INVESTIGATING THE DETRIMENTAL EFFECT OF HOMOCYSTEINE AND THE REGULATION OF TRANSSULFURATION PATHWAY IN KIDNEY ISCHEMIA-REPERFUSION INJURY

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The University of Manitoba
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In partial fulfillment of the Requirements for the degree of
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Department of Animal Science
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Investigating the Detrimental Effect of Homocysteine and the Regulation of Transsulfuration Pathway in Kidney Ischemia-Reperfusion Injury

BY

Gamika A. Prathapasinghe

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

Doctor of Philosophy

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ABSTRACT


Investigating the detrimental effect of homocysteine and the regulation of transsulfuration pathway in kidney ischemia-reperfusion injury. Major Professor; Dr. Karmin O.

Ischemia followed by reperfusion is a major cause for renal injury in both the native kidney and renal allograft. Hyperhomocysteinemia is independently associated with allograft loss in kidney transplanted patients. The aim of the first part of the study was to determine the role of homocysteine (Hcy) in ischemia-reperfusion-induced renal injury. The left kidney of a Sprague-Dawley rat was subjected to ischemia alone or ischemia followed by different reperfusion periods. Hcy levels were elevated 2.9- and 1.5-fold in kidneys subjected to ischemia alone or ischemia-reperfusion, respectively. Administration of anti-Hcy antibodies into the kidney not only abolished ischemia-reperfusion induced oxidative stress and cell death in kidneys but also restored renal function after 1-h of reperfusion. Further investigation revealed that elevation of Hcy level in the kidney upon ischemia-reperfusion was due to reduced activity of cystathionine-β-synthase (CBS), a key enzyme in Hcy metabolism. The aim of the second part of the study was to investigate the effects of pH and nitric oxide (NO) on the CBS activity in the kidney during ischemia-reperfusion. The pH was markedly reduced in kidneys upon ischemia due to metabolic acidosis. Injection of alkaline solution into the kidney partially restored the CBS activity. Further analysis revealed that the reduction of
CBS activity during reperfusion was accompanied by a significant elevation of nitric oxide synthase activity and NO metabolites (nitrate and nitrite) in the kidney tissue. Injection of a NO scavenger, restored the CBS activity in kidneys subjected to ischemia-reperfusion.

The aim of the third part of the study was to characterize the endogenous production of H_{2}S by the rat kidney. Of total CBS activity, 1/3 was committed in the H_{2}S producing pathway. Both Hcy and cysteine (Cys) were utilized as substrates in this pathway however, Cys was found to be the limiting substrate. Ischemia-reperfusion resulted in a significant reduction of the CBS activity in the H_{2}S producing pathway as well. These results suggested that metabolic acidosis during ischemia and accumulation of NO metabolites during reperfusion contributed to reduced CBS activity in both the standard transsulfuration pathway as well as the H_{2}S producing pathway leading to an elevation of renal Hcy levels, which in turn, played a detrimental role in the kidney.
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I would also like to thank my committee members Dr. Grant N. Pierce, Dr. Karin, Wittenberg and Dr. Jim D. House for their advice, suggestions made at the committee meetings and critical review of the draft of this thesis. My sincere appreciation to Dr. Yaw L. Siow for his suggestions and technical support.

On this note I would like to extend warmest of heartfelt thanks to fellow graduate students Connie Woo, Vathsala Edirimanne, San-young, Stephanie Armstrong, Wu Nan and Jennifer Kroeker who offered a helping hand in times of need. Your kindness and willingness to assist in any way was highly appreciated.

Finally, I am forever thankful and in debt to my wife Vathsala, and my parents who have stood beside me in mind, body and spirit as I took on the challenges and obstacles of this research. Their encouragement, helpfulness and support have helped me not only to overcome but also to persevere and excel. Thank you all for your support in the completion of this degree.
To

my beloved Grandparents,

Don Dionis Gunaratne (late grandfather)

Dona Daisy Gunaratne (grandmother)

Dona Sisiliana Wickramanayake (late step grandmother)
PUBLICATIONS


4. Woo CW, Prathapasinghe GA, Edirimanne VER, Hwang S., Siow YL and O K. Effect of hyperhomocysteinemia and folic acid supplementation on liver function. 6th Conference on Homocysteine Metabolism, World Congress on Hyperhomocysteinemia, Saarbrücken, Germany, June 5-9, 2007


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<td>ARF</td>
<td>Acute renal failure</td>
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<tr>
<td>ATN</td>
<td>Acute tubular necrosis</td>
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<td>BHMT</td>
<td>Betaine:Homocysteine methyltransferase</td>
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<td>CBS</td>
<td>Cystathionine-β-synthase</td>
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<td>CGL</td>
<td>Cystathionine-γ-lyase</td>
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<tr>
<td>Cys</td>
<td>Cysteine</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>ESRD</td>
<td>End stage renal disease</td>
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<td>GSH</td>
<td>Glutathione</td>
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<td>Hcy</td>
<td>Homocysteine</td>
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<td>IP</td>
<td>Ischemia preconditioning</td>
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<td>MDA</td>
<td>Malondialdehyde</td>
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<td>MTHFR</td>
<td>Methylenetetrahydrofolate reductase</td>
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<td>NAC</td>
<td>N-acetyl cysteine</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<td>NOS</td>
<td>Nitric oxide synthase</td>
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<td>PLP</td>
<td>Pyridoxal-5-phosphate</td>
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<tr>
<td>PTIO</td>
<td>2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SAH</td>
<td>S-adenosylhomocysteine</td>
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<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
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<td>TUNEL</td>
<td>Terminal deoxyribonucleotide transferase (TdT)-mediated dUTP Nick-End labeling</td>
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CHAPTER 1

INTRODUCTION
Kidney transplantation is the preferred therapeutic option for patients with end stage renal disease (ESRD). It offers greater longevity and quality of life than either peritoneal dialysis or hemodialysis in addition to being the most cost-effective treatment for ESRD (Winkelmayer et al., 2002). However, due to the shortage of suitable kidneys for transplantation, there is a long waiting list of patients. For example, at the end of 2004, there were 57,910 patients in this list in the United States. Yet, only 16,481 of renal transplantations were performed in the following year and of those 9,913 kidneys were originated from non-heart beating (cadaveric) donors. The transplant outcome is less successful in the recipients of non-heart beating kidneys when compared to the recipients of kidneys from living donors (US, Department of Health and Human Services 2005). Kidneys from non-heart beating donors subjected to a greater duration of normothermic (warm) ischemia which is a major factor limiting the availability of suitable kidneys for transplantation (Toledo-Pereyra, 1989; Callaghan & Bradley, 2006). The re-establishment of blood flow after transplantation initiates tissue injury. It interrupts the regulation of oxygen metabolism leading to intense production of oxygen free radicals. The integrity of vascular endothelium is compromised by the action of oxygen free radical on endothelial cells. Thus, oxidative stress is considered as one of the contributors in the pathogenesis of acute renal injury or progressive renal damage (Autor et al., 1984).

A recent study indicated that elevated plasma total homocysteine (Hcy) was independently associated with increased kidney allograft loss in humans (Winkelmayer et al., 2005). Hcy, at elevated levels, is associated with oxidative stress in extra-renal tissues (Wang et al., 2002; Au-Yeung et al., 2004), as well as in the kidney (Zhang et al., 2004;
Diez et al., 2005). The free or reduced sulfhydryl group of Hcy is highly reactive at the physiological pH. In the presence of molecular oxygen Hcy undergoes thiol oxidation reactions (Hoffer et al., 2001). Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide are produced during autooxidation of Hcy (Misra, 1974). It has been suggested that autooxidation of Hcy represents one of the mechanisms contributing to Hcy-induced cell injury (Starkebaum & Harlan, 1986; Welch et al., 1997; Wall et al., 1980). The levels of peroxynitrite and oxygen-derived free radicals are significantly elevated in kidneys isolated from hyperhomocysteinemic rats (Zhang et al., 2004). A recent epidemiological study has revealed a positive association between the plasma Hcy level and development of chronic kidney disease in the general population (Ninomiya et al., 2004). Hyperhomocysteinemia is a common clinical finding in patients with chronic kidney diseases and occurs almost uniformly in patients with end-stage renal disease (Stam et al., 2005; Bostom & Culleton, 1999).

Hcy is an intermediate amino acid formed during the metabolism of methionine. Hcy can be metabolized via two major pathways, namely, remethylation pathway and transsulfuration pathway (Finkelstein et al., 1971). In the remethylation pathway, Hcy is converted to methionine by using folate or betaine as methyl group donor. In the transsulfuration pathway, Hcy is irreversibly catabolized to cysteine (Cys). Although kidney contains enzymes that are responsible for Hcy metabolism via remethylation and transsulfuration pathways (Finkelstein et al., 1971), it is estimated that the majority of Hcy is metabolized through the transsulfuration pathway (House et al., 1997). In mammals, cystathionine-β-synthase (CBS; EC 4.2.1.22) catalyzes the rate-limiting step in the transsulfuration pathway (Kery et al., 1994; Taoka et al., 1998).
Mammalian CBS is a heme containing and pyridoxal-5'-phosphate (PLP; vitamin B₆) dependent enzyme (Kery et al., 1994). Heme in the CBS may remain either in Fe(III) or Fe(II) forms depending on the tissue pH (Pazicni et al., 2004). The optimal activity of CBS in vitro has been recorded at pH 8.5 at which Fe(II) form of heme predominates. Thus, the redox sensitive regulation of CBS is believed to be brought about by the heme group (Pazicni et al., 2004). Normothermic ischemia causes metabolic acidosis in the affected organ (Stanley et al., 1992). This may affect the activity of CBS and the Hcy level during ischemia. In addition to pH, it has been reported that recombinant human CBS binds NO in the ferrous Fe(II) state of heme resulting in the inactivation of the enzyme (Taoka & Banerjee, 2001). Kidney tissue NO level is significantly increased during ischemia as well as reperfusion periods (Salom et al., 2005; Saito & Miyagawa, 2000; Prathapasinghe et al., 2007). A severe increase in plasma NO level after 6 h of reperfusion of the kidney, due to the up-regulation of iNOS activity, has been reported (Chatterjee et al., 2003). Excessive production of NO, particularly from iNOS, or exogenous supply of NO before ischemia was found to play a detrimental role in ischemia-reperfusion induced renal injury (Basireddy et al., 2006; Parlakpinar et al., 2006; Chatterjee et al., 2003).

Development of cardiovascular disease (CVD) which includes ischemic heart disease, peripheral vascular disease, cerebrovascular disease and cardiac failure) accounts for approximately half of all deaths in kidney recipients with a functioning graft (Briggs, 2001). A 10-fold higher relative risk of dying of CVD has been reported in kidney recipients aged between 25-34 years when compared with age and gender matched control group (Foley et al., 1998). Recently, H₂S has received attention as an important
signaling molecule in the cardiovascular system. H$_2$S is synthesized in mammalian tissues by the two transsulfuration pathway enzymes namely, CBS and cystathionine-$\gamma$-lyase (CGL; EC 4.4.1.1) (Wang, 2002). Hcy and Cys are utilized as substrates for these reactions. H$_2$S has cytoprotective effects by upregulating endogenous antioxidant systems (Yan et al., 2006). Furthermore, it lowers blood pressure in the rat (Zhao & Wang, 2002; Cheng et al., 2004). H$_2$S plays a role in the regulation of vascular function both in health and diseases (Wang, 2002). Conversely, CBS deficiency leads to hyperhomocysteinemia which, in turn, causes endothelial dysfunction and hypertension in the rat (Yan et al., 2004). In disease conditions like diabetes, in which the vascular function is impaired, the biosynthesis and release of H$_2$S is increased (Yusuf et al., 2005). Conversely, type-II diabetes in rats with no renal complications, the plasma Hcy level is decreased and the activity of transsulfuration pathway enzymes are increased (Wijekoon et al., 2005). These findings suggest an inverse relation between plasma Hcy and H$_2$S levels.

In view of these facts, we designed our experiments first, to determine the role of Hcy in the pathogenesis of renal ischemia-reperfusion injury, second, to determine the effect of the metabolic acidosis and the tissue NO level on the activity of CBS which is the key enzyme in the Hcy catabolism in the rat kidney, third, to quantify the endogenous H$_2$S synthesis in the kidney, the substrate(s) and the enzyme(s) involved, and the effect of renal ischemia-reperfusion on plasma H$_2$S homeostasis. Male Sprague-Dawley rats with body weight between 250-300g were used as the animal model and the clamping of left renal artery for 15 to 60 minutes followed by 1 to 24 h reperfusion was employed to induce renal ischemia-reperfusion injury. We observed a significant increase in the kidney tissue and plasma Hcy level during ischemia and reperfusion periods,
respectively. Hcy was found to play a detrimental role in the pathogenesis of renal ischemia-reperfusion injury. The metabolic acidosis during ischemia caused the initial (reversible) inhibition of CBS activity and the interaction of CBS and NO during reperfusion prevented the regaining of CBS activity during reperfusion. CBS catalyzed the endogenous synthesis of H₂S in the kidney tissue. Both Cys and Hcy were utilized as substrates. Contrarily to Hcy, both tissue and plasma H₂S levels were significantly decreased in response to renal ischemia-reperfusion.
CHAPTER 2
LITERATURE SURVEY
2.1 METABOLISM OF HOMOCYSTEINE

Hcy is a non-protein amino acid produced as an intermediate of the methionine metabolic pathway. Methionine is one of the eight nutritionally essential/indispensable amino acids and it is the only indispensable sulfur-containing amino acid in mammals. Cys is the other sulfur-containing amino acid which is synthesized endogenously from methionine (Finkelstein et al., 1988). Both methionine and Cys are normally consumed as constituents of dietary proteins in contrast to Hcy which is available in trace amounts in food (Di Buono et al., 2001). The structures of the three sulfur-containing amino acids are depicted in Figure 2.1.

The metabolic pathways of sulfur-containing amino acids are shown in Figure 2.2. Methionine metabolic pathway can be categorized into three major segments namely transmethylation, remethylation and transsulfuration (Fig. 2.2). Liver is known to be the major organ of methionine metabolism and it contains all the enzymes shown in Figure 2.2. A few other organs which include kidney, small intestine and pancreas also contain the enzymes for the transmethylation, remethylation and transsulfuration, however, with relatively low levels of activity compared to liver (Finkelstein, 1990). The foremost functions of transmethylation, remethylation and transsulfuration are to provide methyl groups for methylation reactions, to conserve the methionine carbon skeleton via remethylation and to irreversibly catabolize excess methionine, respectively (Stipanuk, 2004). In addition, transsulfuration is a source of Cys for the synthesis protein, glutathione (GSH) and taurine. Several minor pathways also metabolize methionine. For example, methionine is transaminated to its keto acid, \(\alpha\)-keto-\(\gamma\)-methiolbutyrate. The other possible alternative pathways are polyamine synthesis and the cleavage of
Methionine, homocysteine (Hcy) and cysteine (Cys) are sulfur-containing amino acids. Hcy and Cys are derived from the metabolism of methionine. Thus, methionine is the only sulfur-containing amino acid which is dietarily essential. Both Hcy and Cys contain free sulfhydryl group (SH).

Figure 2.1  Structures of sulfur-containing amino acids

Methionine

Homocysteine

Cysteine
S-adenosylmethionine (SAM) into methylthioadenosine, which may be converted further, into homoserine thiolactone (Stipanuk, 2004).

2.1.1 TRANSMETHYLATION PATHWAY

Methionine is converted into Hcy by the transmethylation pathway. In the initial reaction of the transmethylation pathway, methionine condenses with ATP, yielding the phosphorylated SAM (Mudd et al., 1965). This reaction is catalyzed by S-adenosylmethionine synthase (SAM synthase; EC 2.5.1.6). The isoforms of this enzyme and the affinity of them towards methionine are discussed in a separate paragraph under allosteric regulation of Hcy metabolism. The next reaction is catalyzed by a large number of transmethylases by which the methyl group of SAM is transferred to anyone of the large number of methyl acceptors which include DNA, RNA and protein methylation, creatine synthesis and phosphotidylcholine synthesis (Finkelstein, 1990). As a result, SAM is converted to S-adenosylhomocysteine (SAH) which is, then, reversibly hydrolyzed to Hcy and adenosine by S-adenosylhomocysteine hydrolase (SAH hydrolase; EC 3.3.1.1) (Finkelstein, 1974).

2.1.2 REMETHYLATION PATHWAY

Metabolism of Hcy is at a key junction of the metabolic pathway of sulfur-containing amino acids and it links the methionine metabolic cycle with folate metabolism. The carbon skeleton of Hcy derived from methionine can be conserved by remethylation. In the remethylation pathway Hcy can be remethylated to methionine via two different
Proteins

Methionine

THF

S-Adenosyl-methionine

Methionine synthase

Remethylation

ATP

Dimethylglycine

BHMT

S-Adenosylhomocysteine

Methylated acceptor

Cystathionine

Cystathionine synthase

β-synthase

Transsulfuration

Cystathionine γ-lyase

α-ketobutyrate+NH₃

Cysteine

Glutathione

Protein

Taurine

Serine

Glycine

Vit. B₄

Methylene THF

Methyl THF

Methenyltetrahydrofolate reductase

Figure 2.2 Metabolic pathways of sulfur-containing amino acids

Metabolism of sulfur-containing amino acids comprises three major pathways. Methionine is converted to S-adenosylmethionine (SAM) by SAM synthase as the initial step in the transmethylation pathway. After donating the methyl group to anyone of the great number of methylation reactions, SAM is converted to S-adenosylhomocysteine (SAH). SAH is then, reversibly hydrolyzed to homocysteine (Hcy) and adenosine. The intracellular and extracellular Hcy concentrations are kept low by the remethylation and transsulfuration pathways. The carbon skeleton of Hcy is preserved back to methionine via the action of either methionine synthase or betaine:Hey methyltransferase (BHMT). The reactions in the transsulfuration pathway irreversibly catabolize Hcy into cysteine which is the limiting amino acid for the glutathione biosynthesis.
routes. By methionine synthase (E.C.2.1.1.13), which has a ubiquitous distribution in mammalian tissues, Hcy is remethylated to methionine by utilizing the methyl group of \( N^5 \)-methyltetrahydrofolate (5-MTHF) (Finkelstein et al., 1971; Xue & Snoswell, 1985). This enzyme is important not only as a reaction in remethylation pathway, but also as the focus that connects Hcy, vitamin B\(_{12}\) (Cobalamin) and folate metabolism. The mammalian methionine synthase contains tightly bound vitamin B\(_{12}\) as the prosthetic group. Methionine is generated as a result of the methyl group transferred from methylated cobalamin to Hcy. A small amount of SAM is needed as a catalyst for the initial priming of enzyme bound cobalamin (Burke et al., 1971; Cantoni et al., 1982). Methionine synthase in the rat liver has a \( K_m \) value of 60 \( \mu \)M for Hcy (Finkelstein, 1974).

In addition to methionine synthase, some tissues possess another route for the remethylation of Hcy to methionine. This reaction is catalyzed by betaine:Hcy methyltransferase (BHMT; E.C. 2.1.1.5). BHMT is a zinc metalloenzyme (Breksa & Garrow, 1999; Evans et al., 2002). Contrary to the ubiquitous distribution of methionine synthase, BHMT has more limited tissue distribution with a significant activity found only in the rat liver (Finkelstein et al., 1971). A significant BHMT activity is found in the liver, kidney and eye lens in humans (Skiba et al., 1982). The methyl group donor of the reaction catalyzed by BHMT is betaine which is an obligatory intermediate in choline catabolism. The reported \( K_m \) values of the rat liver BHMT are 15-21 \( \mu \)M and 49-56 \( \mu \)M for Hcy and betaine, respectively (Finkelstein et al., 1971).
2.1.3 TRANSSULFURATION PATHWAY

Two PLP-dependent enzymes namely, cystathionine-β-synthase (CBS; EC 4.2.1.22) and cystathionine-γ-lyase (CGL; EC.4.4.1.1) catalyze the transsulfuration of Hcy to Cys which is the final product of the transsulfuration pathway (Fig. 2.2). The initial and the rate-limiting step of the transsulfuration pathway is catalyzed by CBS (Banerjee et al., 2003). This reaction is physiologically irreversible (Sakamoto et al., 2002). In this reaction, serine is condensed with Hcy and it provides the carbon skeleton of Cys (Finkelstein & Martin, 1986). CBS is a type-II (β) family enzyme of the PLP-containing enzymes and it is unique in being dependent on two cofactors, PLP and heme (Kery et al., 1994). Human CBS has a subunit molecular weight of 63 kDa and exists as of a tetramer of identical subunits (Taoka et al., 1998). The Km of rat liver CBS for Hcy is 800 μM (Finkelstein, 1974). The CGL is also a PLP-dependent enzyme which catalyses the γ-cleavage of cystathionine (Fig. 2.2). This is the final step of the transsulfuration pathway where cystathionine is cleaved to Cys, α-ketobutyrate and NH₃ (Fig. 2.2). Cys has a number of different metabolic fates, depending on the tissue. These include its incorporation into proteins as well as GSH and synthesis of taurine (Stipanuk, 2004). Only very few organs such as liver, kidney, pancreas and small intestine, possess the capability to synthesize Cys from methionine or Hcy (Mudd et al., 1965; Finkelstein, 1990). Therefore, the synthesis of Cys from methionine and Hcy is limited to these tissues. Other tissues depend on the exogenous supply through the diet or export from any of those four organs, for their Cys requirement (Mudd et al., 1965). Thus, transsulfuration is essential as a source of Cys in addition to its role as a mode of elimination of excess Hcy.
2.2 THE REGULATION OF HOMOCYSTEINE METABOLISM

The distribution of Hcy between the transsulfuration and remethylation pathways is regulated in many ways which include dietary regulation, allosteric regulation, hormonal regulation and redox regulation.

2.2.1 DIETARY REGULATION

Dietary levels of protein, methionine and Cys have a great impact on the distribution of Hcy between remethylation and transsulfuration pathways. Additionally, the dietary factors such as folate, Vitamin B₂, B₆ and B₁₂ also play regulatory role in the homeostasis of Hcy. The role of these factors in Hcy-lowering is discussed in section 2.5.2.

2.2.1.1 Effect of Dietary Methionine on CBS and Methionine Synthase Activity

At basal methionine level in the diet, Hcy moieties cycle through the remethylation pathway about 1.5-2.0 times before they are catabolized through the transsulfuration pathway. Conversely, the cycling of Hcy moiety is increased two-fold when dietary methionine content is reduced by half. When the dietary methionine level is high, Hcy cycling falls below basal levels (Eloranta et al., 1990). Since Hcy can be remethylated to methionine, CBS has to compete for the available Hcy with the remethylation enzymes (Finkelstein & Martin, 1986). A study using rat liver extract and Hcy as the substrate at their in vivo concentrations has revealed that CBS has the lowest affinity for Hcy when compared with methionine synthase and BHMT (Finkelstein & Martin, 1986). Hcy catabolism through the transsulfuration pathway is severely impaired (by 53%) on adaptation from high protein to a low protein diet. Supplementation of methionine in the diet increases the CBS activity with a concomitant decrease in the hepatic levels of
methionine synthase. In contrast to the diminished activity of CBS upon adaptation to a low protein diet, the hepatic CBS activity is increased by 12-fold when the dietary methionine level is increased from 0.3 to 3.0% (Finkelstein & Martin, 1986). The demand for hepatic serine which is the co-substrate of this reaction is increased. Hence, the hepatic content of serine is decreased when the dietary methionine level is increased above 0.3% (Finkelstein & Martin, 1986).

2.2.1.2 Dietary Regulation of BHMT Activity

BHMT activity is inhibited in vitro by SAM (Finkelstein & Martin, 1984). Liver BHMT activity is increased in rats fed a high protein diet (Finkelstein et al., 1971). The response of BHMT to methionine in the diet is biphasic; the supplementation of a low level of methionine (0.3%) to a methionine-free diet in rats causes a significant decrease in the enzyme activity. In contrast, there is a marked increase in rat liver BHMT activity in response to a 1% increment of methionine in the diet (Finkelstein et al., 1982). When rats are fed a methionine deficient diet, the liver BHMT activity is increased and in this situation BHMT takes part in methionine conservation (Finkelstein et al., 1982).

2.2.1.3 Sparing Effect by Dietary Cysteine

Since transsulfuration is physiologically irreversible, Cys is not a precursor of methionine. However, the supplementation of Cys in the diet can replace the dietary requirement in humans for methionine by more than half (Di Buono et al., 2003; Finkelstein et al., 1988). The supplementation of 0.8% of Cys in the diet results in a significant decrease in SAM and a two-fold increase in the serine content in the rat liver. The outcome is 70% sparing of the dietary requirement for methionine (Finkelstein et al., 1988). The enzymatic basis for this phenomenon which is discussed elsewhere, is the
marked decrease in hepatic CBS due to the low SAM level and the resulting absence of a potent allosteric stimulus on the CBS enzyme (Di Buono et al., 2001; Finkelstein & Mudd, 1967). This is consistent with the early findings that liver CBS activity is impaired in rats fed a low methionine diet with added Cys to meet the sulfur amino acid requirement (Finkelstein & Mudd, 1967). Cys, which is derived from the transsulfuration pathway or provided through dietary sources, is used for synthetic reactions which include incorporation into proteins and utilization as a precursor in the synthesis of the tripeptide GSH and the synthesis and catabolism of taurine (Stipanuk, 2004). Among the critical functions of these metabolic products of Cys, synthetic reactions, osmotic regulation, antioxidative function, detoxification and involvement in nervous system functions are prominent (Stipanuk, 2004). However, excess Cys is associated with some cytotoxic and neurotoxic effects (Montine et al., 1997; Reis et al., 2000). Elevated plasma Cys has been suggested to be an independent risk factor for cardiovascular disease (El-Khairy et al., 2001).

2.2.2 ALLOSTERIC REGULATION

The intracellular concentration of SAM is one of the determinants of distribution of Hcy between remethylation and transmethylation pathways. SAM which is the high energy sulfonium compound synthesized at the first reaction in the transmethylation pathway (Fig. 2.2), is an allosteric modulator of two enzymes namely the SAM synthase and the CBS in the remethylation and transsulfuration pathways, respectively (Stipanuk, 2004). There are three isoforms of SAM synthases. Two of them, SAM synthase –I (α) and III (β) are specific to the liver. SAM synthase –I has a higher affinity for methionine and is
believed to function at normal physiological conditions (Hoffman et al., 1979). SAM synthase –III has the lowest affinity among all three SAM synthase isozymes for methionine, and is thought to function under conditions of high intake of methionine (Finkelstein et al., 1978). SAM synthase-II (γ) is found in hepatic as well as in extrahepatic tissues. It has the lowest Kₘ for methionine and is strongly inhibited by SAM (Hoffman et al., 1979). This kind of product inhibition is extremely important, because rapid synthesis of SAM would be fatal, perhaps as a consequence of ATP depletion (Hardwick et al., 1970). Because of this low Kₘ and the resulting high affinity of the γ isoform for methionine, the velocity of SAM synthesis would be at its maximal in extrahepatic tissues (Stipanuk, 2004). However, the SAM synthase-III can respond to a high dietary methionine load and as a result the hepatic SAM level is increased (Finkelstein et al., 1978). This, in turn, will determine the route that the Hcy moiety takes at the remethylation and the transsulfuration junction (Selhub, 1999). SAM allosterically inhibits methylenetetrahydrofolate reductase (MTHFR; EC. 1.1.99.15), which catalyzes the formation of 5-MTHF from N⁵,₁⁰ methylenetetrahydrofolate, one of the two methyl donors for the remethylation of Hcy to methionine (Finkelstein & Martin, 1984). On the other hand, SAM activates CBS in the transsulfuration pathway. Experimental overloading of dietary methionine causes attenuation of remethylation enzymes and a stimulation of CBS activity (Finkelstein & Martin, 1986). Conversely, a deficiency of dietary methionine elevates blood Hcy concentrations because of decreased synthesis of SAM and dysregulation of sulfur amino acid metabolism (Ingenbleek & Young, 2004). In patients with inherited SAM synthase deficiency a decreased activity of CBS is seen, possibly due to the lack of SAM mediated allosteric activation of CBS (Stabler et al.,
The SAM-mediated allosteric activation of the CBS occurs via the binding of SAM with the C-terminal domain of the CBS enzyme. Mammalian CBS contains an N-terminal heme (protoporphyrin IX) - containing domain and a catalytic domain in addition to the C-terminal domain. The catalytic domain binds to PLP (Banerjee et al., 2003; Taoka et al., 1998).

