNOS antibody	Calbiochem
Anti-nitrotyrosine antibody	Cayman Chemical
anti-rabbit IgG antibody	Cell Signaling Technology
HRP-conjugated anti-mouse antibody	Cell Signaling Technology
ALT kit	Genzyme Diagnostics
AST kit	Genzyme Diagnostics
Bovine serum albumin	BSA EMD
Bromophenol blue	Sigma-Aldrich
Chloroform	Fisher Scientific
Creatinine kit	Genzyme Diagnostics
Cytokines assay kit	Meso Scale Discovery
Diethyl pyrocarbonate, DEPC	Sigma-Aldrich
Dimethyl sufoxide, 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
Eosin Y	Sigma-Aldrich
Ethylenediaminetetraacetic acid, EDTA	Sigma-Aldrich
Ethylene glycol tetraacetic acid, EGTA	Sigma-Aldrich
Ferric chloride, $FeCl_3$	Sigma-Aldrich
Fetal bovine serum, FBS	PAA
First strand buffer 5X	Invitrogen
Formaldehyde (36%)	Sigma-Aldrich
Hanks balanced salt solution, HBSS	Hyclone
Hematoxyline, Harris	Sigma-Aldrich
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	Fisher Scientific
Hydrochloric acid, HCl	Fisher Scientific
Isopropanol	Fisher Scientific
iQ-SYBR green supermix reagent	Bio-Rad
Keratinocyte-serum free medium	Invitrogen
L-Cysteine	Sigma-Aldrich
Leupeptin	Sigma-Aldrich
Mercaptoethanol-β	Sigma-Aldrich
Methanol	VWR
M-MLV-Reverse transcriptase 200 U/ml	Invitrogen
N-(1-Naphthyl)ethylenediamine dihydrochloride, NEDA	Sigma-Aldrich

N,*N*-Dimethyl-*p*-phenylenediamine sulfate salt	Sigma-Aldrich
Nonidet-P	Sigma-Aldrich
O-(Carboxymethyl) hydroxylamine hemihydrochloride, AOAA	Sigma-Aldrich
Oligo(dT)12-18 primer, 0.5 mg/ml	Invitrogen
Pentobarbital solution	Sigma-Aldrich
Phenylmethanesulfonyl fluoride	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 hydrosulfide hydrate, NaHS	Sigma-Aldrich
Sodium hydroxide, NaOH	Fisher Scientific
Sodium nitrate, $NaNO_3$	Fisher Scientific
Sulfanilamide	Sigma-Aldrich
Superoxide dismutase, SOD	Sigma-Aldrich
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	Fisher
Zinc acetate dihydrate	Sigma-Aldrich
Zinc sulfate heptahydrate, $ZnSO_4$	Sigma-Aldrich

Appendix II

Buffers	*Components*
Buffer A	10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 1 mM DTT, pH 7.9
Buffer C	20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT, pH 7.9
Cell Lysis Buffer	50 mM Tris-HCl, 150 mM NaCl, pH 7.4
Phosphate buffered solution, PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4
Protein Lysis Buffer	20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2.1 μM leupeptin, 1 mM PMSF, 1% Triton-X 100
Reaction Buffer	100 mM Tris, 1 M NaCl, 50 mM DTT, 10 mM EDTA, 40% Glycerol, 1 mg/ml BSA, 50 ng/ml double-strand poly (dI-dC), pH 7.5
Running Buffer	25 mM Tris, 0.19 M Glycine, 0.1% SDS
Seperating gel Buffer	1.5 M Tris, 0.4% SDS, pH 8.8
SDS sample Buffer	20 mM Tris, 25% Glycerol, 10% SDS, 0.02% Bromophenol Blue, 5% β-mercaptoethanol
Stacking gel buffer	0.5 M Tris, 0.4% SDS, pH 6.8
Stripping buffer	2% SDS, 95 mM β-mercaptoethanol in 1X TBS
TBE Buffer 10X	89 mM Tris, 89 mM Boric acid, 2 mM EDTA, pH 8.4 TBS 0.02 M Tris, 0.14 M NaCl, pH 7.6
Transfer Buffer	20 mM Tris, 0.15 M Glycine, 20% Methanol

Appendix III

Equipment	*Company*
Centrifuge 5804R	Eppendorf
Centrifuge 5810	Eppendorf
Dry Bath Incubator	Fisher Scientific
Forma Direct Heat CO_2 Incubator HEPA Class 100	Thermo Scientific
IMx	Abbot
iQ5 real-time PCR Cycler	Bio-Rad
Lumet LB9507	Berthold Technologies
Microscope Fluorescence IX81	Olympus
Microscope Upright BX43	Olympus
Minispin	Eppendorf
MSD imager 2400	Meso Scale Discovery
Scintillation counter LS 6500	Beckman Coulter
Slab Gel Dryer 2000	ThermoSavant
SpectraMax	Molecular Devices
Spectrophotometer DU 800	Beckman Coulter
Ultracentrifuge Optima MAX	Beckman Coulter
Waterbath Isotemp 205	Fisher Scientific