Role of Hyperhomocysteinemia in the Regulation of Oxidative Stress and Inflammatory Responses in the Kidney: Protective Effect of Folic Acid Supplementation

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Abstract

Hyperhomocysteinemia, a condition of elevated blood homocysteine (Hcy) level, is an independent risk factor for cardiovascular disease. Folic acid supplementation can effectively reduce blood Hcy levels. Recent studies have demonstrated that hyperhomocysteinemia is also associated with kidney disease. However, the underlying mechanisms remain unclear. The overall objective of the study was to investigate the biochemical and molecular mechanisms of Hcy-induced kidney injury and the effect of folic acid supplementation on Hcy-induced kidney injury.

Hyperhomocysteinemia was induced in Sprague-Dawley rats by feeding a high-methionine diet for 12 weeks. An elevation of serum total Hcy level was observed in hyperhomocysteinemic rats. Hyperhomocysteinemia-induced superoxide anion production via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation resulted in oxidative stress in the kidney. Reduction of oxidative stress by inhibiting superoxide anion production effectively ameliorated hyperhomocysteinemia-induced kidney injury.

Inflammatory responses such as increased chemokine expression have been implicated as one of the mechanisms of kidney disease. Monocyte chemoattractant protein-1 (MCP-1) is a potent chemokine that is involved in the inflammatory response in kidney disease. Nuclear factor-kappa B (NF-κB) plays an important role in upregulation of MCP-1 expression. We investigated the effect of hyperhomocysteinemia on MCP-1 expression and the molecular mechanism responsible for such an effect in rat kidneys as well as in human kidney proximal tubular cells.

Supplementation of folic acid in the diet significantly reduced serum total Hcy levels and effectively inhibited hyperhomocysteinemia-induced superoxide anion production resulting in amelioration of oxidative stress-mediated kidney injury. This, in turn, attenuated MCP-1 expression in the kidney of hyperhomocysteinemic rats. These results suggested a protective role of folic acid supplementation in hyperhomocysteinemia-induced kidney injury.

In conclusion, our studies have clearly demonstrated that **(1)** hyperhomocysteinemia causes oxidative stress and kidney injury; **(2)** hyperhomocysteinemia-mediated oxidative stress stimulates the expression of a potent chemokine MCP-1; **(3)** folic acid supplementation can offer a protective effect against kidney injury during hyperhomocysteinemia. Therefore, targeting the overproduction of superoxide anion may represent a promising strategy against kidney disease. Folic acid supplementation may offer a protective effect against oxidative stress and oxidative stress-mediated chemokine expression.

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Publications

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Abbreviations

AGEs, Advanced glycation end products

ATCC, American type culture collection

BHMT, Betaine: homocysteine methyltransferase

CBS, Cystathionine-β-synthase

CGL, Cystathionine-γ-lase

DFE, Dietary reference intake

DMEM, Dulbeco's modified Eagle's medium

DNA, Deoxyribonucleic acid

DRI, Dietary reference intake

dTMP, deoxythymidylate

dUMP, deoxyuridylate

EDTA, Ethylenediaminetetraacetic acid

EMSA, Electrophoretic mobility shift assay

ESRD, End stage of renal disease

FAD, Flavin adenine dinucleotide

GAPDH, Glyceraldehyde 3-phosphoate dehydrogenase

GFR, Glomerular filtration rate

Hcy, Homocysteine

HDL, High-density lipoprotein

HK-2, Human kidney cortex proximal tubular cells

H_2S, Hydrogen sulfide

Keap1, Kelch-like ECH-associated protein 1

LDL, Low-density lipoprotein

LPS, Lipopolysaccharide

Met, Methionine

MDA, Malondialdehyde

MCP-1, Monocyte chemoattractant protein-1

mRNA, messenger ribonucleic acid

MPO, Myeloperoxidase

NHANES, National health and nutrition examination survey

NOS, Nitric oxide synthase

NOX, NADPH oxidase

5-MTHF, 5-Methyltetrahydrofolate

MTHFR, Methylenetetrahydrofolate reductase

NADPH, Nicotinamide adenine dinucleotide phosphate

NF-κB, Nuclear factor kappa-B

Nrf2, Nuclear factor-erythroid-2-related factor 2

PBS, Phosphate buffered saline

PCFT, Proton-coupled folate transptor

PCR, Polymerase chain reaction

RDA, Recommended Daily Allowance

RNA, Ribonucleic acid

ROS, Reacive oxidative species

SAH, S-adenosyl homocysteine

SAM, S-adenosyl methionine

SD, Standard deviation

SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresi

SEM, Standard error of the mean

SGHMT, Serine:glycine hydroxymethytransferase

SNPs, Single nucleotide polymorphisms

SOD, Superoxide dismutase

TBARS, Thiobarbituric acid reactive substances

tHcy, Total homocysteine

I. Introduction

1.1 Hyperhomocysteinemia

Hyperhomocysteinemia is a metabolic disorder that is characterized by an elevated level of homocysteine (Hcy) in the circulation. Hcy is a non-protein amino acid produced in cells as an intermediate of the methionine metabolic pathway. Hyperhomocysteinemia is a known risk factor for a number of diseases which include thrombosis, coronary, cerebral, and peripheral vascular diseases (McCully, 2007). In healthy humans, the plasma total Hcy concentration in the circulation is in the range of 5-15 μM (Bostom *et al.*, 2000). The term "homocysteine" refers to both the reduced (sulfhydryl) and the oxidized (disulfide) forms of Hcy. Most of the reduced form of Hcy that enters into the circulation becomes oxidized. There are three forms of the oxidized Hcy: 1) Hcy can combine with another Hcy molecule, the process named "auto-oxidation", and form "homocystine"; 2) Hcy can bind with a cysteine molecule and form "homocysteine-cysteine mixed disulfide"; or 3) Hcy can oxidize the sulfhydryl group on proteins such as plasma albumin to form "protein-bound homocysteine mixed disulfides". It has been observed that the concentrations of low molecular weight of homocystine and homocysteine-cysteine mixed disulfide in serum is about 5-10 % of the total Hcy concentration whereas protein-bound homocysteine mixed disulfides make up about 80-90 % of the total Hcy concentration. Plasma albumin is known as a major carrier of Hcy in human plasma. 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.*, 2004b).