2.2.3 HORMONAL REGULATION

At the transcriptional level, hepatic CBS gene expression is hormonally regulated. Insulin is found to decrease the expression of the CBS gene while glucagon and glucocorticoid hormones increase CBS gene expression (Jacobs et al., 1998; Jacobs et al., 2001; Ratnam et al., 2002; Goss, 1986). The overexpression of CBS in children with Down’s syndrome results in significantly reduced plasma levels of Hcy, methionine, SAH and SAM. Plasma cystathionine and cyst(e)ine are significantly increased in these patients (Finkelstein, 2000). Moreover, sex hormones are involved in the regulation of transsulfuration pathway by acting on the CBS gene expression in the kidney, in particular (Vitvitsky et al., 2004). In mice, renal CBS activity is two-fold higher in males than in females. Castration of male mice results in the reduction of renal CBS expression (Vitvitsky et al., 2004). Interestingly, the effect of testosterone on renal CBS activity differs between species. In mice and hamster, testosterone increases renal CBS activity while rabbit does not show any such difference (Vitvitsky et al., 2007). Conversely, human and rat CBS activity in the kidney is greater in females suggesting testosterone exerts a negative effect in these two species (Vitvitsky et al., 2007).
2.2.4 REDOX REGULATION

One of the main functions of the transsulfuration pathway is to provide the limiting substrate, Cys that is required for the GSH synthesis (Mosharov et al., 2000). GSH is the most abundant intracellular redox buffer and its concentration can range from 1 to 10 mM depending on the tissue type (Mosharov et al., 2000; Beatty & Reed, 1980). Oxidizing conditions diminish the activity of methionine synthase by oxidizing the cofactor intermediate, cobalamin and/or by oxidizing the Cys residues in the essential zinc binding site of the enzyme (Matthews & Daubner, 1982; Goulding & Matthews, 1997). BHMT which is the zinc metalloenzyme in the remethylation pathway is reversibly inhibited by ligand-oxidation and zinc displacement (Millian & Garrow, 1998). Conversely, the key enzyme in the transsulfuration pathway is activated under oxidizing conditions (Taoka et al., 1998). Therefore, the redox regulation of remethylation and transsulfuration pathways secure the availability of Cys for the GSH synthesis by an autocorrective mechanism (Mosharov et al., 2000). The activation of CBS in response to oxidizing stimuli has been tested in vitro. Experiments using human hepatoma cells in culture have shown 1.6- and 2.1-fold increase in cystathionine production in response to 100 μM H₂O₂ or tertiary-butylhydroperoxide, respectively (Mosharov et al., 2000). Similarly, the endogenously produced ROS by pro-inflammatory agents such as tumor necrosis factor-α (TNFα) or lipopolysaccharide (LPS) have increased the CBS activity in cell culture and in mice, respectively (Zou & Banerjee, 2003). Tiron, which is an inhibitor of superoxide anion production, suppresses the cleavage of full-length CBS enzyme. Thus, it has been postulated that the superoxide is involved in the oxidative signaling pathway leading to endoproteolytic cleavage and activation of CBS (Zou & Banerjee, 2003). Conversely, the
presence of antioxidants such as water-soluble derivatives of vitamin E, catalase and superoxide dismutase decrease the activity of the transsulfuration pathway (Vitvitsky et al., 2003). This redox sensitivity of CBS is a function of a change in the oxidation state of the heme cofactor (Taoka et al., 1998). Deletion of the heme binding domain of the CBS enzyme results in the loss of redox sensitivity (Taoka et al., 2002).

2.3 ALTERNATIVE REACTIONS CATALYZED BY TRANSSULFURATION PATHWAY ENZYMES

The standard cellular function of CBS is to catalyze the initial step in the transsulfuration pathway in which serine is condensed with Hcy to produce cystathionine and water (Fig. 2.3.A) (Kery et al., 1994; Taoka et al., 1998). The CGL cleaves cystathionine into Cys, α-ketobutyrate and NH₃ (Fig. 2.2). Both of these enzymes are β-family of PLP-containing enzymes which have the ability to catalyze alternative β-replacement and β-elimination reactions from a variety of different substrates (Fig. 2.3) (Chen et al., 2004). H₂S is produced in place of H₂O in these alternative reactions catalyzed by CBS (Fig. 2.3). CBS could catalyze the synthesis of H₂S from Cys by a β-elimination or α,β-elimination reactions which are reported to occur in mouse brain as well as in rat liver and kidney (Kimura, 2002; Stipanuk & Beck, 1982). In addition, β-replacement reactions could also be a source of H₂S from the reaction between Cys and Hcy or Cys and 2-mercaptoethanol, the latter is not expected to occur in vivo (Jhee et al., 2000). In an alternative reaction, CGL utilizes Cys as a substrate in the production of H₂S (Fig. 2.3.D).
Figure 2.3 Potential alternative reactions catalyzed by cystathionine-β-synthase and cystathionine-γ-lyase in addition to standard transsulfuration pathway (based on ideas from (Chen et al., 2004)
2.4 HOMOCYSTEINE METABOLISM IN THE KIDNEY

Healthy rat kidneys remove approximately 20% of plasma Hcy in a single pass through the kidney (Bostom et al., 1995). Hcy which has a molecular mass of 135 Da, is well within the filtration range of normal glomeruli (House et al., 2000). Similar to other amino acids, the filtered Hcy is intensely reabsorbed (Refsum et al., 1985; Ueland, 1995; Bostom et al., 1995; Guttormsen et al., 1993). About 1-2% (3.5 – 9.8 μmol/day) of Hcy in the glomerular filtrate is excreted (Refsum et al., 1998b; Svardal & Ueland, 1987). In humans, 6% of arterial Hcy is eliminated in a single pass through the kidney (Blom & De Vriese, 2002). The daily filtered Hcy by human kidneys was calculated, assuming the plasma free Hcy concentration is 3 μM and the normal glomerular filtration rate (GFR) is 125 ml/min, to be around 0.5 mmol. Thus, it was calculated about 70% of Hcy in plasma is removed and metabolized by the kidney (Refsum et al., 1998a). However, no significant difference in Hcy concentration across kidneys was observed in fasting patients with coronary artery disease (van Guldener et al., 1998). This discrepancy may be attributed to the low level of Hcy elimination by kidney during fasting and/or the fact that 80% of plasma Hcy is bound to proteins in humans compared with 30% in rats (Brosnan, 2001). Tubular uptake mechanisms for Hcy are achieved by at least two methods. Kinetic studies in the rat kidney revealed low-Km/high-affinity and high-Km/low-affinity systems for Hcy uptake (Foreman et al., 1982). In response to an acute hyperhomocysteinemia, the rat kidney exhibits appreciable capacity and greater rate of Hcy extraction (House et al., 1998). This phenomenon has not been tested in humans since arteriovenous studies on humans are lacking. The only published study in humans showed no significant renal extraction of Hcy under fasting conditions (van Guldener et
al., 1998). Fed and fasted states affect Hcy protein binding and, as a result, the glomerular filtration of Hcy. Following a high protein meal, the plasma Hcy levels, the free fraction of plasma Hcy in particular, was significantly increased (Friedman et al., 2001). The impact of free vs. bound Hcy in the plasma on renal filtration is further emphasized by the effect of the exogenously administered N-acetylcysteine (NAC) on aminothiol protein binding and renal excretion. The free sulphydryl group of exogenously administered NAC interacts with other endogenous sulphydryl containing compounds which include Hcy, displacing them from their protein binding sites. As a result, a mixed disulfide is formed (Burgunder et al., 1989). Patients treated with NAC orally or intravenously demonstrate sudden and dose-dependent reductions in total plasma Hcy and Cys levels (Hultberg et al., 1994; Ventura et al., 1999; Wiklund et al., 1996). Kidney is among the few organs which contain all the enzymes involved in the methionine metabolic pathway (Fig. 2.2). However, in contrast to humans, the rat kidney does not contain BHMT (Finkelstein, 1990). Compared with the liver, BHMT expression in the human kidney is approximately 300% greater (Selhub, 1999). Although the rat kidney contains a detectable level of methionine synthase, about 78% of Hcy is metabolized through the transsulfuration pathway (Foreman et al., 1982; House et al., 1998; House et al., 1997). The remethylation pathway contributes to less than 2% of the disappearance of Hcy in the rat kidney (House et al., 1997). A significant amount of Hcy is converted to SAH (Foreman et al., 1982). The contribution of the transsulfuration and remethylation pathways in the metabolism of Hcy in human kidney has not been quantified. However, the following observations suggest the major role of transsulfuration in the catabolism of Hcy in the human kidney as well. First, the homozygous genotype for the variant allele of
MTHFR is the TT genotype. The point mutation of MTHFR 677G → T gene variant which is a common polymorphism of the gene encoding for the MTHFR enzyme leads to 25% higher total plasma Hcy concentration (Folsom et al., 1998). Second, folate supplementation (0.5 to 5 mg/day) is a proven Hcy-lowering therapy. However, not more than 27% of plasma Hcy can be eliminated by folate therapy in hyperhomocysteinemic patients (van der Griend et al., 2000; Bonaa et al., 2006). In either situation, the remethylation pathway is involved. Based on these two facts, and 70% of plasma Hcy is cleared by kidneys in humans, it could be argued that, similar to the rat kidney, the transsulfuration pathway may be the major pathway which contributes to the metabolism of Hcy in the human kidney.

2.4.1 IMPAIRED KIDNEY AND HOMOCYSTEINE METABOLISM

Uremia-induced malnutrition, retained toxins, deranged hormonal levels, increased urinary amino acid excretion and impaired renal amino acid metabolism are common findings in patients with kidney disease. These conditions profoundly alter the amino acid handling and elimination by the kidneys in this group of patients (Kopple et al., 1978). As a result, the plasma levels of some essential amino acids such as tryptophan, leucine, isoleucine, valine, and lysine are decreased. Conversely, the plasma levels of Cys, citrulline, methylhistidine, glycine, hydroxyproline and all of the nonessential amino acid levels are increased in these patients (Kopple et al., 1978). The prevalence of hyperhomocysteinemia in dialysis patients is over 85% (Moustapha et al., 1999). Deficiencies of folate, vitamin B₁₂, vitamin B₆ and reduced clearance of plasma Hcy due to impaired renal function or impaired extrarenal metabolism of Hcy due to uremic toxins
are believed to be the etiology of hyperhomocysteinemia in patients with ESRD (Friedman et al., 2001; Bostom & Lathrop, 1997). However, the causes of hyperhomocysteinemia in patients in the earlier stages of chronic kidney disease are less clear (Bostom et al., 2001). Treatment with folic acid and vitamin B₁₂ supplementation is highly effective in correcting hyperhomocysteinemia in the general population. However, dialysis patients do not respond to vitamin therapies even at supra-physiological doses (Shemin et al., 2001). The resistance to treatment increases as renal function declines in this group of patients (Sunder-Plassmann et al., 2000). Therefore, it was suggested that hyperhomocysteinemia in kidney diseases is the result of defective clearance of plasma Hcy rather than increased efflux of Hcy into plasma from tissues (Guttormsen et al., 1997). Oral and intravenous loading of Hcy in patients with severe renal insufficiency have revealed that the clearance of plasma Hcy is reduced by 70% (Guttormsen et al., 1997). This may either be due to the impaired intrarenal Hcy clearance and/or uremic-toxin mediated extrarenal impairment of Hcy metabolism. The paucity of data has made it impossible to specify the causative organ and the definitive pathway of hyperhomocysteinemia in renal diseases. However, based on available data, Friedman et al suggested that the loss of metabolically active kidney mass rather than extrarenal tissue might be the cause of hyperhomocysteinemia (Friedman et al., 2001). The inverse relationship between plasma Hcy level and the GFR, which is consistent throughout the nonuremic range of kidney function, is supportive of the suggestion that it is reduced renal function, not the accumulation of uremic toxins, that causes hyperhomocysteinemia (Friedman et al., 2001). Nonetheless, it is not clear which enzymes in the Hcy metabolic pathways in the kidney tissue are affected in dialysis patients.
2.5 HYPERHOMOCYSTEINEMIA

Elevated levels of plasma Hcy, a condition known as hyperhomocysteinemia, is a known risk factor for a number of diseases which include coronary, cerebral, and peripheral vascular diseases and thrombosis (McCully, 2007). In healthy humans, plasma total Hcy concentration which reflects the combined pool of free reduced, free oxidized and bound Hcy in the circulation, is in the range of 5-15 μM (Bostom et al., 2000). Plasma Hcy levels are increased to 16-30 μM, 31-100 μM and greater than 100 μM in mild, moderate and severe hyperhomocysteinemia, respectively (Refsum et al., 2004). In 1962, thirty years after the discovery of Hcy by DuVigneaud, the first case of a congenital anomaly associated with Hcy was found. It was observed in mentally challenged children who had a high level of Hcy in blood as well as in urine (Carson & Neill, 1962; Gerritsen & Waisman, 1964). In 1969, McCully first proposed that Hcy caused atherothrombotic disease based on observations in children with several distinct rare inborn errors of Hcy metabolism (McCully, 1969). In a subsequent animal study, McCully and Wilson demonstrated extensive aortic atherothrombotic abnormalities and venous thrombosis leading to pulmonary embolism and infarction in rabbits fed large doses of Hcy or methionine. These observations led them to propose the “Hcy theory of atherosclerosis” (McCully & Wilson, 1975). Subsequent mechanistic studies revealed that Hcy can cause atherothrombosis by promoting oxidative stress, endothelial cell damage and endothelial dysfunction, inflammation, thrombosis and cell proliferation (Harker et al., 1983; Welch et al., 1997; Eikelboom et al., 1999; Kaul et al., 2006).
2.5.1 CAUSES OF HYPERHOMOCYSTEINEMIA

Hyperhomocysteinemia can result from either increased production or decreased removal of Hcy. A number of factors which include inborn errors in methionine metabolic enzymes, deficiencies of various B vitamins which play important roles as cofactors in this cycle, chronic renal failure and certain chemotherapeutic agents are responsible for decreased rate of removal of plasma Hcy (Kang, 1996; Selhub, 1999; Guttormsen et al., 1996; Kluijtmans et al., 2003). Inborn errors in the remethylation pathway, especially the defective MTHFR activity is the most common cause of genetic hyperhomocysteinemia (Klerk et al., 2002). Nonetheless, inborn errors in CBS enzyme are the most severe homocysteinemia and resultant homocysteinuria (Pogribna et al., 2001). In CBS knockout mouse models, plasma Hcy levels are increased by 40-fold in homozygous and 2-fold in heterozygous mutants when compared with the wild type (Watanabe et al., 1995). The deficiency of folate, vitamin B₆ and vitamin B₁₂ accounts for approximately 60% of cases of hyperhomocysteinemia in the general population (Cuskelly et al., 2001; Guttormsen et al., 1996). Hcy metabolism can be affected by certain chemotherapeutic agents such as theophylline, carbamazepine and valproate. Theophylline is a vitamin B₆ antagonist whereas valproate has antifolate effects. Therefore, hyperhomocysteinemia resulting from these drugs is secondary to vitamin deficiencies (Karabiber et al., 2003).

2.5.2. HOMOCYSTEINE-LOWERING THERAPIES AND BENEFICIAL EFFECTS

The supplementation of folic acid (0.5 – 5 mg/day) results in a 25% decrease in plasma total Hcy in mild to moderate hyperhomocysteinemic patients (van der Griend et al., 2000). Vitamin B₁₂ supplementation causes 7% reduction whereas vitamin B₆ treatment
alone only reduces hyperhomocysteinemia resulted from a post-methionine loading test (van der Griend et al., 2000). Fasting Hcy level is reduced by 12-20% by betaine supplementation (Brouwer et al., 2000). The mandatory folate fortification to white flour, cereal grains and related products introduced in the 1990s resulted in a significant decrease in plasma Hcy levels and the risk for neural tube defects in the United States, Canada and Chile (Jacques et al., 1999; Ray, 2004). Retrospective meta-analysis suggested that a prolonged reduction of plasma total Hcy level by 3-4 μM may substantially reduce the risk of arterial cardiovascular disease of about 30% (Boushey et al., 1995). Subsequent prospective studies in which plasma Hcy levels were measured before the clinical onset of disease, depicted a general association of Hcy with the risk for subsequent ischemic heart disease (Eikelboom et al., 1999; Kaul et al., 2006).

Conversely, recent studies did not see any beneficial effect of Hcy-lowering on inflammatory, endothelial dysfunction, or hypercoagulability markers which are implicated in atherothrombosis (Durga et al., 2005; Dusitanond et al., 2005). The Swiss Heart Study which was aimed at evaluating the impact of Hcy-lowering therapy on major adverse cardiac events was unable to show a significant impact on myocardial ischemia or death. However, it showed a significant risk reduction in major adverse cardiac events when compared with the placebo group (Schnyder et al., 2002). In another placebo controlled study, Hcy-lowering by multivitamin therapy which successfully reduced plasma Hcy level, resulted in a paradoxical increase in restenosis. Remarkably, a slight beneficial effect was observed in patients with elevated plasma Hcy (Lange et al., 2004). Despite the significant reduction in plasma Hcy levels, treatment with folic acid ands Statin (fluvastatin) did not show any beneficial effects on cardiovascular events in a
patient population which comprised men and women between 30 to 85 years with a history of post-acute myocardial infarction (Liem et al., 2004). The long-term treatment with B vitamins in the Norwegian Vitamin Trial (NORVIT) successfully reduced the plasma Hcy level by 27%, yet it was unable to suggest any beneficial effect in any subgroups of the 3,749 total patient population. Moreover, this study suggested harmful effects of combined B vitamin therapy (Bonaa et al., 2006). The HOPE-2 (Heart Outcomes Prevention Evaluation-2) trial was another meta-study which comprised of a patient population of men and women 55 years of age or older who had history of vascular diseases. This study also showed no beneficial effect of Hcy-lowering on cardiovascular events. However, this study showed a 25% reduction of stroke in the patient population (Lonn et al., 2006). Taken together, the results of NORVIT and HOPE-2 trials do not show any beneficial effect of Hcy-lowering on the risk of cardiovascular disease. Instead, they have suggested that Hcy-lowering may tend to cause harm by increasing the risk of cancer and cardiovascular disease (Bonaa et al., 2006; Lonn et al., 2006). McCully’s Hcy theory in atherosclerosis was based on strong, consistent and independent association between elevated Hcy levels observed in retrospective studies (McCully & Wilson, 1975). The atherosclerotic risk modification by Hcy-lowering based on prospective randomized interventions was not considered during the early studies. Therefore, some researchers suggest Hcy is a consequence rather than a cause of cardiovascular disease (Dudman, 1999). However, the vitamin therapy (mostly folate in these studies) reduces the plasma Hcy by enhancing Hcy flux through the remethylation pathway. As a result, the distribution of Hcy to the transsulfuration pathway is compromised. The suggested harmful effect of Hcy-lowering by folate
therapy will be addressed under the beneficial effects of H$_2$S, the endogenous synthesis of which is catalyzed by transsulfuration pathway enzymes, as discussed in Chapter 6.

2.6 REDOX STATUS AND FREE AND BOUND THIOLS IN THE PLASMA

Hcy, Cys, cysteiny1 glycine (Cys-Gly) and GSH are the major thiols present in the plasma. They exist in the free form and bound to plasma proteins. In the presence of an electron acceptor such as molecular oxygen, the sulfhydryl group becomes oxidized (Misra, 1974; Blom, 2000). This kind of autooxidation results in the formation of disulfide bonds between the same type of thiol molecules or with another thiol (Misra, 1974; Blom, 2000). Autooxidation of Hcy, Cys and GSH will result in the formation of free oxidized forms namely homocystine, cystine and oxidized GSH (GSSG), respectively. Similarly, the thiol mixed disulfides which include the Hcy-Cys mixed disulfide and the Hcy-GSH mixed disulfide, result from the oxidation of different sulfhydryl containing amino acids. Several factors influence the rate of the autooxidation of thiols. The copper-binding plasma protein, ceruloplasmin, and albumin are important catalysts in the oxidation of thiols in the plasma (Starkebaum & Harlan, 1986; Feldman et al., 1982; Sengupta et al., 2001a). The copper ion attached to ceruloplasmin is responsible for the autooxidation of L-Cys, Cys-Gly and GSH. However, neither ceruloplasmin nor copper ion bound to ceruloplasmin is a significant physiological catalyst for the autooxidation of L-Hcy (Sengupta et al., 2001a). Instead, the major plasma protein, albumin is the one which mediates the autooxidation of Hcy by thiol:disulfide exchange reaction. Copper attached to albumin is responsible for only about 20% of the total autooxidation of Hcy (Sengupta et al., 2001a).
2.6.1 THIOL-ALBUMIN INTERACTION

The bulk of Hcy and Cys present in human plasma are bound to plasma proteins (Blom, 2000; Blom & De Vriese, 2002). The free reduced Hcy is extremely low in healthy individuals and thus the free reduced Hcy in the circulation accounts for less than 1% of the total value (Blom & De Vriese, 2002). The free oxidized Hcy is about 30% and the plasma protein-bound fraction is almost 70% (Blom & De Vriese, 2002). Cys is the most abundant type of all aminothiols in human plasma. The total Cys concentration is about 250 μmol/L. Of this, approximately 65% is in the albumin-bound form and of the rest, 3-4% is in the free reduced form (Mansoor et al., 1993). In contrast to the free and bound fractions of Cys and Hcy in human plasma, about 60% of plasma total Cys is in the free form; not bound to albumin in the rat (Malloy et al., 1981). The plasma total Cys-Gly concentration in humans is about 30 μmol/L and of it, about 17 μmol/L is bound to plasma proteins (Mansoor et al., 1993). The free reduced fraction is about 10% (Mansoor et al., 1993). GSH is the least abundant thiol in the human plasma, with a concentration of about 6 μmol/L. More than 80% of GSH in human plasma is in the free reduced form (Mansoor et al., 1993). However, GSH is an abundant thiol in mammalian cells that functions in the maintenance of redox status, scavenging of ROS and conjugation of xenobiotics (Kaplowitz et al., 1985).

Albumin accounts for more than 50% of total protein in human plasma. It ranges from 0.6 to 0.75 mM (40-45 g/L) (Curry et al., 1998). In young rats, the plasma albumin concentration is reported to be 33 g/L (Peters & Davidson, 1986). In human plasma, albumin is the predominant Hcy-binding protein. More than 90% of total bound Hcy in the plasma is bound to albumin (Sengupta et al., 2001b). The maximal Hcy binding
capacity of albumin in the human plasma is approximately 140 μmol/L of plasma total Hcy. This may represent the saturation of all the available Hcy-binding sites in plasma albumin (Hortin et al., 2006). Thus, one third of the total albumin molecules in the plasma are disulfide-bonded with plasma thiols (Hortin et al., 2006). Hcy replaces Cys from the albumin-bound Cys and hence an equivalent amount of protein bound Cys is decreased as a result of this Hcy albumin interaction (Ueland et al., 1996).

2.6.1.1 Mechanisms of Thiol-Albumin Interaction

Albumin is exclusively synthesized by the liver and is a non-glycosylated, single-chain polypeptide. The formation of 17 intra-chain disulfide bonds between 34 of the total of 35 structural Cys residues defines the final structure of the tightly folded three domain structure of albumin. As a result of this intra-chain disulfide bonding, one Cys residue at Cys34 is left unbound. This free Cys34 residue accounts for the bulk of bound thiols (-SH) in plasma (Doumas & Peters, 1997). Once synthesized in the liver, nascent albumin is secreted into the circulation in the free thiolate anion form (Doumas & Peters, 1997). This highly reactive thiolate anion is able to make disulfide bonds with plasma thiols at physiological pH (Hortin et al., 2006). In the albumin molecule, Cys34 exists in two forms; one in exposed and the other in the buried form (Christodoulou et al., 1994; Christodoulou et al., 1995). According to this model, proposed by Christodoulou et al., the Cys34 is in close proximity to Histidine (His39), in the buried form (Christodoulou et al., 1994; Christodoulou et al., 1995). This makes a stable salt bridge. The exposed form is stabilized with the formation of a disulfide bond with Hcy or Cys. According to in vitro studies using human serum albumin, the binding of Hcy displaces Cys from albumin. However, stoichiometrically, the formation of new albumin-bound Hcy does not equal
the Cys released from albumin. Interestingly, a higher amount of Cys is released in response to a formation of a lesser amount of albumin-bound Hcy (Togawa et al., 2000). This difference is explained by the formation of albumin thiolactone anion (Sengupta et al., 2001b). The bulk of the Hcy added and the amount of Cys, which is released from albumin-bound form, then undergo autooxidation; homocystine, cystine and Hcy-Cys mixed disulfide are formed (Sengupta et al., 2001b).

According to a model proposed by Sengupta et al. (Sengupta et al., 2001a), L-Cys in human plasma is oxidized to cystine by ceruloplasmin. Liver releases nascent albumin thiolate anion into the circulation. It attacks Cys to form albumin-Cys$^{34}$-S-S-Cys. L-Hcy in the circulation reacts with this Cys-bound albumin, resulting in the formation of the Hcy-Cys mixed disulfide. As a result, albumin returns to its thiolate anion form which then attacks the Hcy-Cys mixed disulfide. Albumin-Cys$^{34}$-S-S-Hcy and the Cys thiolate anion are formed as the final outcome of this reaction cascade. In addition to the formation of Hcy-Cys mixed disulfide, Hcy can be autooxidated to homocystine by a reaction catalyzed by copper co-coordinated to His$^{39}$ of albumin but not by ceruloplasmin. Albumin thiolate anion can react with this homocystine to make some additional albumin-Cys$^{34}$-S-S-Hcy (Sengupta et al., 2001a). In the measurement of total plasma Hcy or Cys, the protein-bound fraction is reduced to the free form by a suitable reducing agent. However, it does not include thiols bound to protein by an amide linkage, such as compounds formed by the reaction of Hcy thiolactone with the lysine residues of protein (Jakubowski, 1999).
2.6.2 HOMOCYSTEINE MEDIATED OXIDATIVE STRESS

As depicted in the Figure 2.4, thiol oxidation reactions promote the formation of ROS due to the yield of two protons (H\(^+\)) and two electrons (e\(^-\)) (Starkebaum & Harlan, 1986; Hayden & Tyagi, 2004). The sulphydryl group of Hcy or Cys has a pKa of 10 or 8.5, respectively. Thus, Hcy is far more reactive at physiological pH than Cys (Zou & Banerjee, 2005). Further, the complicated re-arrangement of Hcy into Hcy thiolactone creates a chemically reactive cyclic thioester which acylates free amino groups in proteins containing lysine side-chains. The formation of homocysteinylated proteins contributes to the oxidative stress (Hayden & Tyagi, 2004). Both in vitro and in vivo assessment of lipid peroxidation has provided evidence for the pro-oxidative properties of Hcy in hyperhomocysteinemic conditions (Voutilainen et al., 1999; Lentz, 1997). Hyperhomocysteinemia following an oral methionine load causes increased lipid peroxidation (Domagala et al., 1997; Voutilainen et al., 1999). The intracellular ROS formation in endothelial cells is enhanced in response to an extracellular bolus administration of Hcy (Heydrick et al., 2004). Copper and ceruloplasmin which catalyze the autooxidation of Hcy are found to enhance the oxidation of LDL by Hcy (Exner et al., 2002; Heinecke et al., 1993). Urinary levels of 8-isoprostaglandin F\(_{2\alpha}\) which is a product of nonenzymatic oxidation of arachidonic acid is increased in homocysteinuric patients (Davi et al., 2001). Supplementation of vitamin E is effective in reducing the Hcy-mediated peroxidation of arachidonic acid in these patients (Davi et al., 2001). A moderate level of Hcy (10-50 \(\mu\)M) can induce the release of arachidonic acid from platelets and enhance the formation of thromboxane B\(_2\) and ROS (Signorello et al., 2002). Additionally, Hcy inhibits several antioxidant enzymes such as GSH peroxidase,
2Hcy-SH + O₂ → Hcy-S-S-Hcy + 2H⁺ + 2e⁻ → ROS Superoxide anion
Hydroxyl radical
Hydrogen peroxide

Hcy-SH + R-SH + O₂ → Hcy-S-S-R + H₂O + H₂O → ROS

Hcy-SH + R₁-S-S-R₂ ← → R₁-S-S-Hcy + R₂-SH → ROS

R = any organic compound in the plasma with a sulfhydryl group accessible to react with homocysteine (Hcy). It may be proteins, cysteine, glutathione, gamma-glutamylcysteine or cysteinyl-glycine

**Figure 2.4  Thiol oxidation reactions**
The free sulfhydryl group of thiol is highly reactive in the physiological pH. Thiol oxidation reactions yield two protons (H⁺) and two electrons (e⁻) leading to the generation of reactive oxygen species such as superoxide anion, hydrogen peroxide and free hydroxyl radical. (based on ideas from (Hayden & Tyagi, 2004))
superoxide dismutase, natural killer-enhanced factors \( \beta \), and proliferation-associated glucoprotein in human umbilical vein endothelial cells and it exacerbates the Hcy induced oxidative stress (Outinen et al., 1999).

2.7 ISCHEMIA-REPERFUSION INJURY IN THE KIDNEY

Kidneys are paired organs located in the posterior abdomen. Blood supply to the kidney is by the renal artery which divides into two branches called anterior and posterior branch arteries at the hilar region (Tisher & Madsen 1991). Both kidneys represent less than 1% of body weight yet they receive 20% of cardiac output and are responsible for approximately 10% of total oxygen consumption by the whole human body (Gullans & Hebert, 1996). In healthy kidneys such high oxygen demand is required to sustain mitochondrial respiration and for the synthesis of energy for the renal tubular transport process (Snowdowne et al., 1985). Ischemia-reperfusion, a result of temporary discontinuation and resumption of blood supply to the kidney during renal transplantation, shock state from various causes and renal artery stenosis, is a major cause of acute renal failure (ARF) (Ploeg et al., 1992).

2.7.1 RENAL ISCHEMIA

There are two types of ischemia that may occur during a surgical procedure such as renal transplantation in which the renal blood supply is temporary interrupted. Warm ischemia (the period between donor asystole and the beginning of cold storage) occurs when kidneys are collected from cadaveric or non-heart beating donors for transplantation in an immune-matched recipient (Kootstra et al., 1991). Intolerance of the kidney to
normothermic ischemia is a major factor which limits the number of suitable kidneys for transplantation. Thus, once the kidneys are collected from a cadaver it has to be preserved in a cold preservation medium (Belzer & Southard, 1988). Kidney preservation is the supply line of cadaveric kidneys for transplantation. It allows time to ship the kidney around the country to be transplanted to the best tissue typed recipient who is desperately in need of a healthy kidney. Thus, the kidneys harvested from cadavers undergo both warm and cold ischemia (Belzer & Southard, 1988). The kidneys harvested from living donors are immediately preserved in cold preservation medium and hence are not subjected to warm ischemia (Ploeg et al., 1988). Both cold and warm ischemia predispose the transplanted kidneys to ARF which is responsible for post-transplant morbidity and the early loss of the transplanted kidney (Fig. 2.5) (Bonventre, 1993). The recipient of the cadaveric donor kidneys have 30-60% incidence of ARF while recipients of living-related donors has 10% (Toledo-Pereyra, 1989; Chatterjee, 2007). Ischemia primes the kidney tissue to the reperfusion damage which is much greater than the damage caused by ischemia alone (Fig. 2.6). The longer the duration of ischemia, the greater the reperfusion injury (Baker et al., 1985). In humans, less than 1 h of warm ischemia is usually associated with temporary dysfunction, while longer than 3 hours results in irreversible damage (Fig. 2.5). Cold ischemia times of greater than 30 hours are detrimental (Beckman, 1990).
Figure 2.5  Sequence of events taking place in the pathogenesis of ischemic injury which predispose the affected kidneys to acute renal failure
(Based on ideas from (Toledo-Pereyra, 1989))
2.7.2 REPERFUSION AND THE GENERATION OF REACTIVE OXYGEN SPECIES

The kidney is an organ which is capable of generating ROS and also vulnerable to the damaging effects of ROS (Rondoni & Cudkowicz, 1953; Guidet & Shah, 1989; Nath et al., 1995; Hansson et al., 1983; Paller et al., 1984; Baud & Ardaillou, 1986; Shah, 1989). Relatively low amounts of ROS which include superoxide anion, hydrogen peroxide, nitric oxide (NO), peroxynitrite and hydroxyl radical are generated in the normal kidney with no adverse outcome (Baud & Ardaillou, 1986). Low concentrations of ROS are physiologically important as cellular signaling molecules (Forman et al., 2004). However, excessive production can be an etiological factor for the ARF (Hansson et al., 1983; Paller et al., 1984; Baud & Ardaillou, 1986; Shah, 1989; Nath & Salahudeen, 1990). There is substantial evidence supporting the involvement of ROS in the initiation and the progression of the functional and structural damage observed in ARF (Baker et al., 1985). The detrimental role of ROS in ischemia-reperfusion injury was first suggested by in vivo experiments employing free radical scavengers and antioxidants which demonstrated a protective effect against injury (Paller et al., 1984; Koyama et al., 1985). Varied vasoactive effects are exerted on the vascular bed by specific ROS (Tesfamariam, 1994; Katusic, 1996; Peters et al., 2000). Superoxide anion is usually vasoconstrictive due to its capacity to scavenge NO which is an established vasorelaxant (Paller & Neumann, 1991). The generation of ROS during the reperfusion is well documented (Paller & Neumann, 1991; Prathapasinghe et al., 2007). A study involving mitochondria harvested from kidneys subjected to ischemia-reperfusion demonstrated
Figure 2.6  Production of reactive oxygen species by the kidney during reperfusion
that the rate of ROS production is increased 1.5-fold and 4-fold during the ischemic and reperfusion phases, respectively (Gonzalez-Flecha & Boveris, 1995).

2.7.2.1 Sources of ROS during Renal Ischemia-Reperfusion

Animal experiments with occlusive clamping of the renal artery have contributed greatly to the understanding of the effect of ROS on ARF. The pathogenic role of ROS is mainly attributed to the superoxide anion produced from increased xanthine oxidase activity after an ischemic insult (Fig. 2.6) (McCord, 1985). Hypoxanthine, which is a product of ATP breakdown during ischemia, is the substrate for the xanthine oxidase which exists predominantly in the form of xanthine dehydrogenase in healthy tissues. In kidneys, the conversion of xanthine dehydrogenase to xanthine oxidase requires approximately 30 minutes of ischemia (McCord, 1985; Carafoli, 1987). The inhibited ATP synthesis during ischemia causes a deranged membrane ion gradient. This leads to an influx of calcium ions into the cells. Intracellular Ca$^{2+}$ is usually maintained around 0.1-0.2 μM which is approximately 10,000-fold lower than the extracellular Ca$^{2+}$ concentration (Carafoli, 1987). Even though there are several calcium-dependent mechanisms likely to be involved in ischemia-reperfusion injury in the kidney, it is often difficult to isolate a single pathway to define its primary role in the tissue injury. In one such pathway, elevated cytosolic Ca$^{2+}$ activates proteases that are capable of transforming xanthine dehydrogenase into xanthine oxidase by proteolytic mechanism (Malis & Bonventre, 1986). Reintroduction of molecular oxygen during reperfusion causes hypoxanthine to be oxidized by xanthine oxidase that produces superoxide anion. Administration of superoxide dismutase which scavenges superoxide anion or inhibition of xanthine oxidase by allopurinol or oxipurinol have shown reno-protective properties in animal
experimental models (Hansson et al., 1983; Paller et al., 1984). However, this type of beneficial effects of xanthine oxidase inhibitors or superoxide scavengers on reno-protective effect in ischemia-reperfusion injury has not been uniformly observed (Gamelin & Zager, 1988; Zager & Gmur, 1989). For example, neither xanthine oxidase inhibition nor intrarenal loading of hypoxanthine, the substrate for xanthine oxidase reaction, was able to alter the severity of the ischemia-reperfusion induced ARF in the rat (Zager & Gmur, 1989).

The plasma membrane-enzymes such as lipoxygenase, NADPH oxidase and cyclooxygenase are also sources of ROS during ischemia-reperfusion injury (Fig. 2.6). Arachidonic acid is the major substrate for the synthesis of prostaglandins, thromboxanes and leukotrienes. ROS intermediates are formed during the synthesis of these products from arachidonic acid (Kuehl & Egan, 1980). The NADH/NADPH oxidase system, which is a component of leukocyte defense mechanism, is present in the renal tubular epithelial cells as well. Activation of this enzyme is also a putative source of ROS in the renal parenchyma (Fig. 2.5) (Cui & Douglas, 1997). Soluble cytosolic components which include thiols, hydroquinones and catechols can also be sources of ROS. These compounds can undergo redox reactions producing ROS, superoxide anion in particular, in the process (Freeman & Crapo, 1982). Additionally, thiols and ascorbate can reduce chelated Fe$^{3+}$ to Fe$^{2+}$ which can later be autooxidized, producing superoxide anion and H$_2$O$_2$ during subsequent spontaneous or catalyzed dismutation of superoxide (Fridovich & Freeman, 1986). The cytochrome P-450 system which is located in the endoplasmic reticulum and nuclear membranes can also be a source of ROS as a result of the oxidation of free fatty acids (Paller & Jacob, 1994). Given the large number of oxidases that they
contain and their abundance in renal proximal tubular cells, peroxisomes are also a potential contributor to the ROS pool during ischemia-reperfusion injury (Gulati et al., 1993). On the other hand, the reperfusion injury due to oxidative stress can also arise from impairment of the antioxidant defense mechanisms which include tissue GSH synthesis and the activities of antioxidant enzymes such as superoxide dismutase, catalase and GSH peroxidase (Singh et al., 1993).

2.7.3 ACUTE RENAL FAILURE
Despite the tremendous advances in critical care medicine, ARF subsequent to ischemia-reperfusion remains unchanged over the past several decades (Furuichi et al., 2002). ARF represents an acute loss of renal function incurred by ischemic or nephrotoxic insult. It results in the accumulation of nitrogenous wastes such as urea and creatinine in the circulation (Thadhani et al., 1996; Lieberthal, 1998). Usually, this is a clinically reversible condition. In the initiation phase of ARF, the renal function, as measured by GFR, is severely impaired. The persistent reduction in the GFR together with aberrant hemodynamic responses and cell injury, in the form of mild necrosis, are evident during the maintenance phase of ARF (Racusen, 1997). The GFR is further decreased with progression of the condition. This stage is histologically characterized by the loss of brush border, vacuolization and flattening of proximal tubules, and cellular necrosis especially in the S3 segment of the proximal tubules (Venkatachalam et al., 1978; Racusen, 1997). In addition, edematous renal interstitium with infiltrated blood cells may be observed in the renal medulla. Apoptotic cells are found in the distal tubular epithelial
cells and the lumen of the distal tubule is occluded by casts (Venkatachalam et al., 1978; Racusen, 1997).

### 2.7.3.1 Pathogenesis of Acute Renal Failure

The intracellular and molecular mechanisms in the pathogenesis of ischemia-reperfusion mediated ARF are complex and multifactorial (Chatterjee, 2007). The ischemia-reperfusion induced cellular injury occurs both in ischemia as well as reperfusion phases. Ischemia causes hypoxia, metabolic acidosis and accumulation of potentially toxic metabolites in the kidney tissue (Tejchman et al., 2006). Oxygen requirement is restored with the onset of reperfusion and the toxic metabolites are exported from the kidney to the circulation (Nath & Norby, 2000).

### 2.7.3.2 Effect of the Depletion of ATP on the Pathogenesis of ARF

Altered cytoskeletal function and structure that largely result from a decrease in tissue ATP concentration reflect the sublethal injury. These changes impair the polarized transport properties of the proximal tubule, leading to numerous functional changes such as back leak of the glomerular ultrafiltrate (Fig. 2.5) (Toledo-Pereyra, 1989). This injury can be repaired once the offending insult is removed. Cellular Na⁺, K⁺ and Ca²⁺ homeostasis are disrupted due to the severe ATP depletion with the extension of ischemia (Fig. 2.5) (Bonventre, 1993). Further, it causes an accumulation of phospholipid metabolites, and produces generalized protein dephosphorylation with protein redistribution and aggregation (Bonventre, 1993; Kobryn & Mandel, 1994; Tsukamoto & Nigam, 1997). The proximal tubular cells are susceptible to necrosis due to these nonspecific events. According to one theory, the proximal cell necrosis can occur as the result of ATP depletion-induced opening of "death channels" which are located in the
plasma membrane (Dong et al., 1998). The presence of high levels of glycine and decreased pH due to metabolic acidosis during ischemia normally keep this death channel closed (Bonventre, 1993). Conversely, the recovery of pH during reperfusion allows the opening of the death channel in cells which are unable to replenish ATP as a result of non-oxidant and oxidant damage to mitochondrial oxidative phosphorylation that occurred during the ischemic and reperfusion phases, respectively (Gonzalez-Flecha & Boveris, 1995; Weinberg et al., 2000).

2.7.3.3 Effect of ROS in the Pathogenesis of ARF

Reperfusion amplifies the ischemia-induced cascade of cellular injury by ROS-dependent mechanisms that lead to necrotic and apoptotic cell death (Fig. 2.6) (Lieberthal, 1998; Weinberg, 1991; Bonventre, 1993; Edelstein et al., 1997). ROS can cause either sublethal or lethal cell injury, the latter is reflected by irreversible processes that culminate in either necrosis or apoptosis (Lieberthal, 1998; Weinberg, 1991; Bonventre, 1993; Edelstein et al., 1997). The progression of the injury is continued due to the generation of a large amount of ROS from multiple sources (such as activation of xanthine oxidase, NADPH oxidase, cyclooxygenase etc.,) during reperfusion. The detrimental effect of ROS is amplified during reperfusion by the release of catalytically active iron and increased production of NO from iNOS which causes the formation of peroxynitrite from the reaction between superoxide anion and NO (Fig. 2.6) (Paller, 1994; Baliga et al., 1993; Ling et al., 1998; Noiri et al., 2001). ROS can alter the basic constituents of cells and cell organelles, i.e. lipids, carbohydrates, proteins and nucleic acids (Nath & Norby, 2000). Peroxidation of lipids in plasma and intracellular membranes by ROS perturbs membrane fluidity and permeability affecting the ion and solute transport (Nath & Norby, 2000).
The adverse effect of ROS on proteins is brought about through mechanisms such as oxidation of sulfhydryl groups, carbonyl formation and deamination (Nath & Norby, 2000). As a result, the structural proteins and enzymes are altered and the outer medulla of the kidney displays impaired activity, impaired turnover rates and sulfhydryl content of sodium-potassium ATPase (Kako et al., 1988). Cytoskeletal proteins such as integrins are destabilized by ROS leading to the loss of cellular attachments to neighboring extracellular matrix (Lieberthal, 1998; Gailit et al., 1993). The mitochondrial ATP synthase complex is inhibited by hydrogen peroxide (Chatterjee et al., 1999a). In addition, hydrogen peroxide inhibits glyceraldehyde-3-phosphate hydrogenase in the glycolytic pathway (Spragg et al., 1985). Consequently, the cellular stores of ATP which are required for processes that are indispensable for cellular vitality are depleted (Lieberthal, 1998; Weinberg, 1991; Bonventre, 1993; Edelstein et al., 1997). Iron-containing enzymes, which include TCA cycle enzymes aconitase and mitochondrial electron transport chain enzymes, are inactivated by NO (Stadler et al., 1991). Inhibition of iNOS activity and the iNOS knockout mice show a significant reduction of ischemia-reperfusion induced ARF suggesting the involvement of NO in the pathogenesis (Noiri et al., 1996; Ling et al., 1999). Oxidant injury is related to the interaction between iron and NO. This has been demonstrated by in vitro experiments in which NO donors induced oxidative stress and cellular injury. The administration of iron chelators ameliorated the NO mediated cellular injury (Liang et al., 2000).

2.7.3.4 Role of Inflammation in ARF

Inflammatory reactions, in response to ischemia-reperfusion, are also contributory in the development of ARF (Bonventre, 2007; Kelly, 2006). Renal inflammation leads to the
death of renal cells and is associated with allograft rejection in kidney transplant patients (Albrecht et al., 2000; Boros & Bromberg, 2006). The removal of inflammatory mediators from plasma by dialysis reduces the risk of ARF (Inthorn & Hoffmann, 1996; Montoliu, 1997). The inflammatory response to ischemia-reperfusion leads to a significant degree of oxidative stress and contributes to the pathogenesis of ARF (Kaysen, 2000; Stenvinkel, 2003; Salvemini et al., 2006). A large number of biologically active inflammatory mediators (which include bradykinin, histamine and platelet-activating factor and pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, tumor necrotic factor (TNF)-α and monocyte chemotactic protein (MCP)-1) are produced in response to the ischemia-reperfusion (Sung et al., 2002; Dong et al., 2007; Thurman, 2007). In addition, pro-inflammatory cells such as polymorphonucleocytes, proximal tubular cells, glomerular mesangial cells and other pro-inflammatory proteins including intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 are also involved in the inflammatory reactions (van Kooten et al., 1999; Daha & van Kooten, 2000; Chatterjee et al., 1999b; Muller et al., 1996; Burne-Taney & Rabb, 2003).

2.7.3.5 Necrotic vs. Apoptotic Cell Death in ARF

Necrotic cell death in the proximal tubules is more prominent than apoptotic death during the initial stage of reperfusion (Bonegio & Lieberthal, 2002). This is because apoptotic cell death is an energy-dependent process. The ATP required to support the full apoptotic program must be substantially glycolytic in origin since the mitochondrial respiration is severely impaired due to the insult from ischemia and mitochondrial de-energization that occurs as apoptosis progresses (Zimmermann et al., 2001). The other reason is the limited production of ATP from glycolysis from the proximal tubules before injury (Guder &
Ross, 1984). Nonetheless, possibly the most fundamental reason that necrotic cell death is more prominent than the apoptosis may be the down regulation of bax and bak, the two pro-apoptotic proteins (Krajewski et al., 1996; Gobe et al., 2000). These pro-apoptotic proteins act at the outer mitochondrial membrane in the intrinsic pathway for the initiation of apoptosis via the release of cytochrome c and other proteins from the mitochondrial intermembrane space (Zimmermann et al., 2001; Gobe et al., 2000; Krajewski et al., 1996). Conversely, the distal tubules (which are more glycolytic than the proximal tubules) are more resistant to necrosis and prone to apoptosis (Schumer et al., 1992; Oberbauer et al., 1999).

A brush border protease called meprin, which is expressed in S3 segment and is the major matrix-degrading enzyme in rat renal tubules, also contributes to necrotic cell death due to its strong toxic effect (Carmago et al., 2002). In addition, the activation of caspases and calpain may contribute to development of necrosis (Kaushal et al., 1998). The expression of pro-apoptotic proteins and caspases are increased with progression of reperfusion (Kaushal et al., 1998). These pro-apoptotic factors are increased due to the elevated production of ROS (Ueda et al., 2000; Chakraborti et al., 1999). The caspase inhibitors can ameliorate the ischemia-reperfusion induced apoptosis in animal models (Daemen et al., 1999).

2.7.4 TREATMENT FOR THE ISCHEMIA-REPERFUSION MEDIATED ARF

A large number of therapeutic strategies have been investigated for their ability to reduce renal ischemia-reperfusion-mediated ARF (Table 2.1). Even though these substances show promise in animal models, none of them has been able to reduce the ARF and
mortality in kidney transplant patients (Chatterjee, 2007). The reasons for not showing beneficial effects in real clinical settings could be explained in two ways; first, due to the multifactorial nature of the pathogenesis of ARF for which a single-drug therapy would never bring complete protection. Second, a major factor involved in the pathogenesis of ischemia-reperfusion-mediated ARF is still elusive (Star, 1998; Lameire & Vanholder, 2001). Therefore, the aim of this study was to identify other factor(s) that might be involved in the pathogenesis of ischemia-reperfusion-mediated ARF. Since Hcy is a pro-oxidant and pro-inflammatory amino acid, and the kidney is a major organ which is involved in Hcy metabolism, we sought to examine the impact of ischemia-reperfusion on kidney tissue and the involvement of Hcy in the kidney injury. Further, we studied the effect of ischemia-reperfusion on the CBS activity in the standard transsulfuration pathway as well as in the alternative pathway which is involved in endogenous H₂S production in the kidney. The specific hypothesis and objectives of the study are cited under the relevant chapter which describes the experimental procedures and results. In general, this study was divided into 3 major parts;

Part I – to study the kidney tissue and plasma Hcy levels during renal ischemia-reperfusion and the detrimental role of Hcy in the pathogenesis of ischemia-reperfusion mediated renal injury (Chapter 4)

Part II – to determine the effect of kidney tissue pH and NO level during ischemia and reperfusion, respectively, on the inhibition of CBS activity (Chapter 5)

Part III – to determine the contribution of transsulfuration pathway enzymes (CBS and CGL) in endogenous H₂S production in the kidney and the effect of ischemia-reperfusion on CBS activity in the H₂S producing pathway (Chapter 6)
<table>
<thead>
<tr>
<th>Substance</th>
<th>Mechanism of action</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Calcium channel blockers</td>
<td>Prevent $\text{Ca}^{2+}$ influx and $\text{Ca}^{2+}$ dependant conversion of xanthine dehydrogenase to oxidase. Prevent the activation of phospholipases which potentiate arachidonic acid metabolism and mitochondrial injury.</td>
<td>(Frei et al., 1987; Wagner et al., 1987) (Green et al., 1989)</td>
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<tr>
<td>Protease inhibitors</td>
<td>Prevent the protease dependent conversion of xanthine dehydrogenase to oxidase.</td>
<td>(Kher et al., 2005)</td>
</tr>
<tr>
<td>ATP-MgCl₂</td>
<td>Improves cellular respiratory function.</td>
<td>(Cruthirds et al., 2005)</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>ROS scavenger.</td>
<td>(Cruthirds et al., 2005)</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>Xanthine oxidase inhibitor. Decrease the production of ROS.</td>
<td>(Lopez-Neblina et al., 1995)</td>
</tr>
<tr>
<td>Iron chelators</td>
<td>Minimize the production of $\text{OH}^-$ via Fenton reaction.</td>
<td>(Haraldsson et al., 1995)</td>
</tr>
<tr>
<td>Membrane stabilizers</td>
<td>Prevent mitochondrial injury.</td>
<td>(McAnulty et al., 2004)</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>Decreases thromboxane level by inhibiting cyclooxygenase.</td>
<td>(Klausner et al., 1989)</td>
</tr>
<tr>
<td>Iloprost</td>
<td>Prostacyclin analogue which increases the prostaglandin: thromboxane ratio.</td>
<td>(Sistolieri et al., 2005)</td>
</tr>
<tr>
<td>Mannitol</td>
<td>Decreases thromboxane A₂ production.</td>
<td>(Haraldsson et al., 1995)</td>
</tr>
<tr>
<td>BN 52021</td>
<td>Platelet Activating Factor (PAF) antagonist.</td>
<td>(Torras et al., 1993)</td>
</tr>
<tr>
<td>Antiendotheline antibody</td>
<td>Prevents PAF.</td>
<td>(Espinosa et al., 1996)</td>
</tr>
<tr>
<td>Glutathione, N₄Acetylcysteine</td>
<td>Antioxidant &amp; precursor of GSH synthesis, respectively.</td>
<td>(Sehirli et al., 2003)</td>
</tr>
<tr>
<td>Thyroid hormone</td>
<td>Stimulates mitochondrial respiration.</td>
<td>(Boydstun et al., 1995)</td>
</tr>
<tr>
<td>Atrial Natriuretic Peptide</td>
<td>Increases GFR by producing efferent vasoconstriction.</td>
<td>(Pollock &amp; Opgenorth, 1993)</td>
</tr>
<tr>
<td>Vitamin C &amp; E</td>
<td>Antioxidant</td>
<td>(Lee et al., 2006; Avunduk et al., 2003)</td>
</tr>
<tr>
<td>Castration</td>
<td>Activation of manganese SOD.</td>
<td>(Kim et al., 2006)</td>
</tr>
<tr>
<td>Treatment</td>
<td>Effect</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>Immunosuppression and prevents apoptosis</td>
<td>(Zhu et al., 2002)</td>
</tr>
<tr>
<td>Monoclonal antibody to Mouse Factor B</td>
<td>Prevents the activation of complement in apoptotic and necrotic pathways</td>
<td>(Thurman et al., 2006)</td>
</tr>
<tr>
<td>Catecholamines</td>
<td>Reduces monocyte infiltration and the expression of major histocompatibility class (MHC) II and P-selectin</td>
<td>(Schaub et al., 2004; Gottmann et al., 2006)</td>
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<tr>
<td>Erythropoietin</td>
<td>Antiapoptotic action</td>
<td>(Sharples et al., 2004)</td>
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<tr>
<td>Melatonin</td>
<td>Antioxidant and ROS scavenger</td>
<td>(Kacmaz et al., 2005)</td>
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<tr>
<td>Ischemia preconditioning</td>
<td>Increased NO release</td>
<td>(Jefayri et al., 2000)</td>
</tr>
<tr>
<td>Cobalt Chloride, Statins</td>
<td>Induces Heme Oxygenase activity</td>
<td>(Garcia Salom et al., 2007; Gueler et al., 2007)</td>
</tr>
<tr>
<td>Mofetil and Rapamycin</td>
<td>Anti-proliferative effect on B &amp; T lymphocytes; inhibit activation, proliferation &amp; migration of vascular smooth muscle cells</td>
<td>(Jolicoeur et al., 2003)</td>
</tr>
<tr>
<td>Tetrahydrobiopterin</td>
<td>Anti-inflammatory effect and prevents NOS uncoupling</td>
<td>(Huisman et al., 2002)</td>
</tr>
<tr>
<td>L-N6-(1-iminoethyl)lysine (L-NIL) &amp; GW274150</td>
<td>Inhibit inducible NOS</td>
<td>(Chatterjee et al., 2002; Chatterjee et al., 2003)</td>
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CHAPTER – 3

MATERIALS AND METHODS
3.1 MATERIALS

3.1.1 CHEMICALS AND REAGENTS
See Appendix I

3.1.2 EQUIPMENT
See Appendix II

3.2 ANIMAL MODEL
Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing 250-300 g were used in all experiments.

3.2.1 INDUCTION OF RENAL ISCHEMIA AND REPERFUSION
Rats were anesthetized with an injection of pentobarbital sodium (50mg/kg) intraperitoneally. Rats were kept on a heat pad and the rectal temperature was maintained at 37°C throughout the experimental procedure. The left renal pedical was carefully cleared of fascia. Renal ischemia was induced by clamping the left renal artery for 15 min to 1 h with a non-traumatic vascular clamp (Fine Science Tools, Vancouver, Canada) as previously described (Sung et al., 2002). Rats subjected to ischemia alone were sacrificed after the kidneys were harvested and collected in ice-cold potassium phosphate buffer. In the ischemia-reperfusion experiments, the clamp was removed after ischemia. The resumption of the blood supply (reperfusion) to the kidney was confirmed visually. The reperfusion was for a 1 to 24 h depending on the experimental plan. Rats remained sedated throughout the reperfusion period. In experiments in which reperfusion was for more than 1 h, the right nephrectomy was performed at the onset of reperfusion of the left
kidney. At the end of the reperfusion period, the left kidney was harvested and collected in ice-cold potassium phosphate buffer. 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. A blood sample was drawn into a heparinized syringe from the abdominal aorta before the rats were sacrificed. Plasma was separated by centrifugation of blood at 3,000 x g for 20 min at 4°C. Kidneys in ice-cold potassium phosphate buffer were bisected. One-half was preserved in 10% formalin for paraffin embedding. The other half was used for enzyme essays and biochemical analysis. All procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and approved by the University of Manitoba Protocol Management and Review Committee.

3.2.2 DETERMINATION OF THE ROLE OF HOMOCYSTEINE IN RENAL ISCHEMIA-REPERFUSION INJURY

3.2.2.1 Intrarenal Arterial Administration of Anti-Homocysteine Polyclonal Antibodies

In one set of experiments, rats were injected with rabbit anti-Hcy polyclonal antibodies (250 µl, 1:20 dilution v/v; Chemicon International, Temecula, CA) or non-immune rabbit IgG (250 µl, 1:20 dilution v/v; Santa Cruz Biotechnology, CA) through the left renal artery, (caudal to the clamp) immediately after the induction of ischemia. Intrarenal arterial injection of anti-Hcy antibodies immediately after the induction of ischemia would allow immediate access of antibodies to the kidney to neutralize Hcy generated
inside the kidney during ischemia-reperfusion. Intrarenal administration minimized antibody dilution in the circulation and minimized systemic immune response.

3.2.2.2 Intravenous Administration of L-Homocysteine

In another set of experiments, rats received freshly prepared L-Hcy (2.5 mg/kg) or vehicle (buffer used to prepare L-Hcy) via the left femoral vein when reperfusion started. L-Hcy was prepared from L-Hcy thiolactone which was dissolved and hydrolyzed with 5M NaOH to remove the thiolactone group (Uerre & Miller, 1966). After incubating at 37°C for 5 min, the preparation was neutralized with 1M KH2PO4 (Uerre & Miller, 1966).

3.2.3 DETERMINATION OF THE EFFECT OF pH ON RENAL CBS ACTIVITY

3.2.3.1 Effect of Ischemia and Reperfusion on Kidney Tissue pH

Rats were subjected to 15 to 60 min ischemia alone or 45 min ischemia followed by 1 to 24 h reperfusion. Kidney tissue homogenate (10%, w/v) was prepared in de-ionized water and the pH of the tissue homogenate was determined by an Accumet pH meter (Fisher Scientific, Quebec, Canada). The right kidney was removed at the onset of reperfusion of the left kidney in all rats which were subjected to more than 1 h of reperfusion.

3.2.3.2 Effect of pH on Renal CBS Activity

The effect of pH on CBS was assessed in vitro in 10% (w/v) normal kidney tissue prepared in 0.05 M potassium phosphate buffer at different pH. The CBS activity was measured with a reaction mixture at the corresponding pH. The nature of the effect of pH on CBS activity was determined. Kidney tissue homogenate (10%, w/v) was prepared at pH 6.5 and pre-incubated for 45 min at 37°C. The pH was brought up to 8.5 in one set
while the other was kept at pH 6.5. After 15 min of further pre-incubation, the CBS activity was determined in a reaction mixture at each corresponding pH.

3.2.3.3 Effect of an Alkalizing Agent on CBS Activity During Ischemia and Reperfusion

Rats were subjected to 45 min ischemia alone or ischemia followed by 6 h reperfusion. Rats received 250 µl of 10 mM (NH₄)₂HPO₄ (I-45min+(NH₄)₂HPO₄) as an alkalizing agent, dissolved in saline via the left intrarenal arterial route immediately after the induction of ischemia. The CBS activity was determined in the kidney tissue homogenate.

3.2.4 DETERMINATION OF THE EFFECT OF NITRIC OXIDE ON CBS ACTIVITY DURING REPERFUSION

3.2.4.1 Effect of Nitric Oxide on CBS Activity

The effect of NO was assessed in a 10% (w/v) kidney tissue homogenate that was prepared in 0.05 M potassium phosphate buffer at pH 6.9. Different concentrations of sodium nitroprusside (SNP) were used as the source of NO (Eitle et al., 1998). The effectiveness of SNP as a spontaneous NO donor was determined by measuring the nitrate and nitrite level in the solution by the Griess reaction (Fig. 3.1). The CBS activity was determined at pH 8.5. The nature of the NO mediated inhibition of CBS activity was assessed with an NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) in vitro (Goldstein et al., 2003). Kidney tissue homogenate (10%, w/v) that was prepared in 0.05 M potassium phosphate buffer at pH 8.5 was incubated either with
Figure 3.1    SNP as an NO donor
10% kidney tissue homogenate was incubated with different concentrations of SNP for 15 min at 37°C. The concentration of NO metabolites was assessed by Griess reaction. Results are expressed as mean ± s.e.m. (n = 2).
200 μM PTIO or 200 μM SNP for 15 min at 37°C. In the next 15 min an equimolar amount of SNP was added to the homogenate which was incubated with PTIO and vice versa. The CBS activity was determined.

3.2.4.2 Effect of an Nitric Oxide Scavenger on CBS Activity during Reperfusion

In the ischemia-reperfusion group, rats were given a dose of 250 μl of 1 mM PTIO as the NO scavenger at the onset of reperfusion via the left femoral vein (I-45min/R-6h+PTIO). In addition, some rats which received 250 μl of 10 mM (NH₄)₂HPO₄ during ischemia were allowed for 6 h reperfusion with or without an injection of 250 μl of 1 mM PTIO during reperfusion (I-45min/R-6h+(NH₄)₂HPO₄+PTIO and I-45min/R-6h+(NH₄)₂HPO₄, respectively). A sham-operated group of rats were subject to the same surgical procedure without inducing ischemia-reperfusion and were sacrificed at corresponding time points. Untreated ischemia-reperfusion group received a similar volume of normal saline via corresponding routes as the vehicle.

3.2.5 ISCHEMIA PRECONDITIONING EXPERIMENTS

Studies of the effect of preconditioning on ischemia-reperfusion injury was tested, rats were subjected to four cycles of 5 min renal ischemia separated by 10 min reperfusion periods before 1 h of renal ischemia followed by 1 h reperfusion (IP+I-60min/R-60min).
3.3 BIOCHEMICAL ANALYSIS

3.3.1 DETERMINATION OF PLASMA AND KIDNEY TISSUE HOMOCYSTEINE LEVELS

The Hcy concentrations in the plasma and kidney tissue homogenate were measured with the IMx Hcy assay which was based on fluorescence polarization immunoassay technology (Abbott Diagnostics, Abbott Park, IL) (Au-Yeung et al., 2004; Woo et al., 2005). Kidney tissue homogenate (1:2 w/v) was prepared in 0.8 N HClO₃ containing 10 mM EDTA. Hcy was converted to SAH by the IMx method and the level of SAH was determined by fluorescence polarization immunoassay technology.

3.3.2 DETERMINATION OF PLASMA CREATININE LEVEL

Kidney function was evaluated by measuring the plasma creatinine level. The plasma creatinine level was determined by the WAKO creatinine kit (Wako Chemical Industries) (Zhu et al., 2002). The addition of a picric acid and an alkaline solution to deproteinized plasma results in the formation of reddish orange color via the reaction of creatinine. The absorption of the colored solution is determined at λ=520 nm.

3.3.3 DETERMINATION OF REACTIVE OXYGEN SPECIES

3.3.3.1 Kidney Tissue Superoxide Anion Level

The level of superoxide anion in the kidney was determined as previously described (Zou et al., 2001). A portion of kidney was homogenized in 1:4 volumes of buffer containing 20 mM HEPES, 1 mM EDTA, and 0.1 mM PMSF followed by centrifugation at 3,000 x
g for 10 min at 4°C. The supernatant kidney homogenate was incubated in a reaction mixture containing 10 μM dihydroethidium for 30 min at 37°C. The superoxide anion in the kidney homogenate caused oxidation of dihydroethidium leading to the formation of ethidium which was detected at an excitation of λ = 475 nm and an emission of λ = 610 nm using a fluorometer (Spectra Max Gemini, Molecular Devices, Sunnyvale, CA). The fluorescent signal produced by ethidium was proportionate to the level of superoxide anion present in the kidney.

3.3.3.2 Kidney Tissue and Plasma Nitric Oxide Level

The measurement of nitrite and nitrate was used to assess the NO levels in kidney tissue. The kidney tissue was homogenized in a buffer (50%, w/v) containing 20 mM Tris (pH 7.4) and 2 mM EDTA. Supernatant was collected after 10 min of centrifugation at 1,500 x g at 4°C. The supernatant was deproteinized with 0.3 N NaOH and 5% ZnSO₄ and centrifuged at 10,000 x g for 20 min at room temperature. To measure NO level in the blood, plasma was used in place of the supernatant of the tissue homogenate. The amount of nitrite and nitrate in the supernatant was determined with Griess reaction method based on the azo coupling reaction (Tarpey et al., 2004). In brief, the supernatant was incubated with nitrate reductase and cofactors, FAD and NADPH to reduce nitrate to nitrite. After 25 min incubation at 37°C the reaction was stopped by adding lactate dehydrogenase and sodium pyruvate to oxidize NADPH. Then, 12.5 mM sulfanilamide in 6N HCl and 12.5 mM N-(1-naphthyl)ethylenediamine were added to the nitrite solution to complete the azo coupling reaction. The diazoamino benzene in the reaction mixture was measured by spectrophotometer at an absorbance of λ = 543 nm. NaNO₂ at different concentrations was used as standards. Figure 3.2 shows the kidney tissue NO metabolite level in
Nitrite and nitrate levels were determined by Griess reaction at different protein concentrations of kidney tissue homogenate.
response to the protein concentration. A sample of approximately 3 mg protein was used in the experiments to determine NO metabolite level in the kidney tissue homogenate in subsequent experiments.

3.3.4 ENZYME ASSAYS

3.3.4.1 Measurement of CBS Activity in the Standard Transsulfuration Pathway

The assay was based on a method developed by Mudd et al and modified by Taoka et al (Mudd et al., 1965; Taoka et al., 1998). A 10% (w/v) tissue homogenate was prepared in 0.05 M potassium phosphate buffer (pH 6.9) followed by centrifugation at 18,000 x g for 30 min at 4°C. The supernatant was carefully separated and the reaction was carried out in a reaction mixture containing 125 mM Tris-HCl (pH 8.5), 2.1 mM EDTA, 0.146 mM L-cystathionine, 41.7 mM DL-Hcy, 0.316 mM SAM, 2.1 mM propargylglycine and 0.42 mM PLP. The reaction was initiated by adding 30 mM [14C] serine (2 μCi/ml) (PerkinElmer, Boston, MA) to the reaction mixture. After 1 h incubation at 37°C, ice-cold 15% trichloroacetic acid was added to stop the reaction. The reaction mixture was then centrifuged at 3,700 x g for 5 min and the supernatant was saved. An aliquot of the supernatant was passed through a Bio-Rad AG 50W-X4 anion exchange column containing 200-400 mesh hydrogen-form resin. The column was washed in the following order; 2 times with 4 ml of water, 6 times with 4 ml of 1 N HCl and 4 times with 4 ml of water. Then, labeled cystathionine was eluted once with 5 ml of 3 N NH₄OH. Eluates were collected and 1 ml aliquots of the collected elute was transferred into 10 ml scintillation vials. Four milliliters of ScintiVerse Xylene solution (Fisher Scientific, NJ, USA) was added into each vial. The radioactivity was counted the next day, by using a
Beckman liquid scintillation counter. The radioactivity of 10 µl of 300 mM [14C] serine was also determined in 4 ml of ScintiVerse. Each determination was corrected by blank values obtained by assays, both with labeled substrate-free reaction media and acid denatured homogenates. The production of [14C] cystathionine as a measure of the CBS activity, in response to the graded increment of CBS enzyme (protein) and the incubation time are depicted in Figures 3.3 and 3.4, respectively. Samples containing approximately 0.6 mg of protein were incubated for 1 h in the determination CBS activity in the standard transsulfuration pathway in subsequent experiments.
Figure 3.3 The CBS activity as a function of the kidney tissue protein concentration
CBS activity in the standard transsulfuration pathway was determined at different protein concentrations of kidney tissue homogenate.
Figure 3.4 The CBS activity as a function of the incubation time
CBS activity in the standard transulfuration pathway in kidney tissue was determined at different incubation time points.
3.3.4.2 Measurement of CBS Activity in H₂S Producing Pathway

The assay was based on a method described by Stipanuk and Beck (Stipanuk & Beck, 1982). A 5% (w/v) tissue homogenate was prepared in 0.05 M potassium phosphate buffer (pH 6.9) followed by centrifugation at 18,000 x g for 30 min at 4°C. Supernatant was saved and the reaction was carried out in a reaction mixture containing 10 mM L-Cys, 10 mM DL-Hcy, 0.05 mM PLP and 0.03 mM SAM prepared in 0.1 mM potassium phosphate buffer (pH 7.4) at 37°C for 30 min in a shaker water bath. Reactions were performed in 25 ml Erlenmeyer flasks fitted with septum stoppers and plastic center wells. Center wells were filled with 0.5 ml of 15:1 (v/v) mixture of 1% (w/v) zinc acetate and 12% (w/v) NaOH and a folded 2 cm x 2.5 cm piece of filter paper to trap H₂S. The reaction was stopped by adding 0.5 ml of 30% (w/v) trichloroacetic acid. After 60 min of further incubation, filter papers were transferred to test tubes containing 3.5 ml water. 0.4 ml of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 M HCl and 0.4 ml of 30 mM FeCl₃ in 1.2 M HCl were added. The absorbance of the resulting solution was measured at λ = 670 nm. The H₂S production in response to the graded increment of CBS enzyme (protein) and the incubation time are depicted in Figures 3.5 and 3.6, respectively. Samples containing approximately 1.2 mg of protein were incubated for 1 h in the determination CBS activity in the H₂S producing pathway in subsequent experiments. To determine the specific role of transulfuration pathway enzymes to the production of H₂S, hydroxylamine and D,L-propargylglycine were used as inhibitors of CBS and CGL, respectively. The tissue and plasma H₂S levels were determined in the same way except the 30% trichloroacetic acid was added before the incubation period. To determine the plasma H₂S level, 100 µl of plasma was incubated with 0.4 ml of 15:1 (v/v)
Figure 3.5 The rate of H$_2$S production as a function of the kidney tissue concentration
CBS activity in the H$_2$S producing pathway in kidney tissue was determined at different protein concentrations.
Figure 3.6 The rate of H₂S production as a function of the incubation time. CBS activity in the H₂S producing pathway in kidney tissue was determined at different incubation time points.
of 1% (w/v) zinc acetate and 12% (w/v) NaOH for 10 min at room temperature. The plasma H$_2$S level was determined by adding 3.5 ml of de-ionized water, 0.4 ml of 20 mM $NN$-dimethyl-$p$-phenylenediamine sulfate in 7.2 M HCl and 0.4 ml of 30 mM FeCl$_3$ in 1.2 M HCl.

### 3.3.4.3 Measurement of NOS activity

Total NOS activity in the kidney was measured by arginine to citrulline conversion assay (Griscavage et al., 1993). In brief, 10% (w/v) kidney tissue homogenate was prepared in 50 mM TEA-HCl (pH 7.4) containing 0.1 mM EGTA, 0.1 mM EDTA, 0.5 mM DTT, 1 μM pepstatin A and 2 μM leupeptin. Homogenates were prepared at 4°C and centrifuged at 20,000 x g for 1 h. Supernatants were assayed for the conversion of $[^3]$H L-arginine to $[^3]$H citrulline at 37°C for 10 min in reaction mixture containing 50 mM TEA-HCl (pH 7.4), 50 μM cold L-arginine and 200,000 dpm of L-[2,3,4-$^3$H] arginine HCl (40-70 Ci/mmol), 100 μM NADPH, 10 μM BH$_4$, 10 μM FAD, 10 μM FMN, 2 mM CaCl$_2$, 1 μg calmodulin. The reaction was terminated by adding 2 ml of ice-cold stop buffer containing 20 mM sodium acetate (pH 5.5), 1 mM citrulline, 2 mM EDTA and 0.2 mM EGTA. Samples were chromatographed on Dowex AG 50W x 8 column with 200 mesh ion-exchange resin (Na$^+$ form). Since the guanidinium group in the arginine molecule is positively charged, the conjugation between the double bond and the nitrogen lone pairs enables the formation of multiple H-bonds. Thus, arginine is remained with the resin while citrulline is eluted (Singh et al., 1997). Samples containing approximately 0.5 mg of protein were incubated for 10 min in the determination of total NOS activity in subsequent experiments.
Figure 3.7 The rate of total nitric oxide synthase activity as a function of the kidney tissue protein concentration.

NOS activity in kidney tissue was determined at different protein concentrations.
Figure 3.8 The rate of total nitric oxide synthase activity as a function of the incubation time
NOS activity in kidney tissue was determined for different incubation periods.
3.3.5 DETERMINATION OF RENAL INJURY

3.3.5.1 Lipid Peroxidation in the Kidney Tissue

The degree of lipid peroxidation in the kidney tissue was determined by measuring malondialdehyde (MDA) levels with thiobarbituric acid reactive substances (TBARS) assay (Ohkawa et al., 1979; Sung et al., 2002). A 10% (w/v) kidney homogenate was prepared in 0.1 M KCl solution containing 3 mM EDTA followed by centrifugation at 600 x g for 15 min at 4°C. An aliquot of 0.4 ml supernatant was added to the reaction mixture containing 0.2 ml of 8% SDS, 1.5 ml of 20% acetic acid, 1.5 ml of 0.8% thiobarbituric acid and 0.4 ml of water. After incubation at 95°C for 1 h, the amount of MDA formed in the reaction mixture was measured by spectrophotometer at absorbance of $\lambda = 532$ nm. MDA was used as the standard and results were expressed as a percentage of the sham operated group. The amount of MDA correlates to the degree of lipid peroxidation in the tissue.

3.3.5.2 Immunohistochemical Staining for Nitrotyrosine

A portion of the kidney was immersion-fixed in 10% neutral-buffered formalin (10% formalin, 25 mM NaH$_2$PO$_4$ and 45 mM Na$_2$HPO$_4$) overnight followed by dehydration in ethanol and xylene, and was embedded in paraffin. Sequential 5 μm paraffin-embedded cross sections were prepared. Immunohistochemical staining was performed to detect nitrotyrosine protein adducts in the kidney (Zhang et al., 2004). After deparaffinization, sections were incubated with 2% bovine serum albumin (BSA) as the blocking buffer followed by incubation with primary antibodies, mouse anti-nitrotyrosine antibodies (1:100) (Zymed Laboratories Inc. San Francisco, CA). After overnight incubation with primary antibodies, sections were treated with 0.3% hydrogen peroxide (H$_2$O$_2$) for 20
min at room temperature to inhibit the endogenous peroxidase. Sections were incubated with biotin-conjugated anti-mouse immunoglobulin (1:200, DakoCytomation, Carpentaria, CA) as secondary antibodies followed by peroxidase conjugated-streptavidin (Zymed). Sections were then treated with 3,3-diaminobenzidine (DAB)-H$_2$O$_2$ colorimetric substrate solution. The attached peroxidase catalyzed the H$_2$O$_2$-mediated oxidation of DAB to yield brown color. The area displayed brownish color indicating the nitrotyrosine protein adducts (Zhang et al., 2004). The sections were then dehydrated with ethanol and xylene mounted with Paramount® (Fisher scientific, Fairlawn, NJ, USA). The images were captured using an Axioskop2 MOT microscope (Carl Zeiss Microimaging, Thornwood, NY) with an Axiocam camera and analyzed using Photoshop 6.0 (Adobe, San Jose, CA). For a negative control, a kidney tissue section from ischemia-reperfusion group was incubated overnight with normal IgG as the primary antibody.

**3.3.5.3 Determination of Apoptotic Cells by TUNEL Staining**

Renal ischemia-reperfusion in animal models causes mitochondrial swelling and loss of membrane potential, which leads to both necrotic and apoptotic forms of cell death (25). DNA fragmentation and Terminal Deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-End Labeling (TUNEL) staining was performed in renal sections as an index of cell injury (Gavrieli et al., 1992; Zhu et al., 2002). A portion of kidney was fixed in 10% neutral-buffered formaldehyde overnight and then embedded in paraffin. DNA fragments in the sliced sections were labeled with 3 μM biotin-conjugated dUTP and 0.3 U/μL TdT (Roche Molecular Biochemical, Quebec, Canada) followed by counterstained with Mayer’s Hematoxylin. TUNEL-positive cells were recognized by focal nuclear
staining (Gavrieli et al., 1992; Zhu et al., 2002). Nuclear morphology in TUNEL positive cells were used to differentiate apoptotic cells from necrotic cells. As a positive control, a section of sham-operated kidney was pretreated with DNase to mimic the appearance of apoptotic cells. The images were captured using an Axioskop2 MOT microscope (Carl Zeiss Microimaging, Thornwood, NY) with an Axiocam camera and analyzed using Photoshop 6.0 (Adobe, San Jose, CA).

**3.3.5.4 Histological Staining**

A portion of the kidney was immersion-fixed in 10% neutral-buffered formalin overnight followed by embedding in paraffin. Sequential 5μm paraffin-embedded cross sections were prepared and stained with Hematoxylin and Eosin using standard methods.

**3.3.6 STATISTICAL ANALYSIS**

Results were analyzed using Student’s t test and ANOVA followed by Newman-Keuls multiple comparison test as appropriate. Statistical analyses were conducted using GraphPad Prism 3.02 32 Bit Executable. Data were presented as means ± s.e.m. (number of samples). P value ≤ 0.05 was considered as statistically significant.
CHAPTER 4
DETRIMENTAL ROLE OF HOMOCYSTEINE IN RENAL ISCHEMIA-REPERFUSION INJURY
4.1 RATIONALE AND HYPOTHESIS

Kidney transplantation offers greater longevity and quality of life at a lower cost when compared with other treatment options such as hemodialysis and peritoneal dialysis. Therefore, kidney transplantation is the preferred therapeutic option for patients with ESRD (Winkelmayer et al., 2002). Due to the scarcity of living-related donor organs, the use of cadaveric kidney has increased (Winkelmayer et al., 2005). The graft and patient survival rates after kidney transplantation have improved in the past decades due to the advancement in the immunosuppression therapies (Nankivell et al., 2003). However, it has been estimated that ischemic insult, especially during warm-ischemia, is responsible for 20-30% chronic allograft nephropathy which is now the dominant cause of kidney transplant failure (Ploeg et al., 1992). Ischemia-reperfusion injury is one of the most common causes of delayed function of renal allograft and is associated with poor long-term renal function (Boom et al., 2000). In addition to kidney transplantation, ischemia-reperfusion injury occurs during surgical procedures such as nephrolithotomy, parenchymal sparing surgery for renal tumors and renal arterial surgeries due to complete and prolonged interruption of renal arterial blood flow (Bonventre, 1993).

Prolonged renal ischemia can lead to ARF. Oxidative stress represents one of the important mechanisms underlying renal ischemia-reperfusion injury (Bonventre, 1993). Elevated plasma Hcy level is associated with a number of disease conditions such as cardiovascular disease, cerebrovascular disease and it is a common occurrence in patients who undergo maintenance dialysis (Bostom et al., 1999b; Bostom et al., 1999a; Bostom et al., 1997). The plasma total Hcy has been shown to be associated with arteriosclerotic events in kidney transplant recipients (Ducloux et al., 2000). The plasma total Hcy
concentration in renal transplant recipients and patients with chronic renal insufficiency is 15 μM. It is increased to 24 μM level in patients with ESRD when compared with the 9 μM level found in the population based control group in the study (Friedman et al., 2001). Interestingly, a recent study has indicated that elevated plasma levels of total Hcy are independently associated with increased kidney allograft loss in humans (Winkelmayer et al., 2005). Data are lacking on plasma and tissue Hcy status during renal ischemia-reperfusion injury. In one study, it was shown that the plasma Hcy level was increased by 2.5-fold during reperfusion period in rats which underwent intestinal ischemia (Bar-Or et al., 2004). Ischemia in kidney resulted in an increase of tissue SAH which was subsequently hydrolyzed to Hcy, in the rat (Kloor et al., 2002). In the first part of the study we hypothesize that renal ischemia causes an accumulation of kidney tissue Hcy, which plays a detrimental role in the pathogenesis of ischemia-reperfusion mediated ARF. To our knowledge, this is the first reported study that has determined the putative role of Hcy in the pathogenesis of ischemia-reperfusion injury in the kidney.
4.2 OBJECTIVES:

1. To determine the plasma and kidney tissue Hcy status during renal ischemia as well as reperfusion in the rat

2. To determine the effect of the transsulfuration pathway in the regulation of tissue and plasma Hcy during ischemia-reperfusion

3. To determine the involvement of Hcy in the pathogenesis of renal ischemia-reperfusion injury in the rat

4. To determine the effect of ischemia-preconditioning on kidney tissue CBS activity and Hcy status during ischemia-reperfusion
4.3 RESULTS: (Findings of this part of the study have been published in Am J Physiol (Prathapasinghe et al., 2007). Please see appendix for digital copyright permission. All Figures in this chapter were “used with permission”).

4.3.1 ELEVATION OF HOMOCYSTEINE LEVELS IN THE KIDNEY AND PLASMA DURING RENAL ISCHEMIA-REPERFUSION

Plasma and kidney tissue Hcy levels were determined in rats subjected to 1 h ischemia alone or 1 h ischemia followed by 1 h reperfusion. The Hcy level in kidneys subjected to 1 h ischemia was significantly higher than that in the sham-operated group (24.68 ± 3.05 vs. 8.54 ± 0.52 nmol/g tissue in the sham group; Fig. 4.1). The Hcy level remained elevated in kidneys during the 1 h reperfusion period (12.50 ± 0.69 nmol/g tissue; Fig. 4.1). Ischemia alone did not elicit any significant change in the plasma Hcy level (Fig. 4.2). However, the plasma Hcy level was significantly elevated in rats subjected to ischemia followed by reperfusion (5.18 ± 0.32 vs. 3.35 ± 0.29 µmol/L in the sham group; Fig. 4.2). Hcy metabolism in the kidney is mainly through the transsulfuration pathway in which CBS catalyzes the rate-limiting reaction (House et al., 1997). To investigate whether an increase in the Hcy level in kidneys during ischemia-reperfusion was a result of impaired transsulfuration pathway, the CBS enzyme activity was measured. The CBS activity in kidneys subjected to ischemia alone or ischemia followed by reperfusion was significantly lower than that in the sham-operated group (Fig. 4.3). Reduction of Hcy metabolism due to impaired CBS activity during ischemia-reperfusion could lead to an elevation of Hcy level in the kidney and subsequently in the circulation.
Figure 4.1  Kidney tissue Hcy concentration
Left kidney was subjected to 1 h ischemia or 1 h ischemia followed by 1 h reperfusion (I-1h/R-1h). Homocysteine (Hcy) level in the kidney tissue homogenate was measured. In the control group (Sham), only the laparotomy (the surgical opening of the abdominal cavity) was performed. Results are expressed as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group. #P<0.05 when compared with the value obtained from ischemia alone group. Kidney tissue Hcy level in the sham group was 8.54 ± 0.52 nmol/g tissue.
Figure 4.2  Plasma Hcy concentration

Left kidney was subjected to 1 h ischemia or 1 h ischemia followed by 1 h reperfusion (I-1h/R-1h). In the control group (Sham), only the laparotomy was performed. Plasma homocysteine (Hcy) levels in different groups of rats were measured. Results are expressed as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group. Plasma Hcy concentration in the sham group was 3.35 ± 0.29 µmol/L.
Figure 4.3  Kidney tissue CBS activity
Left kidney was subjected to 1 h ischemia or 1 h ischemia followed by 1 h reperfusion (I-1h/R-1h). In the control group (Sham), only the laparotomy was performed. Kidney tissue Cystathionine-β-synthase (CBS) activity in different groups of rats was measured. Results are expressed as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group. Kidney tissue CBS activity in the sham group was 6.04 ± 0.35 nmol/min/mg protein)
4.3.2 GENERATION OF SUPEROXIDE AND NITRIC OXIDE IN THE KIDNEY

Ischemia-reperfusion resulted in a significant elevation of superoxide in the kidney tissue. Ischemia alone did not cause any change in kidney tissue superoxide level while during the reperfusion, the kidney tissue superoxide level was significantly increased (Fig. 4.4). Kidney tissue NO metabolites, nitrite and nitrate levels were determined by Griess reaction. Ischemia caused an elevation of tissue nitrate and nitrite levels in the kidney tissue homogenate. It remained elevated throughout the 1 h reperfusion period (Fig. 4.5).
Figure 4.4  Kidney tissue superoxide anion level
Left kidney was subjected to 1 h ischemia or 1 h ischemia followed by 1 h reperfusion (I-1h/R-1h). In the control group (Sham), only the laparotomy was performed. Kidney tissue superoxide anion level in different groups of rats was measured. Results are expressed as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group.
Figure 4.5  Kidney tissue nitrite and nitrate level
Left kidney was subjected to 1 h ischemia or 1 h ischemia followed by 1 h reperfusion (I-1h/R-1h). In the control group (Sham), only the laparotomy was performed. Kidney tissue nitric oxide metabolite level in different groups of rats was measured. Results are expressed as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group. Kidney tissue NO metabolite concentration in sham group was 362.98 ± 22.61 nmol/g tissue)
4.3.3 RELATIONSHIP OF CBS ACTIVITY TO KIDNEY INJURY

To determine the relationship of CBS activity to the resulting accumulation of Hcy to renal injury, lipid peroxidation was measured in rats subjected to different ischemia and reperfusion periods (Fig. 4.6 and 4.7). We examined whether ischemia for a shorter period (30 vs. 60 min) had a differential effect on the CBS activity and lipid peroxidation in the kidney. The left kidney was subjected to ischemia for 30 min followed by reperfusion for 1 h. The CBS activity was significantly reduced in kidneys subjected to 30 min ischemia followed by 1 h reperfusion compared with the sham-operated group (Fig. 4.6). This short ischemic insult also caused a significant increase in lipid peroxidation in the kidney (Fig. 4.7). These results suggested that ischemia for 30 min or 1 h followed by reperfusion for 1 h could inhibit CBS activity and induce lipid peroxidation in the kidney.
Figure 4.6  Effect of the duration of ischemia on CBS activity in the kidney
Left kidney was subjected to sham operation, 30 min ischemia followed by 1 h reperfusion (I-30min/R-1h), or 1 h ischemia followed by 1 h reperfusion (I-1h/R-1h). The Cystathionine-β-synthase (CBS) activity was determined in the kidney tissue homogenate. Results are expressed as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group. #P<0.05 when compared with the value obtained from I-30min/R-1h group.
Figure 4.7  Effect of the duration of ischemia on lipid peroxidation in the kidney
Left kidney was subjected to sham operation, 30 min ischemia followed by 1 h reperfusion (I-30min/R-1h), or 1 h ischemia followed by 1 h reperfusion (I-1h/R-1h). The renal injury was assessed by the levels of lipid peroxidation in the kidney tissue homogenate. Results are expressed as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group. #P<0.05 when compared with the value obtained from I-30min/R-1h group. Kidney tissue MDA level in sham group was 1.36 ± 0.09 nmol/mg protein.
4.3.4 EFFECT OF HOMOCYSTEINE ANTIBODY TREATMENT ON LIPID PEROXIDATION AND PLASMA CREATININE LEVEL IN THE KIDNEY DURING ISCHEMIA-REPERFUSION

Peroxynitrite can lead to tissue damage by oxidizing lipids to lipid peroxides. Elevation of MDA levels is an indicator of lipid peroxide formation in the tissue. There was more than a two-fold increase in the MDA level in kidneys subjected to ischemia-reperfusion, reflecting an increased renal lipid peroxidation (Fig. 4.8). Administration of anti-Hcy antibodies into the kidney effectively reduced ischemia-reperfusion induced lipid peroxidation to the basal level found in the sham-operated group (Fig. 4.8). As a control, one group of rats received nonspecific antibodies (IgG) and such a treatment did not reduce ischemia-reperfusion induced lipid peroxidation in the kidney (Fig. 4.8). One-hour ischemia followed by 1 h reperfusion caused a significant increase in plasma creatinine level (0.43 ± 0.02 vs. 0.35 ± 0.01 mg/dl in the sham group). Administration of anti-Hcy antibodies reduced the plasma creatinine level to the basal level observed in the sham-operated group (Fig. 4.9). Administration of nonspecific antibodies (IgG) did not reduce plasma creatinine level in rats subjected to ischemia-reperfusion (Fig. 4.9). These results suggested that elevation of tissue Hcy might contribute to ischemia-reperfusion induced lipid peroxidation in the kidney and renal dysfunction.
Figure 4.8  Effect of Hcy antibody on kidney tissue lipid peroxidation

Left kidney was subjected to sham operation, 1 h ischemia and 1 h reperfusion with or without injection of anti-Hcy antibodies (I-1h/R-1h+HcyAb) or ischemia-reperfusion with injection of non-specific IgG (I-1h/R-1h+IgG). Lipid peroxides in kidneys were determined by measuring the amount of MDA in the tissue homogenate. Values are expressed as percentage increase of control (Sham: $1.363 \pm 0.099$ nmol MDA/mg protein). Results are depicted as mean ± s.e.m. (n=6). *$P<0.05$ when compared with the value obtained from sham-operated group. #$P<0.05$ when compared with the value obtained from I-1h/R-1h group.
Figure 4.9  Effect of Hcy antibody on plasma creatinine level

Left kidney was subjected to sham operation, 1 h ischemia and 1 h reperfusion with or without injection of anti-Hcy antibodies (I-1h/R-1h+HcyAb) or ischemia-reperfusion with injection of non-specific IgG (I-1h/R-1h+IgG). Plasma creatinine level was determined. Results are depicted as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group. #P<0.05 when compared with the value obtained from I-1h/R-1h group. Plasma creatinine level in sham group was 0.43 ± 0.02 mg/dl.
4.3.5 ROLE OF HOMOCYSTEINE IN ISCHEMIA-REPERFUSION MEDIATED NITROTYROSINE PROTEIN ADDUCT FORMATION

Superoxide can interact rapidly with NO to form peroxynitrite, a potent oxidant (Radi et al., 2001). To determine whether there was an increase in peroxynitrite formation in the kidney subjected to ischemia-reperfusion, immunohistochemical staining was performed to detect nitrotyrosine, a biomarker for peroxynitrite. A significant increase in the intensity of nitrotyrosine protein adduct staining was found in kidneys subjected to ischemia-reperfusion (Fig. 4.10 and 4.11), indicating an increase in peroxynitrite formation. On the other hand, little nitrotyrosine protein adduct was detected in sham-operated kidneys (Fig. 4.10 and 4.11). Next, to determine whether Hcy played any role in ischemia-reperfusion induced peroxynitrite formation in the kidney, anti-Hcy antibodies were injected into the left renal artery immediately after the induction of ischemia. Administration of anti-Hcy antibodies effectively abolished ischemia-reperfusion induced peroxynitrite formation in the kidney (Fig. 4.10 and 4.11). As a control, one group of rats was given nonspecific antibodies (rabbit IgG). Administration of nonspecific antibodies into the kidney did not attenuate ischemia-reperfusion induced peroxynitrite formation (Fig. 4.10 and 4.11). These results indicated that elevation of tissue Hcy levels might be responsible for increased peroxynitrite formation in the kidney upon ischemia-reperfusion.
<table>
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<th>I-1h/R-1h +HcyAb</th>
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</table>

**Figure 4.10  Immunohistochemical staining for nitrotyrosine protein adducts in the kidney**

Immunohistochemical staining for nitrotyrosine protein adducts was performed with anti-nitrotyrosine antibodies. Arrows point to the areas positively stained with nitrotyrosine protein adducts.
Figure 4.11  Intensity of the nitrotyrosine staining in the kidney
Left kidney was subjected to sham operation, 1 h ischemia followed by 1 h reperfusion (I-1h/R-1h) or ischemia-reperfusion with administration of anti-homocysteine antibodies (I-1h/R-1h+HcyAb). Immunohistochemical staining for nitrotyrosine protein adducts was performed with anti-nitrotyrosine antibodies. After counterstaining with Mayer's hematoxylin, nitrotyrosine protein adducts were identified under light microscope with a magnification of 400×. As a negative control, immunohistochemical staining was performed by using non-specific rabbit IgG as primary antibodies (I-1h/R-1h+IgG). The intensity of nitrotyrosine staining was quantified. Results are depicted as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group. *P<0.05 when compared with the value obtained from I-1h/R-1h group.
4.3.6 ROLE OF HOMOCYSTEINE IN ISCHEMIA-REPERFUSION INDUCED CELL DEATH

The effect of ischemia-reperfusion on cell death was examined by TUNEL assay. Few TUNEL-positive cells were found in sham-operated kidneys (Fig. 4.12 and 4.13). However, there was a marked increase in the number of TUNEL-positive cells in the cortex and medulla of kidneys subjected to ischemia-reperfusion (Fig. 4.12 and 4.13). It is known that ischemia-reperfusion can cause kidney cell death by necrosis as well as apoptosis. Necrotic cell death is characterized by cell swelling and nuclear swelling, whereas apoptotic cell death is characterized by cell shrinkage and nuclear chromatin condensation (Lieberthal & Levine, 1996; Miller et al., 2000). The morphology of TUNEL-positive cells was examined (Lieberthal & Levine, 1996; Miller et al., 2000). Necrotic cells displayed cell swelling with dispersed nuclear materials while apoptotic cells were smaller in size with condensed nuclei (Fig. 4.12). Most of the TUNEL-positive cells found in the ischemia-reperfusion group were necrotic cells rather than apoptotic cells (Fig. 4.12 and 4.13). Taken together, these results suggested that Hcy, at an elevated level, contributed to ischemia-reperfusion induced cell death both by necrosis and apoptosis in the kidney. Neutralization of tissue Hcy with anti-Hcy antibodies could attenuate 1 h ischemia and 1 h reperfusion induced cell death in kidneys.
Figure 4.12 TUNEL positive cell counts in the kidney cortex

Left kidney was subjected to sham operation, 1 h ischemia followed by 1 h reperfusion (I-1h/R-1h) or ischemia-reperfusion with anti-Hcy antibodies (I-1h/R-1h+HcyAb). Ischemia-reperfusion induced cell death was assessed by TUNEL staining. After counterstaining with Mayer's hematoxylin, TUNEL positive cells were counted under light microscope with a magnification of 400x. Data represent the average of 10 high-power fields under light microscope. Results are depicted as mean ± s.e.m. (n=6). *P <0.05 when compared with the numbers obtained from sham-operated group. #P <0.05 when compared with the numbers obtained from I-1h/R-1h group.
Figure 4.13  TUNEL positive cell counts in the kidney medulla
Left kidney was subjected to sham operation, 1 h ischemia followed by 1 h reperfusion (I-1h/R-1h) or ischemia-reperfusion with anti-Hcy antibodies (I-1h/R-1h+HcyAb). Ischemia-reperfusion induced cell death was assessed by TUNEL staining. After counterstaining with Mayer’s hematoxylin, TUNEL positive cells were counted under light microscope with a magnification of 400×. Data represent the average of 10 high-power fields under light microscope. Results are depicted as mean ± s.e.m. (n=6). *P <0.05 when compared with the numbers obtained from sham-operated group. #P <0.05 when compared with the numbers obtained from I-1h/R-1h group.
4.3.7 ROLE OF HOMOCYSTEINE IN OXIDATIVE STRESS IN THE KIDNEY

To further examine the effect of Hcy on oxidative stress in the kidney, L-Hcy (2.5 mg/kg) or vehicle was injected into rats via the left femoral vein. In the absence of ischemia-reperfusion, injection of Hcy via intravenous route caused a significant increase in renal superoxide (Fig. 4.14) and lipid peroxide levels (Fig. 4.15). Additionally, the lipid peroxidation in the right kidney of left kidney ischemia-reperfused rats was determined. Interestingly, the lipid peroxidation of the right kidney tissue homogenate prepared from rats subjected to ischemia-reperfusion to the left kidney was significantly elevated when compared with the sham-operated group (Fig. 4.16). This may suggest the endogenously elevated Hcy during the reperfusion period exerted the oxidative stress on the right kidney which was not subjected to ischemia-reperfusion. Next, the in vitro effect of Hcy was examined. The addition of L-Hcy to the tissue homogenate prepared from normal kidneys caused a significant increase in superoxide (Fig. 4.17) and lipid peroxide (Fig. 4.18) levels. Such in vitro stimulatory effect by Hcy was in a concentration dependent manner (Fig. 4.17 and 4.18).
Figure 4.14  Effect of Hcy injection on kidney tissue superoxide level
Left kidney was subjected to sham operation, 1 h ischemia and 1 h reperfusion (I-1h/R-1h), injection of 0.25 ml L-homocysteine (2.5 mg/kg) without subjecting to ischemia-reperfusion (Hcy), or injection of 0.25 ml vehicle (buffers used for L-Hcy preparation) via left femoral vein (Vehicle). Levels of superoxide anion in the kidney were determined. Results are depicted as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group.
Figure 4.15  Effect of Hcy injection on kidney tissue lipid peroxidation level
Left kidney was subjected to sham operation, 1 h ischemia and 1 h reperfusion (I-1h/R-1h), injection of 0.25 ml L-homocysteine (2.5 mg/kg) without subjecting to ischemia-reperfusion (Hcy), or injection of 0.25 ml vehicle (buffers used for L-Hcy preparation) via left femoral vein (Vehicle). Levels of lipid peroxidation in the kidney were determined. Results are depicted as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group.
Figure 4.16  Lipid peroxidation of the right kidney of left kidney ischemia-reperfused rats
Left kidney was subjected to 1 h ischemia followed by 1 h reperfusion. In the control group (Sham), only the laparotomy was performed. The lipid peroxidation was determined in the unaffected right kidney tissue homogenate. Results are expressed as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group.
Figure 4.17 In vitro effect of Hcy on superoxide production

Tissue homogenate was prepared from normal kidneys. Aliquots of homogenate were incubated with L-homocysteine (L-Hcy) at various concentrations for 1 h. At the end of incubation, superoxide level in the incubation mixture was measured. Results are expressed as mean ± s.e.m. (n=6). *P <0.05 when compared with the value obtained from samples incubated without L-Hcy.
Figure 4.18  *In vitro* effect of Hcy on lipid peroxidation
Tissue homogenate was prepared from normal kidneys. Aliquots of homogenate were incubated with L-homocysteine (L-Hcy) at various concentrations for 1 h. At the end of incubation, lipid peroxidation level in the incubation mixture was measured. Results are expressed as mean ± s.e.m. (n=6). *P <0.05 when compared with the value obtained from samples incubated without L-Hcy.
4.3.8  EFFECT OF THE DURATION OF ISCHEMIA-REPERFUSION AND RIGHT NEPHRECTOMY ON KIDNEY INJURY

We then examined the effect of a shorter period of ischemia (30 min) in the left kidney followed by reperfusion for 1 or 24 h in combination with right nephrectomy on renal function and oxidative stress. In one set of experiments, ischemia in the left kidney was induced for 30 min followed by reperfusion for 1 h with right nephrectomy. In this group of rats, there was a significant increase in plasma creatinine level and kidney lipid peroxide content compared with the sham-operated group (Table 4.1). Administration of anti-Hcy antibodies to the left kidney exerts a protective effect as both creatinine level and lipid peroxide content were reduced to the levels similar to the sham group (Table 4.1). As a control, one group of rats received nonspecific antibodies (IgG) and such a treatment had no protective effect on renal function (Table 4.1). In another set of experiments, ischemia in the left kidney was induced for 30 min followed by reperfusion for 24 h with right nephrectomy. There was a significant increase in plasma creatinine level and kidney lipid peroxide content compared with the sham-operated group (Table 4.1). Administration of anti-Hcy antibodies to the left kidney did not significantly reduce plasma creatinine levels or kidney lipid peroxide content to that similar to the sham group (Table 4.1). Injection of Hcy resulted in a further increase in plasma creatinine level and lipid peroxidation in kidneys subjected to 30 min ischemia followed by reperfusion for 1 h or 24 h (Table 4.1).
TABLE 4.1 Effect of different ischemia and reperfusion durations on plasma creatinine levels and lipid peroxidation

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<th>Treatment group</th>
<th>Plasma creatinine (mg/dl)</th>
<th>Kidney MDA (% of sham)</th>
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<td><strong>Group A</strong></td>
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</tr>
<tr>
<td>Sham</td>
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<td>100.000±2.196</td>
</tr>
<tr>
<td>I-30min/R-1h</td>
<td>0.450±0.004*</td>
<td>134.617±5.862*</td>
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<td>I-30min/R-1h+HcyAb</td>
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<td>133.443±2.204*</td>
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<tr>
<td>I-30min/R-1h+Hcy</td>
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<td>193.597±22.975**</td>
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<tr>
<td><strong>Group B</strong></td>
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<td>115.053±3.300**</td>
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<tr>
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</tr>
<tr>
<td>I-30min/R-24h+Hcy</td>
<td>2.110±0.369**</td>
<td>150.732±6.389**</td>
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Right kidney was surgically removed (right nephrectomy) at the onset of the reperfusion of left kidney. A). Left kidney was subjected to sham operation (Sham), 30-min ischemia followed by 1 h reperfusion (I-30min/R-1h), 30 min-ischemia followed by1 h reperfusion with anti-Hcy antibodies injected into the left renal artery immediately after the induction of ischemia (I-30min/R-1h+HcyAb), 30 min-ischemia followed by 1 h reperfusion with nonspecific rabbit IgG injected into the left renal artery immediately after the induction of ischemia (I-30min/R-1h+IgG), or 30 min ischemia followed by 1 h reperfusion with L-Hcy (2.5 mg/kg in 250 μl) injection into the left femoral vein when reperfusion started (I-30min/R-1h+Hcy). B). Left kidney was subjected to sham operation (Sham), 30 min ischemia followed by 24 h reperfusion (I-30min/R-24h). With right nephrectomy, the same treatments were applied and rats were sacrificed after 24 h of reperfusion. Plasma creatinine level was measured and lipid peroxides in kidneys were determined by measuring the amount of malondialdehyde (MDA). Results are expressed as means ± s.e.m. (n=4). *P<0.05 when compared with the value obtained from the corresponding sham-operated group. #P<0.05 when compared with the value obtained from the corresponding I/R group.
4.3.9 THE PROTECTIVE EFFECTS OF ISCHEMIA-PRECONDITIONING ARE MEDIATED BY HOMOCYSTEINE-LOWERING MECHANISM

Brief periods of ischemia followed by reperfusion have been shown to have some protective effects to a subsequent ischemic challenge in a variety of organs including the kidney (Islam et al., 1997). The mechanism of such a protection by ischemia preconditioning (IP) is not well understood. However, it is believed that the activation of adenosine A₁ receptors, activation of ATP sensitive K⁺ channels, induction of stress proteins, induction of nitric oxide synthase or scavenging of free radicals by antioxidant enzymes may be involved in the IP mediated attenuation of the tissue injury (Lasley et al., 1993; Heurteaux et al., 1995; Knowlton et al., 1991; Bratell et al., 1990). We determined the kidney tissue Hcy level in rats which were subjected to ischemia-preconditioning alone or ischemia-preconditioning followed by 1 h ischemia and 1 h reperfusion. Ischemia-preconditioning showed a significant decrease in kidney tissue Hcy level when compared with the ischemia-reperfusion group (Fig. 4.19). The decreased Hcy level in the ischemia-preconditioning was the result of improved CBS activity in these two groups (Fig. 4.20). The reno-protective effect of ischemia-preconditioning was determined by measuring the lipid peroxidation in the kidney tissue homogenate. As shown in Figure 4.21, the kidney tissue lipid peroxidation was significantly decreased in the ischemia-preconditioned groups.
**Figure 4.19**  **Effect of ischemia-preconditioning on kidney Hcy level**

Left kidney was subjected to 1 h ischemia followed by 1 h reperfusion (I-1h/R-1h), 4 cycles of 5 min ischemia and 10 min reperfusion followed by 1 h ischemia and 1 h reperfusion (IP+I-1h/R-1h) or 4 cycles of 5 min ischemia and 10 min reperfusion alone (IP). Homocysteine (Hcy) level in the kidney tissue homogenate was measured. In the control group (Sham), only the laparotomy was performed. Results are expressed as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group. #P<0.05 when compared with the value obtained from I-1h/R-1h group.
Figure 4.20  Effect of ischemia-preconditioning on kidney CBS activity
Left kidney was subjected to 1 h ischemia followed by 1 h reperfusion (I-1h/R-1h), 4 cycles of 5 min ischemia and 10 min reperfusion followed by 1 h ischemia and 1 h reperfusion (IP+I-1h/R-1h) or 4 cycles of 5 min ischemia and 10 min reperfusion alone (IP). Cystathionine-β-synthase activity in the kidney tissue homogenate was determined. In the control group (Sham), only the laparotomy was performed. Results are expressed as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group. #P<0.05 when compared with the value obtained from I-1h/R-1h group.
Figure 4.21  Effect of ischemia-preconditioning on kidney lipid peroxidation
Left kidney was subjected to 1 h ischemia followed by 1 h reperfusion (I-1h/R-1h), 4 cycles of 5 min ischemia and 10 min reperfusion followed by 1 h ischemia and 1 h reperfusion (IP+I-1h/R-1h) or 4 cycles of 5 min ischemia and 10 min reperfusion alone (IP). Lipid peroxidation in the kidney tissue homogenate was measured. In the control group (Sham), only the laparotomy was performed. Results are expressed as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group. #P<0.05 when compared with the value obtained from I-1h/R-1h group.
4.4 DISCUSSION

Ischemia followed by reperfusion caused oxidative stress and cell death in the kidney. The novel findings of this part of the study are 1) ischemia-reperfusion impairs Hcy metabolism leading to Hcy accumulation in the kidney and subsequently in the plasma; 2) Hcy, at elevated levels, contributes to ischemia-reperfusion induced lipid peroxidation, apoptotic and necrotic cell death in the kidney; 3) Ischemia-reperfusion mediated inhibition of renal CBS activity was the cause of elevated Hcy level; and 4) the protective effect of ischemia-preconditioning is mediated, in part, via the restored CBS activity and the consequential decrease of kidney tissue Hcy level.

4.4.1 DETRIMENTAL ROLE OF HOMOCYSTEINE IN ISCHEMIA-REPERFUSION INJURY

Several lines of evidence obtained from the present study suggested that Hcy played an important role in ischemia-reperfusion induced oxidative stress in the kidney. First, there was a significant elevation of Hcy level in kidneys subjected to ischemia alone or ischemia followed by reperfusion. This was in accordance with the reduced activity of CBS, an enzyme responsible for regulating the rate-limiting step in Hcy metabolism via the transsulfuration pathway. The CBS activity remained at the diminished level after 24 h of reperfusion. Second, the level of two biochemical markers for oxidative stress (peroxynitrite, lipid peroxide) and a renal functional marker (plasma creatinine level) were increased in rats subjected to ischemia-reperfusion. Administration of anti-Hcy antibodies to kidneys effectively abolished ischemia-reperfusion induced peroxynitrite formation and lipid peroxidation during the first hour of reperfusion. Third, the addition
of Hcy to the kidney homogenate caused a significant increase in lipid peroxidation in a concentration dependent manner.

4.4.2 HOMOCYSTEINE-MEDIATED OXIDATIVE STRESS IN THE PATHOGENESIS

It has been suggested that autooxidation of Hcy and formation of Hcy mixed disulfides may contribute to the ROS pool (Jakubowski et al., 2000). Hcy, when added to plasma, undergoes autooxidation, which was accompanied by the generation of H$_2$O$_2$ or superoxide anion (Heinecke et al., 1987). In the present study, elevation of renal superoxide was observed only during the reperfusion period, even though an increase in renal Hcy level was observed during the ischemia and ischemia-reperfusion periods. This is not surprising because the thiol oxidation reactions require the presence of molecular oxygen and hence Hcy remained in the reduced form until reperfusion started. During reperfusion, reoxygenation occurred in the kidney. In the presence of oxygen, Hcy could undergo thiol oxidation reactions to generate ROS, including superoxide, in the kidney (Fig. 4.22). CBS activity is increased under oxidizing conditions (Taoka et al., 1998; Mosharov et al., 2000). However, according to our observations, it remained inhibited throughout the reperfusion period. This may perhaps be due to an interaction of CBS with a much stronger or irreversible inhibitor which is overproduced during the reperfusion period. The factors which affect the CBS activity are studied in the proceeding chapter. Superoxide avidly reacts with NO to form peroxynitrite. Increased peroxynitrite formation is believed to be responsible for ischemia-reperfusion induced oxidative stress.
Figure 4.22 Postulated mechanism of Hcy mediated ischemia-reperfusion injury in the rat kidney

Tissue Hcy level was elevated during ischemia due to impaired CBS activity which remained inhibited throughout the reperfusion period. With the onset of reperfusion, tissue Hcy was effluxed into plasma where it was autooxidized in the presence of molecular oxygen. NO level was also increased during ischemia and reperfusion. The increased level of superoxide, NO and the production of peroxynitrite may have contributed to the ischemia-reperfusion mediated renal injury. Treatment with Hcy antibody ameliorated the renal injury, presumably by preventing the autooxidation of Hcy. It may have prevented the generation of reactive oxygen species including superoxide.
in the kidney (Walker et al., 2000). It is reported that renal arterial ischemia causes an abrupt increase in tissue NO concentration (Saito & Miyagawa, 2000). Such an increase in renal NO concentration during ischemia originates primarily from thiol-dependant tissue stores (Salom et al., 2005). In the present study, besides an elevation of superoxide level, there was a significant increase in the level of NO metabolites in kidneys subjected either to ischemia or to ischemia-reperfusion. In accordance, there was a significant increase in peroxynitrite formation and lipid peroxidation in the same kidneys. An increase in the number of TUNEL-positive cells in the medulla and cortex after ischemia-reperfusion provided further evidence for renal injury. Administration of anti-Hcy antibodies to the kidney not only abolished lipid peroxidation but also effectively blocked 1 h ischemia/1 h reperfusion-induced necrotic and apoptotic cell death. Anti-Hcy antibody treatment prevented the renal injury, presumably by forming immune complexes with Hcy, which, in turn, made Hcy unavailable for the thiol oxidation reactions. Thus, the generation of ROS might have been attenuated. Taken together, these results suggested that elevation of tissue Hcy levels contributed to oxidative stress and apoptosis in the kidney during 1 h ischemia followed by 1 h reperfusion. However, a single dose of anti-Hcy antibody injection had no effect on lipid peroxidation and plasma creatinine level in rats subjected to a longer duration of reperfusion. In order to assess the renal function of the ischemia-reperfused kidney, right nephrectomy was performed in rats which were subjected to prolonged reperfusion period. The absence of protective effect in this group might be due to diminished CBS activity during the 24 h reperfusion period throughout which the Hcy level in the kidney remained elevated. A single
administration of antibodies might not be sufficient to neutralize excess Hcy accumulated in the kidney during a prolonged period of reperfusion.

On the other hand, results obtained from the present study did not rule out the possibility that mechanisms other than Hcy might be involved in oxidative stress and cell death in the kidney during 24 h reperfusion. The mechanisms by which prolonged ischemia-reperfusion causes renal injury remain to be further investigated in a future study. The kidney is one of the major sites for removal of Hcy from the circulation. It has been estimated that the kidney removes approximately 20% of plasma Hcy in a single pass across the kidney in healthy rats (Bostom et al., 1995; House et al., 1997). In the present study, kidney Hcy level was significantly increased during the ischemic phase while the plasma Hcy level remained unchanged. During the reperfusion phase, an elevation of plasma Hcy level was observed. The elevation of Hcy level in the circulation was likely a result of excess Hcy exported from the kidney during the reperfusion phase. The elevation of Hcy levels in the kidney upon renal ischemia-reperfusion appeared to be due to impaired Hcy metabolism through the transsulfuration pathway. It has been reported that in rat kidney, the majority of Hcy (up to 78%) is metabolized via the transsulfuration pathway while a small amount of Hcy is remethylated to methionine (House et al., 1997). This finding concurs with our current observation that the activity of CBS, the enzyme responsible for the rate-limiting reaction in the transsulfuration pathway, was markedly decreased during ischemia. CBS activity remained at a diminished level throughout the reperfusion phase. Such a reduction in CBS activity caused Hcy accumulation in the kidney. Taken together, these results suggest that accumulation of Hcy in the kidney during ischemia-reperfusion was likely caused by a
reduction of CBS activity. Kidneys subjected to brief episodes of ischemia preconditioning prior to prolonged ischemia have shown some protective effects in the kidney (Islam et al., 1997). The results of current study demonstrated a significant elevation of CBS activity in the kidneys subjected to ischemia-preconditioning. The tissue Hcy level was decreased and the renal injury was ameliorated in this group. Thus, it was revealed that the renoprotective effect of ischemia-preconditioning is in part, brought about by Hcy-lowering mechanism. To the best of our knowledge, the present study demonstrated, for the first time, that Hcy is elevated in the kidney during ischemia-reperfusion.

In summary, our results clearly display a direct link between ischemia-reperfusion induced elevation of tissue Hcy levels and kidney injury. Anti-Hcy antibody administration offers a transient protective effect on ischemia-reperfusion induced oxidative stress and renal dysfunction. Regulation of Hcy metabolism may provide some beneficial effect by protecting kidneys from ischemia-reperfusion induced injury.
CHAPTER 5
INHIBITION OF CYSTATHIONINE-β-SYNTHASE ACTIVITY DURING RENAL ISCHEMIA-REPERFUSION: ROLE OF pH AND NITRIC OXIDE
5.1 **RATIONALE AND HYPOTHESIS**

In the previous chapter we demonstrated that ischemia-reperfusion caused an inhibition of kidney tissue CBS activity and as a result tissue and plasma Hcy levels were significantly elevated. The CBS activity is enhanced under oxidizing environment (Zou & Banerjee, 2003). However, we did not observe a restoration of CBS activity during reperfusion in which superoxide level was significantly increased. Mammalian CBS is a pyridoxal-5'-phosphate dependent enzyme in which heme is a cofactor located in the N-terminal domain (Kery et al., 1994). From acidic to physiological pH, heme in the CBS is in the Fe(III) form while at alkaline pH the Fe(II) form predominates (Pazicni et al., 2004). The optimal activity of CBS *in vitro* was recorded at pH 8.5. Pazicni *et al* found a profound connection between the heme group of CBS and pH in the redox sensitive regulation of enzyme activity (Pazicni *et al.*, 2004). Thus, it has been suggested that the heme regulates CBS activity through changes in iron redox state (Pazicni *et al.*, 2004). Kidney is a major organ with regard to the regulation of acid-base balance (Boron, 2006). It maintains the acid-base balance by bicarbonate reabsorption (Boron, 2006; Yucha, 2004). In addition, systemic acidosis stimulates the phosphate dependant glutaminase and glutamate dehydrogenase which are involved in the generation of bicarbonate and ammonia (NH₃) from glutamine in the proximal tubules (Curthoys & Gstraunthaler, 2001). Increased urinary excretion of ammonium ion is a major component of the kidneys respond to metabolic acidosis (DuBose *et al.*, 1991; Karim *et al.*, 2006).

NO readily reacts with iron that is incorporated into the heme complex or in non-heme iron protein. It has been reported that human CBS binds to NO weakly in the Fe(II) state of heme resulting in the inactivation of the enzyme (Taoka & Banerjee, 2001).
During ischemia, an abrupt increase in the tissue NO level has been reported in the rat kidney (Salom et al., 2005). The onset of reperfusion causes NO to return to basal levels and thus the NO concentration remains at normal level during the very early phase of reperfusion (Salom et al., 2005; Saito & Miyagawa, 2000). However, we previously observed that the NO concentration, as measured by NO metabolites, was elevated during ischemia and remained elevated during the early reperfusion phase (Prathapasinghe et al., 2007). Kidney contains all three isoforms of nitric oxide synthase (NOS). Neuronal (nNOS) and endothelial (eNOS) isoforms are found in the macula densa and renal vasculature, respectively (Chatterjee et al., 2003). The inducible form (iNOS) is expressed in the glomerulus, renal tubule and arcuate arteries of rat kidney (Joles et al., 2002). The iNOS activity was increased dramatically during the first 24 h of reperfusion both in the cortex and medulla of the rat kidney (Komurai et al., 2003). After 6 h of reperfusion of the kidney, the plasma NO level was severely increased due to the up-regulation of iNOS activity (Chatterjee et al., 2003). Excessive production of NO, particularly from iNOS, or an exogenous supply of NO before ischemia was found to play a detrimental role in ischemia-reperfusion induced renal injury (Chatterjee et al., 2003; Basireddy et al., 2006; Parlakpinar et al., 2006). Conversely, the eNOS activity was decreased during reperfusion (Komurai et al., 2003).

Ischemia leads to the development of metabolic acidosis due to the accumulation of lactic acid as the end product of anaerobic glycolysis (Stanley et al., 1992). We hypothesized that the metabolic acidosis resulting from the ischemia, and elevated NO level during reperfusion, are responsible for the inhibition of CBS activity during ischemia and reperfusion, respectively. Therefore, we sought to explore the effect of an
alkalizing treatment during ischemia and NO scavenger during reperfusion on the activity of CBS and ischemia-reperfusion mediated renal injury.

5.2 OBJECTIVES:

1. To determine the kidney tissue pH and NO level during ischemia-reperfusion

2. To determine the effect of pH and NO on the CBS activity

3. To explore the plausibility of retention of CBS activity during ischemia and reperfusion by manipulating the tissue pH with an alkalizing treatment and tissue NO level with a treatment of an NO scavenger
5.3 RESULTS

5.3.1 EFFECT OF pH FLUCTUATION ON KIDNEY CBS ACTIVITY

To determine whether changes in tissue pH had any effect on CBS activity, rat kidneys were subjected to ischemia (15 to 60 min). The kidney tissue pH was decreased after ischemia for 15, 45 or 60 min (Fig. 5.1). In correspondence, the CBS activity was decreased in those kidney tissue samples (Fig. 5.2). Although the kidney tissue pH was elevated during reperfusion (Fig. 5.1), the CBS activity remained low (Fig. 5.2). These results suggested that factors other than acidosis also affected the CBS activity during the reperfusion period. Phosphate and NH₃ are the two major buffering agents present in the urine. Phosphate is freely filtered by glomeruli and travels down the tubules where it combines with H⁺ to form HPO₄²⁻ (Karim et al., 2006; Yucha, 2004). Therefore, (NH₄)₂HPO₄ solution was used as an alkalizing agent to counteract the metabolic acidosis that was developed during ischemia. According to our preliminary experiments, an intrarenal arterial injection of 250 μl of 10 mM (NH₄)₂HPO₄ was required to retain the kidney tissue pH after 45 min ischemia at a value similar to sham-operated kidneys (pH 7.05). In the subsequent experiments, one dose (250 μl) of 10 mM (NH₄)₂HPO₄ was injected to rats via the left renal artery immediately after the onset of ischemia. Such a treatment significantly increased the CBS activity in kidneys subjected to ischemia alone or ischemia-reperfusion when compared with the untreated ischemia group (Fig. 5.3). However, (NH₄)₂HPO₄ treatment with ischemia-reperfusion did not show any statistically significant increase in the CBS activity when compared with the untreated ischemia-reperfusion group (Fig. 5.3).
**Figure 5.1  Effect of the duration of ischemia and reperfusion on tissue pH**

Left kidney was subjected to sham operation or different periods of ischemia. In the ischemia-reperfusion group, rats were subjected to 45 min ischemia followed by 1, 6 and 24 h reperfusion. The tissue pH was determined in the homogenate prepared in de-ionized water. Results are expressed as mean ± s.e.m (n=4). *P≤0.05 when compared with the value obtained from sham-operated group. pH of the kidney homogenate in sham-operated group was 7.05
Figure 5.2 Effect of the duration of ischemia and reperfusion on CBS activity

Left kidney was subjected to sham operation or different periods of ischemia. In the ischemia-reperfusion group, rats were subjected to 45 min ischemia followed by 1, 6 and 24 h reperfusion. CBS activity was determined. Results are expressed as mean ± s.e.m. (n=6). *P≤0.05 when compared with the value obtained from sham-operated group.
Figure 5.3  Effect of an alkalizing treatment during ischemia on CBS activity
Left kidney was subjected to sham operation (Sham), 45 min ischemia (I-45min) or 45 min ischemia followed by 6 h reperfusion (I-45min/R-6h). An injection of (NH₄)₂HPO₄ (I-45min+(NH₄)₂HPO₄) was given through the left renal artery immediately after the induction of ischemia. A group of rats which received (NH₄)₂HPO₄ during ischemia were allowed for 6 h of reperfusion (I-45min/R-6h+(NH₄)₂HPO₄). CBS activity was determined in the kidney tissue homogenate after the ischemia and reperfusion periods. Results are expressed as mean ± s.e.m (n=6). *P≤0.05 when compared with the value obtained from sham-operated group. #P≤0.05 when compared with the value obtained from I-45min group.
In vitro assays determined that the pH mediated inhibition of the CBS activity, was completely reversible once the pH in the tissue homogenate was restored (Fig. 5.4). The effect of pH on the CBS activity was further examined in the in vitro assay. The tissue homogenate was prepared from rat kidneys without surgery and used for CBS activity measurement. The CBS activity was determined in the reaction mixtures with pH ranging from 6.5 to 10. The optimum enzyme activity of CBS was recorded at pH 8.5 (Fig. 5.5). These results suggested that metabolic acidosis during ischemia might contribute to the reduction of CBS activity in the kidney.
Figure 5.4  Reversibility of CBS activity inhibited by pH
10% kidney tissue homogenate was prepared at pH 6.5 and pre-incubated for 45 min at 37°C. The pH was brought up to 8.5 in one set while the other left at the pH 6.5. After 15 min of further pre-incubation the CBS activity was determined in a reaction mixture at corresponding pH. Results are expressed as mean ± s.e.m. (n=3). *P<0.05 when compared with the value obtained from pH 6.5 group.
Figure 5.5  Effect of pH on CBS activity
10% (w/v) kidney tissue homogenate was prepared in 0.05 M potassium phosphate buffer at different pH. The CBS enzyme reaction was carried out with a reaction mixture at the corresponding pH of the homogenate. Results are expressed as mean ± s.e.m. (n=4).
5.3.2 EFFECT OF NITRIC OXIDE ON KIDNEY CBS ACTIVITY

NO is a known inhibitor of CBS activity (Taoka & Banerjee, 2001). The effect of NO on the kidney CBS activity was examined in the presence of various amounts of sodium nitroprusside (SNP), a known NO donor, in the reaction mixture. Addition of SNP to the assay reaction mixture displayed a dose-dependent inhibition of the kidney CBS activity (Fig. 5.6). To further confirm the inhibitory effect of NO on the CBS activity, an NO scavenger (PTIO) was administered to rats. Injection of PTIO via right femoral vein at the onset of reperfusion partially restored the CBS activity (Fig. 5.7). Combined injection of (NH₄)₂HPO₄ during ischemia and PTIO during reperfusion also significantly increased the CBS activity in the kidney (Fig. 5.7). L-NAME was injected at 3 different doses; 10, 3, 1.5 mg/kg. Only at the lowest concentration L-NAME (1.5 mg/kg) was effective in regaining the CBS activity (Fig. 5.7). L-NAME is a known inhibitor of NOS and it decreases total NOS activity in the rat kidney (Pechanova et al., 2006). In contrast to the inhibitory effect of acidic pH on the CBS activity, NO caused an irreversible inhibition (Fig. 5.8). When the tissue homogenate was pre-incubated with SNP, the subsequent addition of PTIO as an NO scavenger was unable to restore the CBS activity (Fig. 5.8). On the other hand, the pre-incubation with PTIO provided a significant protection against the NO mediated inhibition upon the subsequent addition of SNP (Fig. 5.8).
Figure 5.6  Effect of NO on CBS activity

The effect of NO on CBS activity in the transsulfuration pathway was determined in a 10% (w/v) kidney tissue homogenate prepared in 0.05 M potassium phosphate buffer. Different concentrations of sodium nitroprusside (SNP) were used as the source of NO. The CBS activity was measured at pH 8.3. Results are expressed as mean ± s.e.m (n=3). *P≤0.05 when compared with the control group.
Figure 5.7  Effect of NO scavenger during reperfusion on CBS activity
Left kidney was subjected to sham operation (Sham), 45 min ischemia followed by 6 h reperfusion (I-45min/R-6h), 45 min ischemia followed by 6 h reperfusion with an intravenous injection of PTIO (I-45min/R-6h+PTIO) or 45 min ischemia with an intrarenal arterial injection of (NH₄)₂HPO₄ during ischemia followed by 6 h reperfusion with an intravenous injection of PTIO (I-45min/R-6h+(NH₄)₂HPO₄+PTIO) or 45 min ischemia followed by 6 h reperfusion with an intravenous injection of L-NAME (I-45min/R-6h+L-NAME). Kidney tissue CBS activity was measured. Results are expressed as mean ± s.e.m. (n=6). *P≤0.05 when compared with the value obtained from sham-operated group. #P≤0.05 when compared with the value obtained from I-45min/R-6h group.
Figure 5.8  Irreversibility of CBS activity inhibited by NO
10% kidney tissue homogenate was pre-incubated with 200 µM SNP or 200 µM PTIO for 15 min at pH 8.5. Equimolar amount of PTIO was added to the tissue homogenate that was pre-incubated with SNP and vice versa. Following 15 min of further pre-incubation at 37°C, the CBS activity was determined. Results are expressed as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from control group. #P<0.05 when compared with the value obtained group pre-incubated first with SNP.
The nature of the protective effect of PTIO on CBS activity was further examined *in vitro*. Equal molar concentration of PTIO showed the maximum restoration of the NO mediated inhibition of CBS activity (Fig. 5.9). Interestingly, PTIO demonstrated stoichiometrically a greater NO scavenging capacity at higher SNP:PTIO levels (Fig. 5.9). There was no significant difference between the 150 μM and 50 μM PTIO concentrations with regard to the protective effect that they exerted on NO-mediated inhibition of CBS activity. The NO mediated inhibition of CBS activity was significantly greater at alkaline conditions (Fig. 5.10) which, presumably, explained why CBS activity was not restored during the reperfusion period. Next, the levels of NO metabolites were determined in the kidney tissue. A significant increase in the NO metabolite levels was observed in the kidney tissue during reperfusion periods (Fig. 5.11). There appeared to be an inverse relationship between the CBS activity (Fig. 5.2) and NO levels in the kidney during reperfusion periods (Fig. 5.11). The NOS activity was increased during the reperfusion period (Fig. 5.12). Ischemia did not seem to affect the NOS activity (Fig. 5.12), however, the NO metabolite showed a significant increase (Fig. 5.11). The elevation of NO metabolite level during ischemia might be derived from tissue stores (Salom *et al.*, 2005). Neither the alkaline nor PTIO treatment resulted in a decrease in NO metabolite level nor NOS activity in kidney tissue homogenates (Fig 5.11 and 5.12).
Figure 5.9  Stoichiometry of the PTIO:SNP in the NO-mediated inhibition of CBS activity

10% kidney tissue homogenate was prepared in 0.05 M potassium phosphate buffer. CBS activity was measured in a reaction mixture at pH 8.5, containing 200 μM SNP and different concentration of PTIO. Results are expressed as mean ± s.e.m (n=3). *P≤0.05 when compared with the value obtained from control group. #P≤0.05 when compared with the value obtained from SNP alone group.
Figure 5.10  Effect of pH on NO-mediated inhibition of CBS activity

10% kidney tissue homogenate was prepared in 0.05 M potassium phosphate buffer. The CBS activity was measured at the corresponding pH in 10% kidney homogenate prepared in a range of pH in the presence or absence of 200 μM SNP.
Figure 5.11 Effect of the duration of reperfusion on kidney tissue nitrate and nitrite levels

Left kidney was subjected to sham operation (Sham), 45 min ischemia followed by 1, 6 and 24 h reperfusion or 45 min ischemia followed by 6 h reperfusion with an injection of (NH₄)₂HPO₄ during ischemia or PTIO during reperfusion. Kidney tissue nitrite and nitrate levels were determined. Results are expressed as mean ± s.e.m. (n=6). *P≤0.05 when compared with the value obtained from sham-operated group.
Figure 5.12  Effect of the duration of reperfusion on kidney tissue NOS activity
Left kidney was subjected to sham operation (Sham), 45 min ischemia followed by 1, 6 and 24 h reperfusion or 45 min ischemia followed by 6 h reperfusion with an injection of (NH₄)₂HPO₄ during ischemia or PTIO during reperfusion. Kidney tissue NOS activity was determined. Results are expressed as mean ± s.e.m. (n=6). *P<0.05 when compared with the value obtained from sham-operated group.
5.3.3 EFFECT OF NITRIC OXIDE SCAVENGER ON RENAL INJURY AND FUNCTION

There was a significant elevation of Hcy levels in kidneys subjected to ischemia-reperfusion (Fig. 5.13) while PTIO administration reversed the tissue Hcy levels to that of the sham-operated group (Fig. 5.13). Plasma Hcy level was significantly elevated disproportionate to the inhibition of CBS activity in the kidney tissue in ischemia-reperfusion group (Fig. 5.14). This may perhaps be due to the right nephrectomy at the onset of reperfusion period. The PTIO treatment also prevented lipid peroxidation as determined by measuring the level of MDA in the kidney tissue (Fig. 5.15). Kidney function was determined by plasma creatinine measurement. The PTIO treatment showed a significant reduction in plasma creatinine level (Fig. 5.16). Kidney function was further tested by measuring plasma creatinine after 24 h reperfusion. A second dose of PTIO (first dose was administered at the onset of reperfusion via intravenous route) was administered via intraperitoneal route after 12 h of reperfusion to these rats. Plasma creatinine level was significantly decreased in PTIO treated group (0.729±0.018 mg/dl) when compared with 24 h untreated ischemia-reperfusion group (0.867±0.026 mg/dl). NO can react with superoxide anion to form peroxynitrite, a potent free radical. An immunohistochemical analysis was performed to detect nitrotyrosine protein adduct, a biomarker of peroxynitrite. Ischemia-reperfusion caused a significant increase in the intensity of nitrotyrosine protein adduct staining, indicating an increased peroxynitrite formation. Rats treated with PTIO displayed much less nitrotyrosine protein adduct staining in the kidney tissue (Fig 5.17 and 5.18).
Figure 5.13  Effect of NO scavenger on kidney tissue Hcy level
Left kidney was subjected to sham operation (Sham), 45 min ischemia followed by 6 h reperfusion (I-45min/R-6h) or 45 min ischemia followed by 6 h reperfusion with an intravenous injection of PTIO (I-45min/R-6h+PTIO). Kidney tissue Hcy level was determined. Results are depicted as mean ± s.e.m (n=6). *P≤0.05 when compared with the value obtained from sham-operated group. #P≤0.05 when compared with the value obtained from I-45min/R-6h group.
Figure 5.14   Effect of NO scavenger on plasma Hcy level
Left kidney was subjected to sham operation (Sham), 45 min ischemia followed by 6 h reperfusion (I-45min/R-6h) or 45 min ischemia followed by 6 h reperfusion with an intravenous injection of PTIO (I-45min/R-6h+PTIO). Plasma Hcy level was determined. Results are depicted as mean ± s.e.m (n=6). *P≤0.05 when compared with the value obtained from sham-operated group.
Figure 5.15  Effect of NO scavenger on lipid peroxidation
Left kidney was subjected to sham operation (Sham), 45 min ischemia followed by 6 h reperfusion (I-45min/R-6h) or 45 min ischemia followed by 6 h reperfusion with an intravenous injection of PTIO (I-45min/R-6h+PTIO). Kidney tissue lipid peroxidation was determined. Results are depicted as mean ± s.e.m (n=6). *P<0.05 when compared with the value obtained from sham-operated group. #P<0.05 when compared with the value obtained from I-45min/R-6h group.
Figure 5.16  Effect of NO scavenger on plasma creatinine

Left kidney was subjected to sham operation (Sham), 45 min ischemia followed by 6 h reperfusion (I-45min/R-6h) or 45 min ischemia followed by 6 h reperfusion with an intravenous injection of PTIO (I-45min/R-6h+PTIO). Plasma creatinine level was measured. Results are depicted as mean ± s.e.m (n=6). *P<0.05 when compared with the value obtained from sham-operated group. #P<0.05 when compared with the value obtained from I-45min/R-6h group.
Figure 5.17  Effect of NO scavenger on nitrotyrosine protein adduct formation

Left kidney was subjected to sham operation (Sham), 45 min ischemia followed by 6 h reperfusion (I-45min/R-6h) or 45 min ischemia followed by 6 h reperfusion with an intravenous injection of PTIO (I-45min/R-6h+PTIO). Immunohistochemical staining for nitrotyrosine protein adducts was performed with anti-nitrotyrosine antibody. After counterstaining with Mayer’s Hematoxylin, nitrotyrosine protein adducts were identified under light microscope with a magnification of x400. Arrows point to the areas positively stained (brown color) with nitrotyrosine protein adducts.
Figure 5.18  Effect of NO scavenger on nitrotyrosine stain intensity
Left kidney was subjected to sham operation (Sham), 45 min ischemia followed by 6 h reperfusion (I-45min/R-6h) or 45 min ischemia followed by 6 h reperfusion with an intravenous injection of PTIO (I-45min/R-6h+PTIO). Mean intensity of nitrotyrosine staining was quantified. Results are depicted as mean ± s.e.m (n=6). *P≤0.05 when compared with the value obtained from sham-operated group. #P≤0.05 when compared with the value obtained from I-45min/R-6h group.
TUNEL positive cells were present both in the cortex and medulla upon ischemia-reperfusion, indicating cell death (due to apoptosis and necrosis) in the kidney (Fig. 5.19). In contrast to the 60 min ischemia group of the previous chapter, the most of the TUNEL positive cells were apoptotic in the 45 min ischemia followed by 6 h reperfusion group (Fig. 5.19). The PTIO treatment caused a significant reduction in the count of apoptotic and necrotic cells (Fig. 5.19 and 5.20). The gross and microscopic appearance of kidney sections was also examined. Administration of PTIO retained the gross appearance of ischemia-reperfusion kidneys similar to the sham-operated group (Fig. 5.21). Hematoxylin and Eosin stained tissue sections show a massive extravasation of blood cells in the medulla region of the ischemia-reperfusion group (Fig. 5.21). The PTIO treatment caused a marked reduction in blood cell infiltration (Fig. 5.21).
Figure 5.19  Effect of NO scavenger on Apoptotic cell death
Left kidney was subjected to sham operation (Sham), 45 min ischemia followed by 6 h reperfusion (I-45min/R-6h) or 45 min ischemia followed by 6 h reperfusion with an intravenous injection of PTIO (I-45min/R-6h+PTIO). TUNEL-positive cells were identified under light microscope with a magnification of x400. Inset: image of normal cells and apoptotic cells were enlarged. Filled arrow points to the apoptotic cells with condensed nuclear material while open arrow points to normal cell.
Figure 5.20  Effect of NO scavenger on Apoptotic cell count

Left kidney was subjected to sham operation (Sham), 45 min ischemia followed by 6 h reperfusion (I-45 min/R-6 h) or 45 min ischemia followed by 6 h reperfusion with an intravenous injection of PTIO (I-45 min/R-6 h+PTIO). Ischemia-reperfusion induced cell death was assessed by TUNEL staining. After counterstaining with Mayer’s Hematoxylin, TUNEL-positive cells were identified under light microscope with a magnification of x400. The numbers of apoptotic cells were counted. Data represent the average of 10 high-power fields under light microscope. Results are depicted as mean ± s.e.m. *P≤0.05 when compared with the value obtained from sham-operated group. #P≤0.05 when compared with the value obtained from I-45 min/R-6 h group.
Figure 5.21  Effect of NO scavenger on renal morphology

Left kidney was subjected to sham operation (Sham), 45 min ischemia followed by 6 h reperfusion (I-45min/R-6h) or 45 min ischemia followed by 6 h reperfusion with an intravenous injection of PTIO (I-45min/R-6h+PTIO). Gross appearance of a mid-transverse plane of kidney was examined and renal vascular integrity and cellular morphology was examined by Hematoxylin and Eosin staining. Arrows point to the areas with blood cell infiltration.
5.4 DISCUSSION

We previously demonstrated that ischemia-reperfusion induced renal injury was, in part, mediated by Hcy (Prathapasinghe et al., 2007). The reduced CBS activity, initially as a consequence of ischemia, remained low throughout 24 h reperfusion period (Prathapasinghe et al., 2007). In the present study, our results clearly demonstrated two major factors that affected the CBS activity during ischemia and reperfusion. Decreased pH caused the initial inhibition of CBS during ischemia while the elevation of NO levels prevented the regaining of the enzyme activity during reperfusion. Furthermore, the adjustment of tissue pH and the administration of an NO scavenger could retain and recover the CBS activity significantly in kidneys subjected to ischemia-reperfusion. The renal injury was substantially minimized in these rats as reflected by histological changes, reduced lipid peroxidation and cell death as well as improved kidney function.

5.4.1 EFFECT OF AN ALKALIZING TREATMENT DURING ISCHEMIA ON CBS ACTIVITY

First, we examined the effect of pH on renal CBS activity during ischemia-reperfusion. The anaerobic metabolism during ischemia could result in metabolic acidosis in the kidney (Perico et al., 2004). Metabolic acidosis was severe when the ischemia was prolonged in the kidney. The results showed an inverse relationship between the CBS activity and the degree of acidosis (Fig. 5.1 and 5.2). Injection of an alkaline solution (\((\text{NH}_4)_2\text{HPO}_4\)) into the kidney at the onset of ischemia improved the CBS activity marginally, yet a statistically significantly elevated level as compared with the ischemia group (Fig. 5.3). However, such a treatment was unable to restore the CBS activity to the
normal physiological level, perhaps due to the limited availability of free NH$_3$ in the solution. NH$_3$ in a solution can cross cell membranes predominantly in their uncharged form and can consume cytosolic H$^+$, resulting in an increase in intracellular pH (Boron, 2006). Some other factors which include carbon monoxide (CO) may have caused the inhibition of CBS activity during ischemia (Taoka & Banerjee, 2001). CO-mediated inhibition of CBS activity is reversible (Taoka et al., 1999). Therefore, if CO played a role in the impairment of CBS activity during ischemia, it might not exert an inhibitory effect throughout the reperfusion period. Similarly, the pH mediated inhibition of CBS activity was also reversible (Fig. 5.4). The kidney tissue pH during reperfusion was higher than the normal physiological level. The response of the kidney to an acute acidosis is such that the withdrawal of the cause of acute acidosis results in a rise in intracellular pH above its initial level (Boron, 2006). This agrees with the observation in the present study (Fig. 5.1). In the present study, kidney CBS activity exhibited a pH optimum of 8.5 (Fig. 5.5). Interestingly, neither the elevation of tissue pH during reperfusion nor the alkaline treatment at the induction of ischemia was able to restore the CBS activity after 6 h reperfusion period (Fig. 5.1 and 5.3). These results suggested that factor(s) other than pH change might be responsible for reduced CBS activity in the kidney particularly during reperfusion.

5.4.2 EFFECT OF A NITRIC OXIDE SCAVENGER ON CBS ACTIVITY

Next, we investigated whether NO played a role in the reduction of CBS activity in the kidney upon ischemia-reperfusion. In accordance with a previous study, the CBS activity was inhibited \textit{in vitro} when a known NO donor (SNP) was added to the enzyme assay
mixture (Fig. 5.6) (Eitle et al., 1998; Taoka & Banerjee, 2001). The prior addition of an NO scavenger (PTIO) prevented the subsequently imposed NO-mediated CBS inhibition suggesting a low affinity interaction between NO and CBS (Fig. 5.8) (Taoka & Banerjee, 2001). However, PTIO was unable to restore the CBS activity which has already been impaired due to NO suggesting the irreversibility of NO-mediated inhibition of CBS activity (Fig. 5.8). We observed a significant increase in the NOS activity in the reperfusion period and increased levels of NO metabolites in the kidney tissue both during ischemia and reperfusion periods (Fig. 5.11 and 5.12).

In humans, both urinary and serum nitrite and nitrate levels are significantly elevated in renal transplant recipients who subsequently develop acute renal failure. The elevation of NO metabolite levels occur before clinical diagnosis of ARF in these patients and subside with successful therapeutic intervention (Albrecht et al., 2000). Either a dose of PTIO or L-NAME, at very early stage of reperfusion, showed a significant recovery of CBS activity after 6 h of reperfusion when compared with untreated ischemia-reperfusion group (Fig. 5.7). As depicted in Figure 5.10, the NO-mediated inhibition of CBS activity was greater towards an alkaline pH. Therefore, similar to the in vitro observation, it was plausible that an interaction of NO with CBS might be responsible for the reduction of CBS activity during reperfusion. The use of NOS inhibitors have shown promising renal protective effects against ischemia-reperfusion injury in the rat (Chatterjee et al., 2003; Chatterjee et al., 2002). Moreover, iNOS knockout mice were protected against ischemia-reperfusion mediated acute renal failure (Ling et al., 1999). In the present study, PTIO was used to counteract the generation of excess NO. A recent study showed that PTIO treatment prevented the acute renal failure associated with septic shock in the rat (Millar
& Thiemermann, 2002). At higher NO concentrations, the stoichiometric ratio of the interaction between NO and PTIO reaches two while at steady state one NO molecule reacts with one PTIO molecule (Goldstein et al., 2003). As shown in Figure 5.9, quantitatively, a much lower concentration of PTIO was able to counteract the NO-mediated inhibition of CBS activity. In this study, L-NAME was tested at 3 different dose rates (10, 3 and 1.5 mg/kg). Only the lowest concentration (1.5 mg/kg) showed a protective effect (Fig. 5.7). Therefore, it can be postulated that the PTIO scavenges excess NO produced during ischemia-reperfusion (Komurai et al., 2003).

5.4.3 EFFECT OF THE RESTORATION OF CBS ACTIVITY ON RENAL INJURY

Finally, we explored whether correction of kidney tissue CBS activity could ameliorate the ischemia-reperfusion mediated renal injury. The inhibited CBS activity during ischemia-reperfusion caused Hcy accumulation in the kidney tissue during ischemia (Prathapasinghe et al., 2007). Restoration of CBS activity by PTIO treatment resulted in a significant decrease in kidney tissue Hcy level (Fig. 5.13). However, the NO metabolites, as measured by tissue nitrite and nitrate did not show a decrease in PTIO treated group (Fig. 5.11). This discrepancy may be attributed to the nature of the reaction between PTIO and NO. Reaction of PTIO with NO results in the formation of PTI and NO₂ (Akaike et al., 1993). The method we exploited to determine kidney tissue NO is based on the measurement of NO₃ and NO₂ being the metabolites of NO. The restoration of CBS activity had a protective effect against kidney tissue injury as assessed by the formation of nitrotyrosine protein adducts (Fig. 5.17 and 5.18), lipid peroxidation (Fig. 5.15), apoptotic and necrotic cell count (Fig. 5.19 and 5.20). In addition, the renal
Figure 5.22 Postulated mechanism of Hcy mediated ischemia-reperfusion injury in the rat kidney-Effect of alkalizing agent and NO scavenger

Metabolic acidosis caused inhibition of CBS activity during ischemia. An alkalizing treatment during ischemia showed a significant retention of CBS activity only during ischemia. The interaction of CBS with NO during reperfusion prevented the regaining of CBS which was reversibly inhibited by acidic pH. Treatment of NO scavenger facilitated the regaining of CBS activity during reperfusion period. In addition, the PTIO treatment may have contributed in ameliorating the renal injury by preventing the NO-mediated oxidative stress.
function, as determined by plasma creatinine level, was improved when the CBS activity was restored in the PTIO treated group (Fig. 5.16). These findings further support the notion that Hcy, at elevated levels contributes to kidney ischemia-reperfusion injury (Prathapasinghe et al., 2007). The reperfusion resulted in export of intracellular Hcy into the plasma where it could be oxidized into homocystine, Hcy mixed disulfides and ROS (Prathapasinghe et al., 2007; Starkebaum & Harlan, 1986). Thus, the generation of ROS should be an extracellular phenomenon during reperfusion. An increase in levels of ROS was found in the renal venous blood during kidney ischemia-reperfusion (Chien et al., 2001). ROS can rupture the integrity of the blood vessel endothelium (Thies & Autor, 1991). During reperfusion, the integrity of the renal vasculature was compromised and it resulted in massive extravasation of intravascular fluid and blood cells into a previously ischemic area (Toledo-Pereyra, 1989). As shown in Figure 5.21, the renal medulla was extensively infiltrated with blood cells in ischemia-reperfused kidneys. Conversely, treatment with PTIO retained the CBS activity and prevented the extravasation of blood cells. The reno-protective effect of PTIO treatment as measured by lipid peroxidation and histological examination was far greater than its effect on the restoration of CBS activity. NO is a reactive nitrogen species with detrimental effects via the oxidative stress mediated tissue injury. The scavenging of NO by PTIO may have ameliorated the direct cytotoxic effects of NO by itself via the formation of peroxynitrite (Fig. 5.22).

In summary, our data indicate that the inhibition of CBS activity during ischemia-reperfusion is biphasic. Metabolic acidosis exerted an inhibition of CBS activity during ischemia while NO binding caused a further inhibition of CBS activity during reperfusion. Treatment with an NO scavenger significantly increased the CBS activity,
which in turn, ameliorated the ischemia-reperfusion mediated renal injury suggesting a plausible therapeutic potential.
CHAPTER 6

EFFECT OF ISCHEMIA REPERFUSION ON ALTERNATIVE PATHWAYS CATALYZED BY CYSTATHIONINE-\(\beta\)-SYNTHASE IN THE RAT KIDNEY
6.1 RATIONALE AND HYPOTHESIS

A marginal restoration of CBS activity in the PTIO treated rats resulted in, proportionately, a much greater reno-protective effect as determined by lipid peroxidation, nitrotyrosine protein adduct formation, apoptotic cell counts and kidney tissue morphology. It is hypothesized that the scavenging of NO by PTIO, reducing the availability of NO not only for the interaction with CBS but also ameliorated the NO mediated oxidative stress on kidney tissue. NO is a reactive nitrogen species that itself is associated with the pathogenesis of ischemia-reperfusion injury. On the other hand, transsulfuration pathway enzymes are responsible for the endogenous synthesis of H₂S by alternative reactions catalyzed by CBS and CGL in addition to the standard transsulfuration pathway (Szabo, 2007; Chen et al., 2004). Recently, H₂S has gained much attention as an essential gaseous transmitter and its ability to attenuate myocardial ischemia-reperfusion injury in several animal models (Johansen et al., 2006; Zhang et al., 2007; Elrod et al., 2007; Rossoni et al., 2008). Therefore, in this section of the study, our main focus was to explore the involvement of transsulfuration pathway enzymes in the biosynthesis of H₂S in normal and ischemia-reperfused kidneys.

\[ \text{L-Serine} + \text{L-Homocysteine} \rightarrow \text{L-Cystathionine} + \text{H₂O} \]

CBS activity in the transsulfuration pathway

\[ \text{L-Cysteine} + \text{L-Homocysteine} \rightarrow \text{L-Cystathionine} + \text{H₂S} \]

CBS activity in the H₂S producing pathway
The physiological importance of H₂S was first suggested by Abe and Kimura in 1996 (Abe & Kimura, 1996). Until then, H₂S was best known (for at least 300 years) as a toxic gas and environmental pollutant emanating from sewers, swamps and toxic by-products of industrial processes. It has now been established as an important vasodilator and a gasotransmitter similar to NO (Szabo, 2007). Both Hcy and Cys are utilized as substrates for the H₂S synthesis which occurs in the range of 1-10 pmoles per second per mg protein (Chen et al., 2004; Doeller et al., 2005). In the rat, the brain has the highest concentration of H₂S, 300 nmol/g tissue whilst substantial amounts have been reported in liver, kidney and pancreas (Warenycia et al., 1989; Goodwin et al., 1989; Yusuf et al., 2005). The serum H₂S concentration is about 50 µM and 100 µM in rats and humans, respectively (Richardson et al., 2000; Zhao et al., 2001). Thus, it is now becoming increasingly apparent that a substantial level of H₂S is synthesized naturally in the body. Even though it has never been evaluated and compared in one single study group, the plasma or tissue concentration of Hcy and H₂S levels seems to be inversely related. In disease conditions like diabetes, in which the vascular function is impaired, the biosynthesis and release of H₂S is increased, perhaps as an adaptive mechanism (Yusuf et al., 2005). In diabetic rats with no renal complications, the plasma Hcy level is decreased and both enzymes in transsulfuration pathway are increased (Wijekoon et al., 2005). The plasma H₂S level is increased from 23 µM to 31 µM in a mouse model of pancreatitis (Bhatia et al., 2005). In endotoxic, hemorrhagic and septic shock, the plasma H₂S level is significantly increased (Mok et al., 2004; Collin et al., 2005; Li et al., 2005). Both CBS and CGL mRNA are upregulated in these disease conditions (Mok et al., 2004; Collin et al., 2005; Li et al., 2005). The brain H₂S concentration was reportedly low in
Alzheimer’s patients who usually have reduced CBS activity and increased Hcy levels (Eto et al., 2002). Conversely, fibroblasts from Down’s syndrome patients have demonstrated a 150% increase in CBS activity resulting in a significant decrease in plasma Hcy levels (Chadefaux et al., 1985). Interestingly, the H$_2$S production was increased by approximately 2.3-fold in patients with Down’s syndrome (Belardinelli et al., 2001; Kamoun et al., 2003).

Plasma H$_2$S level is reduced by half in patients with coronary heart disease (Jiang et al., 2005). A more than 50% lower level of plasma H$_2$S level has been detected in spontaneously hypertensive rats (Du et al., 2003). H$_2$S plays a role in the regulation of vascular function both in health and disease (Wang, 2002). It mimics the vasodilatory activity of other gaseous mediators such as nitric oxide and carbon monoxide. In vitro, H$_2$S relaxes the pre-contracted rat aorta and dilates mesenteric arterioles in rats (Ali et al., 2006). H$_2$S rapidly travels through cell membranes without relying on specific transporters (Szabo, 2007). Multiple studies have demonstrated the antinecrotic and antiapoptotic effects of H$_2$S at micromolar concentrations (Whiteman et al., 2004; Yan et al., 2006; Rinaldi et al., 2006). Low levels of H$_2$S can up-regulate endogenous antioxidant systems (Yang et al., 2004). However, higher (millimolar) levels of H$_2$S are found to be cytotoxic (Szabo, 2007). Administration of H$_2$S to animals induces transient hypotension (Zhao & Wang, 2002; Cheng et al., 2004). Conversely, CBS deficiency leads to hyperhomocysteinemia which, in turn, causes hypertension and endothelial dysfunction in the rat (Yan et al., 2004).

Therefore, we hypothesized that the inhibition of CBS may impair the endogenous synthesis of H$_2$S in the kidney in contrast to the accumulation of Hcy.
6.2 OBJECTIVES

1. To determine the role of rat kidney in endogenous H$_2$S biosynthesis and plasma H$_2$S homeostasis

2. To determine the contribution of transsulfuration pathway enzymes in endogenous H$_2$S production

3. To characterize the renal H$_2$S biosynthesis
6.3 RESULTS

6.3.1 ENDOGENOUS PRODUCTION OF H₂S IN THE RAT KIDNEY

To determine the effect of ischemia-reperfusion on the endogenous production of H₂S by kidney, rat kidneys were subjected to 45 min ischemia or ischemia followed by 6 h reperfusion (Fig. 6.1). The kidney tissue H₂S level was almost halved in ischemic kidneys when compared with the sham-operated group (Fig. 6.1). It remained significantly lower throughout the 6 h reperfusion period (Fig. 6.1). Plasma H₂S level was not changed during ischemia period, however, it was significantly decreased after the reperfusion period (Fig. 6.2). Next, to determine the substrates utilized in the endogenous production of H₂S, 5% (w/v) kidney tissue homogenates from healthy rats were incubated in the presence of different substrates (Fig. 6.3). Neither Cys nor Hcy alone were capable of producing significant amount of H₂S in the kidney (Fig. 6.3). However, when the Cys and Hcy were added together to the reaction mixture, in equal molar concentrations, a significant production of H₂S was observed (Fig. 6.3). To determine whether the intracellularly produced Cys which is the end product of the standard transsulfuration pathway was utilized in the endogenous H₂S production in the kidney, H₂S production was measured in the presence of a combination of serine and Hcy as substrates (Fig 6.3). Interestingly, this combination of the substrates did not result in an appreciable production of H₂S suggesting that intracellularly produced Cys may not be used as a substrate in the production of H₂S by the kidney.
Fig. 6.1  

Kidney tissue H$_2$S level

Left kidney was subjected to sham operation, 45 min ischemia (I-45min) or 45 min ischemia followed by 6 h reperfusion (I-45min/R-6h). The tissue H$_2$S level was determined. Results are expressed as mean ± s.e.m (n=6). *P≤0.05 when compared with the value obtained from sham-operated group. Kidney tissue H$_2$S level in the sham group was 186.64 ± 17.88 nmol/g tissue.
Fig. 6.2  Plasma H₂S level
Left kidney was subjected to sham operation, 45 min ischemia (I-45min) or 45 min ischemia followed by 6 h reperfusion (I-45min/R-6h). Plasma H₂S level was determined. Results are expressed as mean ± s.e.m (n=6). *P≤0.05 when compared with the value obtained from sham-operated group. Plasma H₂S level in the sham group was 31.28 ± 2.12 μmol/L.
Fig. 6.3  Kidney tissue H₂S production by different substrates
5% (w/v) kidney tissue homogenate was prepared in 0.05 M potassium phosphate buffer. The rate of H₂S production was assessed in the presence of different substrates at 10 mM concentration. Results are expressed as mean ± s.e.m (n=6). The rate of H₂S production when both Cys and Hcy were added together was 2.01 ± 0.08 nmol/min/mg protein.
6.3.2 CBS IS THE ENZYME RESPONSIBLE FOR THE ENDOGENOUS H$_2$S PRODUCTION IN THE RAT KIDNEY

To determine which enzyme(s) of the transsulfuration pathway catalyze the endogenous H$_2$S production in the rat kidney, the H$_2$S production was assessed in the presence of 2 mM hydroxylamine or 2 mM DL-propargylglycine as inhibitors for CBS and CGL, respectively. Hydroxylamine is a carbonyl specific reagent which dissociate PLP from CBS (Braunstein et al., 1971). The H$_2$S production was completely arrested by hydroxylamine (Fig. 6.4). Propargylglycine treatment had no impact on H$_2$S production (Fig. 6.4). As described in the previous chapter, the CBS activity in the standard transsulfuration pathway was significantly inhibited in kidneys subjected to ischemia or ischemia followed by reperfusion. According to the observation made in Figure 6.4, of the enzymes in the transsulfuration pathway, CBS was the only enzyme responsible for the endogenous production of H$_2$S in the rat kidney. Thus, the decrease in the kidney tissue H$_2$S level after ischemia and reperfusion may be attributed to the impaired CBS activity. We then determined the CBS activity in the H$_2$S producing pathway in comparison to that of the standard transsulfuration reactions (Fig. 6.5). Quantitatively, CBS was capable of catalyzing the endogenous H$_2$S production in the kidney tissue homogenate approximately at 1/3 of the rate of the standard transsulfuration reaction (Fig. 6.5). The CBS activity in the H$_2$S producing pathway showed a similar pattern of inhibition in response to ischemia and ischemia followed by different durations of reperfusion (Fig. 6.5).
Fig. 6.4  Kidney tissue H$_2$S production in the presence of inhibitors for CBS and CGL enzymes

5% (w/v) kidney tissue homogenate was prepared in 0.05 M potassium phosphate buffer. The rate of H$_2$S production was assessed with 10 mM Hcy and 10 mM Cys as substrates in the presence 2 mM hydroxylamine or 2 mM propargylglycine as inhibitors for CBS and CGL, respectively. Results are expressed as mean ± s.e.m (n=6). *P≤0.05 when compared with the value obtained from control group.
Fig. 6.5  CBS activity in the standard transsulfuration and H₂S producing pathways
Left kidney was subjected to sham operation or 45 min ischemia or 45 min ischemia followed by 1, 6 and 24 h reperfusion. CBS activity was determined both in the standard transsulfuration pathway and H₂S producing pathway. Results are expressed as mean ± s.e.m (n=6). TS = standard transsulfuration pathway
6.3.3 INTRACELLULAR PRODUCED CYSTEINE IN THE TRANSSULFURATION PATHWAY IS NOT SUFFICIENT TO DRIVE THE ENDOGENOUS H₂S SYNTHESIS IN THE RAT KIDNEY

The kidney is one of only a few organs which contain all enzymes of the transsulfuration pathway (Finkelstein, 1990). Cys is the end product of transsulfuration reactions (Finkelstein et al., 1988). Theoretically a noticeable production of H₂S could be expected if kidney tissue homogenates were incubated in the presence of serine and Hcy which are the substrates for the transsulfuration pathway. However, as depicted in Figure 6.3, no detectable level of H₂S was produced in this combination of substrates in the presence of all co-factors. This made us determine the Km (Michaelis-Menten constant) values for Cys, Hcy, Cys and Hcy together in the H₂S producing pathway. When kidney tissue homogenate was incubated with graded increment of Cys and Hcy in equimolar concentrations, the maximum velocity (Vmax) was recorded as 30.87±0.22 nmol/min/mg protein. The half maximum velocity (Km) was recorded at a concentration of 7 mM for the mixture of Cys and Hcy (Fig. 6.6). The Km value for Cys alone while Hcy was in excess in the reaction mixture was calculated to be 9 mM (Fig. 6.7). As previously observed (Fig. 4.17), a considerable amount of superoxide was generated when tissue homogenate was incubated with high concentrations of Hcy. Despite the fact that CBS is activated under oxidative environment, the affinity of CBS towards Cys was very low. Interestingly, the Km for Hcy when the incubation medium contained Cys in excess, was calculated to be 0.4 mM (Fig. 6.8). Thus, it can be suggested that Cys is the limiting substrate for the endogenous synthesis of H₂S in the kidney and presumably for this
Fig. 6.6 The rate of H₂S production in kidney tissue depending on the equimolar concentrations of Cys and Hcy. 5% (w/v) kidney tissue homogenate was prepared in 0.05 M potassium phosphate buffer. The Michaelis-Menten constant (Km) for both Hcy and Cys in combination as substrates was determined.
Fig. 6.7 The rate of H₂S production in kidney tissue depending on the concentration of Cys while Hcy is in excess. 5% (w/v) kidney tissue homogenate was prepared in 0.05 M potassium phosphate buffer. The Michaelis-Menten constant (Km) for Cys in the presence of excess Hcy was determined.
Fig. 6.8  The rate of H$_2$S production in kidney tissue depending on the concentration of Hcy while Cys is in excess. 5% (w/v) kidney tissue homogenate was prepared in 0.05 M potassium phosphate buffer. The Michaelis-Menten constant (Km) for Hcy in the presence of excess Cys was determined.
reason, intracellularly produced Cys from the transsulfuration reactions was not sufficient to drive the biosynthesis of H₂S.

6.3.4 EFFECT OF NITRIC OXIDE AND pH ON CBS ACTIVITY IN THE H₂S PRODUCING PATHWAY

As shown in the previous chapter, the CBS activity in the standard transsulfuration pathway was greatly affected by tissue pH and NO especially during the ischemia and reperfusion periods, respectively. The effect of NO on the H₂S producing pathway was determined in the presence of a graded increment of SNP. Similar to the standard transsulfuration pathway, CBS in the H₂S producing pathway was also inhibited by NO. Remarkably, the NO-mediated inhibition of CBS in the H₂S producing pathway was much greater than that of the standard transsulfuration pathway (Fig. 5.6 vs. Fig. 6.9). Further, the NO-mediated inhibition of CBS in the H₂S producing pathway was irreversible (Fig. 6.10). Similar to the standard transsulfuration pathway, the prior addition of an NO scavenger prevented the subsequently imposed inhibitory effect of NO on CBS (Fig 5.8 vs. Fig. 6.10). CBS in the H₂S producing pathway recorded an optimum activity at pH 8.5 (Fig. 6.11). The pH mediated inhibition was reversible once the pH was corrected (Fig. 6.12). The NO-mediated inhibitory effect of CBS in the H₂S producing pathway was greater at an alkaline pH (Fig. 6.11). This synergistic effect of alkaline pH on NO-mediated inhibition of CBS in H₂S producing pathway was much more apparent when compared with that in the standard transsulfuration pathway (Fig. 5.10 vs. Fig. 6.11). It was further observed that an equimolar concentration of PTIO could protect the
Fig. 6.9  Effect of NO on CBS activity in the H₂S producing pathway
The effect of NO on CBS activity in the H₂S producing pathway was determined in a 5% (w/v) kidney tissue homogenate that was prepared in 0.05 M potassium phosphate buffer at pH 7.4. Different concentrations of sodium nitroprusside (SNP) were used as the source of NO. The CBS activity in the H₂S producing pathway was measured at pH 7.4. Results are expressed as mean ± s.e.m (n=3). *P≤0.05 when compared with the control group.
Fig. 6.10  Irreversibility of NO mediated inhibition of CBS activity in the H2S producing pathway
5% (w/v) kidney tissue homogenate was pre-incubated for 15 min with either SNP or PTIO at pH 7.4. In the next 15 min of pre-incubation, an equimolar concentration of PTIO was added to the homogenate which was pre-incubated with SNP and vice versa. The CBS activity in the H2S producing pathway was measured. Results are expressed as mean ± s.e.m (n=4). *P<0.05 when compared with the control group. #P<0.05 when compared with the group which was pre-incubated with SNP first.
Fig. 6.11  Effect of pH on NO-mediated inhibition of CBS activity in the H₂S producing pathway
5% (w/v) kidney tissue homogenate was prepared in a range of pH and the CBS activity in the H₂S producing pathway was determined with a reaction mixture at the corresponding pH.
Fig. 6.12  Reversibility of pH mediated inhibition of CBS activity in the H₂S producing pathway
5% (w/v) kidney tissue homogenate was prepared in 0.05 M potassium phosphate buffer at pH 6.5. After pre-incubation for 45 min, the pH was adjusted at 8.5 in one group. The CBS activity in the H₂S producing pathway was determined with a reaction mixture at the corresponding pH. Results are expressed as mean ± s.e.m (n=4). *P≤0.05 when compared with the pH 6.5 group.
Fig. 6.13  Stoichiometry of the PTIO:SNP in the NO-mediated inhibition of CBS activity in the H₂S producing pathway

5% (w/v) kidney tissue homogenate was prepared in 0.05 M potassium phosphate buffer at pH 7.4. CBS activity in the H₂S producing pathway was measured in a reaction mixture containing 200 μM SNP alone or together with a graded increment of PTIO. Results are expressed as mean ± s.e.m (n=4). *P≤0.05 when compared with the control group. #P≤0.05 when compared with the SNP alone group.
NO mediated inhibition of CBS activity in the H₃S producing pathway (Fig. 6.13). Even though lower concentrations of PTIO were also able to prevent the NO-mediated inhibition of CBS activity, it was not as effective as the preventative effect observed in the standard transsulfuration pathway (Fig 5.9 vs. Fig. 6.13).

6.3.5 ROLE OF NITRIC OXIDE ON THE INHIBITION OF CBS ACTIVITY IN THE H₃S PRODUCING PATHWAY DURING ISCHEMIA-REPERFUSION

The plasma NO level was determined by measuring nitrate and nitrite by the Griess reaction. After 6 and 24 h of reperfusion, plasma NO metabolite levels showed a significant increase (Fig. 6.14). The administration of an NO scavenger (PTIO) at the onset of reperfusion restored the CBS activity in the H₃S producing pathway significantly. This demonstrated the involvement of NO in the inhibition of CBS activity in the H₃S producing pathway (Fig. 6.15). The PTIO treatment showed a restoration of CBS activity in the H₃S producing pathway (Fig. 6.15).
Fig. 6.14  Effect of the duration of reperfusion on plasma NO metabolite level
Left kidney was subjected to sham operation (Sham) or 45 min ischemia followed by 1, 6 and 24 h reperfusion. NO metabolites in the plasma were determined. Results are expressed as mean ± s.e.m (n=6). *P<0.05 when compared with the sham-operated group. Plasma NO metabolite level in the sham group was 3.56 ± 0.33 μmol/L.
Fig. 6.15 Effect of NO scavenging on kidney CBS activity in the H₂S producing pathway
Left kidney was subjected to sham operation (Sham) or 45 min ischemia followed by 6 h reperfusion or 45 min ischemia followed by 6 h reperfusion with an intravenous injection of PTIO at the onset of reperfusion. CBS activity in the H₂S producing pathway was determined. Results are expressed as mean ± s.e.m (n=8). *P≤0.05 when compared with the sham-operated group. #P≤0.05 when compared with the ischemia-reperfusion group.
6.3.6 ROLE OF KIDNEY IN THE HOMEOSTASIS OF PLASMA H$_2$S

Plasma H$_2$S level was significantly decreased after 45 min ischemia followed by 6 h reperfusion (Fig. 6.2). To determine the contribution of kidney in the maintenance of plasma H$_2$S level, the arteriovenous (A-V) difference of H$_2$S across the healthy rat kidney was determined. There was no significant A-V difference in H$_2$S level across the kidney (Fig. 6.16). To elucidate the source of plasma H$_2$S, the tissue H$_2$S level, the rate of H$_2$S production by different organs, the substrates utilized for the endogenous H$_2$S production and the transsulfuration enzyme(s) responsible for H$_2$S production by different organs were assessed (Fig. 6.17, Table 6.1 and Table 6.2). Rat liver displayed the highest rate of H$_2$S production (Table 6.1). Liver exported a significant amount of H$_2$S to the plasma as measured by the A-V difference across the liver (Fig. 6.16). The tissue H$_2$S levels were higher in the liver and brain (Fig. 6.17). Nonetheless, the brain had a relatively low rate of H$_2$S production (Table 6.1). Similarly, the rate of H$_2$S production by the rat heart was very low yet it had a significant tissue level of H$_2$S (Table 6.1 and Fig 6.17). Comparable to the kidney, CBS was the only enzyme responsible for the H$_2$S synthesis in the brain (Table 6.1). CGL played a significant role in the H$_2$S production in the liver. However, the bulk of H$_2$S was synthesized from the reaction catalyzed by CBS (Table 6.2). In the heart, CGL was responsible for endogenous H$_2$S production (Table 6.2).
Blood samples were drawn simultaneously from abdominal aorta, renal vein and hepatic vein from healthy rats under anesthesia. Plasma H$_2$S level was determined. Negative A-V balance indicates a net output of H$_2$S across the organ. Results are expressed as mean ± s.e.m (n=6). *P≤0.05 when compared with the values obtained from aorta.
Fig. 6.17  **Tissue H$_2$S levels in different organs**
Brain, heart, liver and kidney from healthy rats were harvested. The tissue H$_2$S level was determined in a 5% (w/v) homogenate prepared in 0.05 M potassium phosphate buffer at pH 6.9. Results are shown as mean ± s.e.m. (n=6).
TABLE 6.1  Rate of H₂S production in different organs from different substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CBS activity in H₂S producing pathway (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
</tr>
<tr>
<td>Hcy</td>
<td>0.197±0.040</td>
</tr>
<tr>
<td>Cys</td>
<td>0.587±0.198</td>
</tr>
<tr>
<td>Hcy+Cys</td>
<td>1.398±0.119</td>
</tr>
<tr>
<td>Hcy+Ser</td>
<td>0.745±0.216</td>
</tr>
</tbody>
</table>

5% tissue homogenate from each organ was prepared in 0.05 M potassium phosphate buffer at pH 6.9. The CBS activity in the H₂S producing pathway was determined. Results are expressed as mean ± s.e.m (n=6).
TABLE 6.2  Rate of H$_2$S production in different organs in the presence of inhibitors for CBS and CGL enzymes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>CBS activity in H$_2$S producing pathway (nmol/min/mg protein)</th>
<th>Brain</th>
<th>Heart</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(2mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>1.398±0.119</td>
<td>0.382±0.078</td>
<td>17.001±1.386</td>
<td>2.011±0.083</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td></td>
<td>0.204±0.038*</td>
<td>0.156±0.047*</td>
<td>1.841±0.206*</td>
<td>0.096±0.039*</td>
</tr>
<tr>
<td>Propargylglycine</td>
<td></td>
<td>1.240±0.446</td>
<td>0.041±0.003*</td>
<td>15.956±1.052</td>
<td>2.184±0.026</td>
</tr>
</tbody>
</table>

5% tissue homogenate from each organ was prepared in 0.05 M potassium phosphate buffer at pH 6.9. The transsulfuration pathway enzymes involved in the H$_2$S production was determined in the presence of 2 mM hydroxylamine as an inhibitor for CBS or 2 mM DL-propargylglycine as an inhibitor for CGL. Results are expressed as mean ± s.e.m (n=6). *P≤0.05 when compared with the control values within the group.
6.3.7 EFFECT OF RENAL ISCHEMIA-REPERFUSION ON H₂S PRODUCTION IN DISTANT ORGANS

As depicted in Figure 6.2, the plasma H₂S level was significantly decreased in rats subjected to 45 min ischemia followed by 6 h reperfusion. However, there was no net output of H₂S from rat kidney into plasma (Fig. 6.16). Therefore, we assessed the effect of renal ischemia-reperfusion on the H₂S synthesis by distant organs. The H₂S synthesis in the liver was assessed in this regard because liver recorded the highest rate of H₂S production among the 4 organs which were studied in this section. In addition, liver exported a significant amount of H₂S in the plasma compartment. We made an interesting observation, i.e. the liver H₂S production was significantly impaired in rats subjected to renal ischemia-reperfusion (Fig. 6.18). This might be a reason by which plasma H₂S level was decreased in renal ischemia-reperfusion rats.
Fig. 6.18 Effect of renal ischemia-reperfusion on H$_2$S production by liver
Left kidney was subjected to sham operation (Sham) or 45 min ischemia followed by 6 h reperfusion. CBS activity in the H$_2$S producing pathway in the liver was determined. Results are expressed as mean ± s.e.m (n=6). *$P$≤0.05 when compared with the sham-operated group.
6.4 DISCUSSION

The novel findings of this part of the study are 1) ischemia-reperfusion impairs endogenous synthesis of H₂S by the kidney; 2) CBS is the sole enzyme in the transsulfuration pathway which catalyzes the biosynthesis of H₂S in the kidney; 3) similar to the standard transsulfuration pathway, the CBS activity in the H₂S producing pathway showed a reversible inhibition by acidosis and irreversible inhibition by NO; 4) renal ischemia-reperfusion resulted in a significant decrease in plasma H₂S which presumably was due to the impairment of H₂S production by the liver as a result of the effect of renal ischemia-reperfusion on distant organs.

6.4.1 ENDOGENOUS PRODUCTION OF H₂S IN THE RAT KIDNEY

The goal of this part of the study was to determine the effect of ischemia-reperfusion on H₂S biosynthesis in the kidney. In agreement with most of the literature on endogenous synthesis of H₂S, CBS catalyzed the renal H₂S biosynthesis (Chen et al., 2004). CGL did not play a significant role in the H₂S production by the rat kidney. Being a type-II family PLP-containing enzyme, CBS catalyzed the H₂S production by utilizing Cys and Hcy as substrates by a β-replacement reaction (Fig. 2.3). Neither Cys nor Hcy alone, as the substrate resulted in an appreciable production of H₂S in the kidney. The kinetic studies revealed a supra-physiological Km value for Cys in the H₂S producing pathway (Fig. 6.6 and Fig. 6.7). This agrees with the reported Km values for Cys or Cys and Hcy in combination by other researchers (Stipanuk & Beck, 1982; Chen et al., 2004). The Km for Hcy when Cys was in excess in the reaction mixture was recorded at 0.4 mM concentration. This value was found to be within the range of Km reported for Hcy in the
standard transsulfuration pathway (Finkelstein, 1974; Chen et al., 2004). Thus, it can be suggested that Cys is the limiting substrate in the H$_2$S producing pathway in the rat kidney. For this reason intracellularly produced Cys as the end product of the standard transsulfuration pathway was not able to drive the H$_2$S production in the kidney (Fig. 6.3). Despite the supra-physiological Km for Cys, 1/3 of the CBS activity (when compared with the standard transsulfuration pathway) was committed to the H$_2$S producing pathway in the rat kidney (Fig. 6.5).

6.4.2 EFFECT OF ISCHEMIA AND REPERFUSION ON CBS ACTIVITY IN THE H$_2$S PRODUCING PATHWAY

Ischemia for 45 min caused a significant decrease in the kidney tissue H$_2$S level suggesting a great impairment in the CBS activity in the H$_2$S producing pathway during ischemia (Fig. 6.1). This observation is another indication that there is a substantial production of H$_2$S by the kidney and Hcy is utilized as a substrate in this reaction, as the latter showed a significant increase during ischemia (Fig. 4.1). The reperfusion did not show any improvement in the H$_2$S production since the CBS activity remained inhibited throughout the reperfusion period (Fig. 6.1). Similar to the standard transsulfuration pathway, the CBS activity in the H$_2$S producing pathway remained inhibited throughout the 24 h reperfusion period (Fig. 6.5). The tissue NO metabolite level (Fig 5.11), NOS activity (Fig. 5.12) and plasma NO metabolite level (Fig. 6.14) were increased during reperfusion. Further, the PTIO treatment to scavenge excess NO in the kidney was able to restore the CBS activity in the H$_2$S producing pathway (Fig. 6.15). Therefore, it may be suggested that the interaction of CBS with NO results in the inhibited activity of CBS in
the H₂S producing pathway during reperfusion. The effects of pH and NO on CBS in H₂S producing pathway were similar to those the standard transsulfuration pathway. However, it is noteworthy that the NO-mediated inhibitory effect on CBS activity in the H₂S producing pathway was remarkably greater at alkaline pH (beyond pH 7.0) (Fig. 6.11). This phenomenon was not as apparent in the standard transsulfuration pathway (Fig. 5.10).

In patients with kidney disease, the increased urinary amino acid excretion and impaired renal amino acid metabolism profoundly alter the amino acid handling and elimination by the kidney. Plasma levels of Cys are increased in these patients (Kopple et al., 1978). Renal ischemia-reperfusion resulted in a significant increase in kidney tissue Cys level at the expense of GSH (Scaduto et al., 1988; Slusser et al., 1990). Thus, it was suggested that the accumulation of Cys resulted from the catabolism of GSH during ischemia-reperfusion (Slusser et al., 1990). However, with the findings of our study, it could be argued that the impaired CBS activity in the H₂S producing pathway may also have contributed to the increased tissue Cys in ischemia-reperfusion kidneys and increased plasma Cys levels in patients with renal diseases. In the case of plasma Hcy, the prevalence of hyperhomocysteinemia in dialysis patients is over 80% (Moustapha et al., 1999). Renal ischemia-reperfusion injury results in a significant increase in plasma Hcy level (Prathapasinghe et al., 2007). According to our findings, the accumulation of Hcy could be attributed to the inhibition of CBS enzyme in both standard transsulfuration as well as H₂S producing pathways.
6.4.3 EFFECT OF RENAL ISCHEMIA-REPERFUSION ON H$_2$S PRODUCTION BY THE LIVER

Ischemia-reperfusion in the rat kidney resulted in a significant decrease in plasma H$_2$S level (Fig. 6.2). This was the most intriguing observation because sulfate is almost entirely eliminated from the body in the urine (Hamadeh & Hoffer, 2001; Hou et al., 2003). The A-V balance studies revealed that there was no net output of H$_2$S across the kidney, suggesting that the kidney was not a major source for plasma H$_2$S (Fig. 6.16). Instead, it was revealed that the liver produced H$_2$S at a much greater rate when compared with the kidney, brain and heart (Fig. 6.17). The liver exported a significant quantity of H$_2$S into the plasma (Fig. 6.16). Remarkably, the CBS activity in the H$_2$S producing pathway was significantly inhibited in the liver of rats subjected to renal ischemia-reperfusion. Plasma NO metabolite level was significantly elevated during the reperfusion period. Therefore, this kind of an inhibitory effect on distant organs may perhaps be mediated by NO. This phenomenon is worth further examination in future studies.

To the best of my knowledge, the present study demonstrated, for the first time, CBS was significantly involved in the biosynthesis of H$_2$S in the kidney. The H$_2$S producing pathway was responsible for approximately 1/3 of total Hcy catabolism (irreversible) by the rat kidney. Ischemia-reperfusion inhibited CBS activity not only in the standard transsulfuration pathway but also in the H$_2$S producing pathway. Ischemia-reperfusion in the kidney had an inhibitory effect on the endogenous H$_2$S synthesis by the liver which, in turn, resulted in decreased plasma H$_2$S level.
CHAPTER 7
GENERAL DISCUSSION AND FUTURE PERSPECTIVES
In the first two parts of the research project, we demonstrated that ischemia-reperfusion impaired Hcy metabolism in the rat kidney causing the accumulation of Hcy in the kidney tissue and plasma. The inhibition of CBS activity in the standard transsulfuration pathway was impaired due to metabolic acidosis during ischemia and due to interaction with NO during reperfusion. In the last part of this research project, we determined not only the standard transsulfuration pathway but also the endogenous H₂S production by the kidney was significantly impaired in response to ischemia-reperfusion in the rat kidney. The H₂S production in the kidney was solely catalyzed by CBS. The inhibitory effect of low pH and NO showed a similar pattern on the CBS in both pathways. However, the NO-mediated inhibition of CBS in the H₂S producing pathway was much greater when compared with that in the standard transsulfuration pathway. Interestingly, the plasma H₂S level was significantly lower in rats that were subjected to renal ischemia-reperfusion and it might perhaps be due to the impaired biosynthesis and export of H₂S by the liver.

Hyperhomocysteinemia has been shown to be associated with arteriosclerotic events in kidney transplant recipients (Ducloux et al., 2000). Even though the effect of Hcy on cardiovascular disease is not directly related to our study, the results of the latest prospective meta-analysis of Hcy will be discussed with regard to the future perspectives. There should be 3 prerequisites to be evaluated before the declaration of Hcy as a risk factor: 1) establishment of a strong and consistent association of hyperhomocysteinemia and adverse disease outcomes; 2) identification of a rational mechanism through which Hcy exerts its detrimental effects on disease pathogenesis; 3) demonstration of the relation of Hcy-lowering to disease risk modification by prospective randomized
interventional trials (Kaul et al., 2006). In the first part of the study we evaluated these three requirements by determining the tissue and plasma Hcy level following a renal ischemia-reperfusion episode. Using polyclonal Hcy antibodies and exogenous administration of L-Hcy, we were able to either ameliorate or exacerbate the ischemia-reperfusion injury in the rat kidney, respectively. Thus, we have introduced the “Hcy theory in renal ischemia-reperfusion injury”.

In contrast to our findings, the role of Hcy in the pathogenesis of cardiovascular disease has been questioned with the findings of the NORVIT and HOPE-2 studies (Bonaa et al., 2006; Lonn et al., 2006). Intriguingly, these studies suggested Hcy-lowering may perhaps enhance the risk of cardiovascular disease and cancer. In another similar study a slight beneficial effect was observed in patients with elevated plasma Hcy (Lange et al., 2004). In an editorial review, Loscalzo raised two questions in this regard (Loscalzo, 2006). 1) “Does the failure of Hcy lowering therapy to reduce the rates of cardiovascular events suggest that the Hcy hypothesis is incorrect? And if so, is Hcy a surrogate for another, metabolically related species that is the true atherogenic culprit? 2) If Hcy is an atherogenic determinant, do the results of these trials suggest that vitamin therapy has other, potentially adverse effects that offset its Hcy-lowering benefit?”. However, there is an ample amount of data which support the association of hyperhomocysteinemia with an elevated risk of atherothrombosis (McCully & Wilson, 1975; McCully, 2007). In our study, we also demonstrated the involvement of Hcy in ischemia-reperfusion injury. With the observation that a significant portion of the rate-limiting enzyme in the transsulfuration pathway is committed to the endogenous H2S production in the kidney, we were compelled to consider the second part of the first
question raised by Loscalzo, i.e. is Hcy a surrogate for another, metabolically related species that is the true atherogenic culprit? (Loscalzo, 2006). As we observed in the last part of our study, H$_2$S production which is metabolically related to Hcy, was significantly impaired during renal ischemia-reperfusion. Moreover, a recent study reported that administration of exogenous H$_2$S at the onset of reperfusion resulted in a significant decrease in the myocardial infarct size in mice which were subjected to ischemia-reperfusion of the heart (Elrod et al., 2007). Therefore, it could be argued not only the elevation of Hcy but also the consequential decrease in the H$_2$S level may also have played in concert in the pathogenesis of ischemia-reperfusion injury.

The meta-analysis of Hcy-lowering on cardiovascular disease outcome used folate as an Hcy-lowering therapy which channeled Hcy through the remethylation pathway. In the remethylation pathway, folate is utilized as a co-substrate and vitamin B$_{12}$ and B$_6$ act as cofactors at methionine synthase and serine hydroxymethyl transferase reactions, respectively. In addition, vitamin B$_6$ is a cofactor for both CBS and CGL in the transsulfuration pathway (Finkelstein, 1990). As observed in the NORVIT study, the vitamin B$_6$ supplemented group did not show a reduction in plasma Hcy level probably because the addition of a cofactor in excess cannot increase the rate of an enzyme reaction (Bonaa et al., 2006). Nonetheless, a co-substrate can increase the rate of an enzymatic reaction as long as the other substrate/s and cofactors are adequate. This was observed in both NORVIT and HOPE-2 studies in which the folate supplementation resulted in a significant reduction in plasma total Hcy level (Bonaa et al., 2006; Lonn et al., 2006). As a result of folate supplementation, the distribution of Hcy between
remethylation and transsulfuration pathways is altered and less Hcy is available for the catabolism through the transsulfuration pathway.

Since Hcy is a substrate for the synthesis of H$_2$S endogenously, it could be argued that Hcy-lowering by folate might lead to the decrease in plasma H$_2$S level which may, in turn, be the reason for masking the beneficial effect of Hcy-lowering. An inadequate H$_2$S levels can induce hypertension and subsequently cardiovascular events. Presumably for this reason, recent prospective studies observed that Hcy-lowering can exacerbate cardiovascular outcomes. Conversely, our study demonstrated a reno-protective effect when Hcy was lowered by restoring CBS activity. Therefore, the pathway in which Hcy was metabolized in the attempt of Hcy-lowering may be the matter of significance rather than just lowering of Hcy. Future studies, therefore, should focus on restoring CBS activity to near normal levels immediately after the ischemia. Such an attempt will prevent the accumulation of Hcy and at the same time it will restore the endogenous production of H$_2$S. The other aspect that a future study should focus upon is the use of exogenous H$_2$S in the form of either NaHS or Na$_2$S in an attempt to ameliorate ischemia-reperfusion injury. As an alternative approach, a dose of exogenous Cys together with PTIO could also be tested. As we observed in the third part of the project, PTIO restored the CBS activity in the H$_2$S producing pathway to a near normal level. Therefore, the administration of Cys with PTIO will drive Hcy in the H$_2$S producing pathway. Such an attempt will prevent the accumulation of Hcy and at the same time it will result in the synthesis of H$_2$S. NAC has been used in many renal ischemia-reperfusion models (Nitescu et al., 2006; Di Giorno et al., 2006). The therapeutic effect of NAC against ischemia-reperfusion injury was suggested to be mediated via the synthesis of Cys which,
in turn, was utilized in the GSH synthesis (Nitescu et al., 2006; Nath & Norby, 2000). However, none of these studies has examined the effect of NAC treatment on the kidney tissue Hcy status and endogenous production of H₂S.

Finally, it is worth determining the applicability of our findings, i.e., alkalizing and/or NO scavenger treatments in real clinical settings. The inclusion of PTIO in the University of Wisconsin (UW) solution which is a widely used tissue preservation media would be the most plausible way to test our findings in clinical situation. It is notable that one of the ingredients of the UW solution is adenosine (1.34g/l = 5mmol/L; pH 7.4) (Southard & Belzer, 1995; Chiang, 2001). As illustrated in Figure 2.3, adenosine can drive Hcy back into SAH which exerts a feedback inhibition on transmethylation of SAM. This reverse reaction of Hcy to SAH is favored in the normal physiological pH (Stipanuk, 2004). Even though the use of adenosine as an ingredient in the UW solution has never been considered in relation to Hcy metabolism, it may perhaps have given the UW solution the Hcy-lowering properties already.
1. Homocysteine played a detrimental role in the pathogenesis of ischemia-reperfusion mediated injury in the kidney

Kidney tissue Hcy concentration was significantly elevated during ischemia. The reperfusion caused the efflux of Hcy into plasma, resulting in hyperhomocysteinemia. The inhibition of CBS activity was responsible for the tissue accumulation of Hcy in the kidney. Polyclonal anti-Hcy antibodies prevented the ischemia-reperfusion mediated renal injury and kidney function. Exogenous administration of L-Hcy resulted in renal injury. The reno-protective effects observed in ischemia-preconditioning were brought about by Hcy-lowering mechanism.

2. The inhibition of cystathionine-β-synthase activity was biphasic during renal ischemia and reperfusion

Metabolic acidosis was the cause of impaired CBS activity during ischemia. The interaction of CBS with NO prevented the restoration of CBS activity during the reperfusion period. An alkalizing treatment during ischemia showed a significant retention of CBS activity. An NO scavenger showed a significant restoration of CBS activity during reperfusion. Kidney tissue Hcy levels were significantly decreased in the NO scavenger treated group and renal injury was significantly ameliorated.

3. Ischemia-reperfusion resulted in an inhibition of endogenous H₂S biosynthesis which was catalyzed by cystathionine-β-synthase

Of the total CBS activity, 1/3 was committed in the endogenous H₂S production. Both Cys and Hcy were utilized as substrates in the production of H₂S in the rat kidney. Ischemia-reperfusion showed a similar pattern of inhibition of CBS activity in the H₂S producing pathway similar to the CBS in the standard transsulfuration pathway.
Plasma H$_2$S levels were significantly decreased after a 6 h reperfusion period presumably due to the inhibition of H$_2$S biosynthesis by the liver. Renal ischemia-reperfusion has an inhibitory effect on the CBS activity in the H$_2$S producing pathway not only in the kidney itself but also in the liver.
CHAPTER 9

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LIST OF REFERENCES

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CHAPTER 10

APPENDICES
APPENDIX -1

Chemicals and reagents

Chemical | Vendor
---|---
14C (U) L-Serine | PerkinElmer
2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) | Sigma-Aldrich
2-Thiobarbituric acid | Sigma-Aldrich
3,3',5,5'-tetramethylbenzidine | Fisher
Acetic Acid, Glacial | Sigma-Aldrich
Ammonium phosphate | Fisher
Anti-Homocysteine plyphonal antibodies | Chemicon
Bio-Rad Protein Assay | Bio-Rad
biotin-conjugated dUTP | Roche
biotin-conjugated TdT | Roche
Bovine Serum Albumin (BSA) | EMD
Calcium Chloride | Fisher
Cobalt (II) hydrochloride hexahydrate | Sigma-Aldrich
Creatinine kit (CRE) | Wako
Cystathionine | Sigma-Aldrich
Cytoscint | Fisher
DL-Homocysteine | Sigma-Aldrich
DL-Propargylglycine | Sigma-Aldrich
DNA, Salmon testes | Sigma-Aldrich
Dnase I | Amersham
Ethidium Bromide | Sigma-Aldrich
Ethylene glycol tetraacetic acid | Sigma-Aldrich
Ethylene diaminetetraacetic acid | Sigma-Aldrich
Ferric Chloride | Sigma-Aldrich
Flavin adenine dinucleotide Disodium salt Hydrate | Sigma-Aldrich
Flavin mononucleotide | Sigma-Aldrich
Hematoxylin, Harris | Sigma-Aldrich
Hematoxylin, Mayers | Sigma-Aldrich
Heparin | Sigma-Aldrich
HEPES | Sigma-Aldrich
Homocysteine HPLC kit (4C reagents) | Sigma-Aldrich
Hydrochloric acid | Sigma-Aldrich
Hydrogen peroxide 30% | Sigma-Aldrich
Hydroxylamine hydrochloride | Sigma-Aldrich
L-[2,3,4-3H] arginine monochloride | Sigma-Aldrich
L-Cysteine | Sigma-Aldrich
Leupeptin | Sigma-Aldrich
L-Homocysteine thiolactone | Sigma-Aldrich
L-Lactate Dehydrogenase | Sigma-Aldrich
L-NAME (Nitro-L-arginine methyl ester) | Sigma-Aldrich
L-Serine | Sigma-Aldrich
Malonaldehyde bis-(dimethyl acetyl | Sigma-Aldrich
Manganese (II) Chloride tetrahydrate | Sigma-Aldrich
Methanol | VWR
mouse anti-nitrotyrosine antibodies
N-(1-Naphthyl)ethylenediamine dihydrochloride (NEDA)
N-ethylmaleimide (NEM)
Nitrate Reductase
NN-dimethyl-p-phenylenediamine sulfate
Orthovanadate
Pentobarbital
Pepstatin A
Phenylmethanesulfonyl fluoride (PMSF)
Potassium Chloride
Potassium Phosphate dibasic (K2HPO4)
Potassium Phosphate monobasic (KH2PO4)
Proteinase K
Pyridoxal-5'-phosphate hydrate
Resin AG 50W-X8 Sodium form
Resin AG 50W-X8 Hydrogen form
S-adenosylmethionine (SAM)
Sodium Azide
Sodium Chloride
Sodium Citrate
Sodium dodecyl sulfate (SDS)
Sodium hydrosulfide hydrate
Sodium Hydroxide
Sodium L-ascorbate
Sodium Nitrite
Sodium Nitroprusside
Sodium Orthovanadate
Sodium phosphate dibasic (Na2HPO4)
Sodium phosphate monobasic (NaH2PO4)
Sodium Pyruvate
Sodium thiosulfate
Sulfanilamide
Trichloracetic acid
Tris
Triton X-100
Xylene
Zinc Acetate dihydrate
β-Nicotinamide adenine dinucleotide 2'-phosphate reduced (NADPH)
β-Nicotinamide adenine dinucleotide, reduced form

Zymed
Sigma-Aldrich
Sigma-Aldrich
Sigma-Aldrich
Sigma-Aldrich
Sigma-Aldrich
Sigma-Aldrich
Sigma-Aldrich
Sigma-Aldrich
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Invitrogen
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### Equipment

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<th>Model</th>
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<tr>
<td>Axioskope2 MOT microscope</td>
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<td>Centrifuge 5804R</td>
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<td>IMx</td>
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<td>LS 6500 Multi-purpose Scintillation counter</td>
<td>Beckman Instruments</td>
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<td>Microtome</td>
<td>Therma</td>
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<td>MRX TC Revelation</td>
<td>Dynex</td>
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<td>Spectra Max Gemini</td>
<td>Molecular devices</td>
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<td>Spetrophotometer DU 800</td>
<td>Beckman Coulter</td>
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APPENDIX –III

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March 20, 2008

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