In 1932, the name of “homocysteine” was first proposed by Du Vigneaud (Mudd *et al.,* 2000). 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 their blood as well as in their 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). Subsequent studies have demonstrated that hyperhomocysteinemia can cause atherothrombosis by promoting oxidative stress, endothelial dysfunction, inflammation, thrombosis, and cell proliferation (Harker *et al.,* 1983; Welch *et al.,* 1997; Eikelboom *et al.,* 1999; Kaul *et al.,* 2006).

1.1.1 Homocysteine (Hcy) Metabolism

The metabolism of Hcy is linked to methionine and one-carbon metabolism. The need for one-carbon methyl groups is universal and most cells in the body actively metabolize methionine through the methionine cycle (Finkelstein, 1998). The generation of one-carbon methyl groups results in the production of Hcy. Although most cells produce Hcy during the methionine cycle activity, they differ in the expression of pathways available for the removal of Hcy. Most cells and tissues express at least one remethylation pathway for Hcy removal. However, transsulfuration pathway activity is limited in its distribution. Although less data is available, human cells and tissues also have unequal potentials for metabolizing Hcy (Finkelstein, 1990; Finkelstein & Harris, 1973; Finkelstein *et al.,* 1971; Finkelstein *et al.,* 1978).

Hcy is a sulfhydryl group-containing amino acid formed during the metabolism of methionine **(Figure 1.1.1)**. Methionine is one of the eight nutritionally indispensable amino acids and is the only indispensable sulfur-containing amino acid in mammals. Methionine is converted to Hcy via the "transmethylation pathway" **(Figure 1.1.2)**. Hcy is either converted to methionine via the "remethylation pathway" or is further catabolized to cysteine via the "transsulfuration pathway" **(Figure 1.1.2).** Cysteine is another sulfur-containing amino acid and is synthesized endogenously from methionine (Finkelstein *et al.*, 1988) **(Figure 1.1.2)**. Both methionine and cysteine 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 metabolic pathway of methionine provides a methyl group through the formation of S-adenosylhomocysteine (SAH) from S-adenosylmethionine (SAM) **(Figure 1.1.2)**. Methylation, which is an attachment or substitution of a methyl group on various substrates, is a critical chemical reaction in biological systems. It contributes to epigenetic inheritance via protein methylation, which is the major post-translational modification of protein, and DNA methylation that is important for gene regulation. Liver and kidney are known to be the major organs of methionine metabolism and these organs contain all the enzymes shown in **Figure 1.1.2**. A few other organs including the small intestine and pancreas also contain enzymes for the transmethylation, remethylation, and transsulfuration, however, at relatively low levels of activity as compared to the liver and the kidney (Finkelstein, 1990). The important 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 cysteine for

the synthesis of protein, glutathione, and taurine. Several minor pathways also metabolize methionine. For example, methionine is transaminated to its keto acid, α-keto-γ-methiolbutyrate (Stipanuk, 2004).

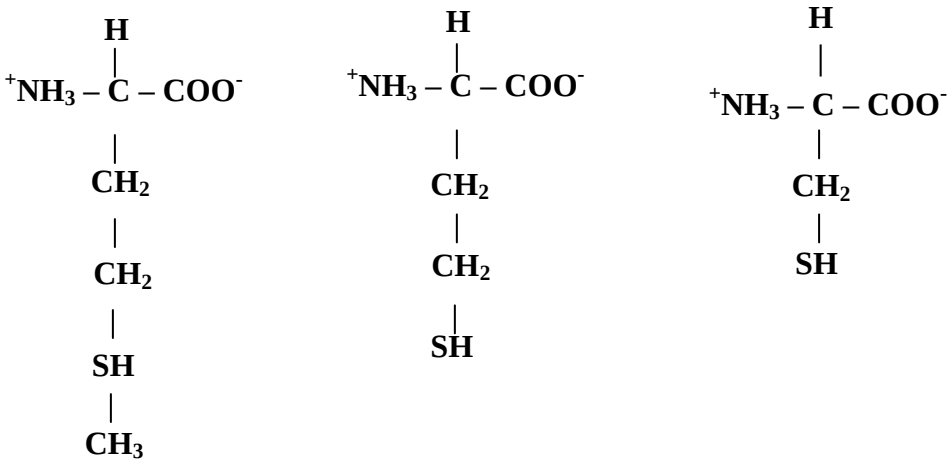

Figure 1.1.1 Structures of sulfur-containing amino acids

Methionine, homocysteine (Hcy), and cysteine are sulfur-containing amino acids. Hcy and cysteine are derived from the metabolism of methionine. Therefore, methionine is the only sulfur-containing amino acid, which is a dietary essential amino acid. Both Hcy and cysteine contain a free sulfhydryl group. (Based on ideas from Selhub et al., 1999; Stipanuk et al., 2004)

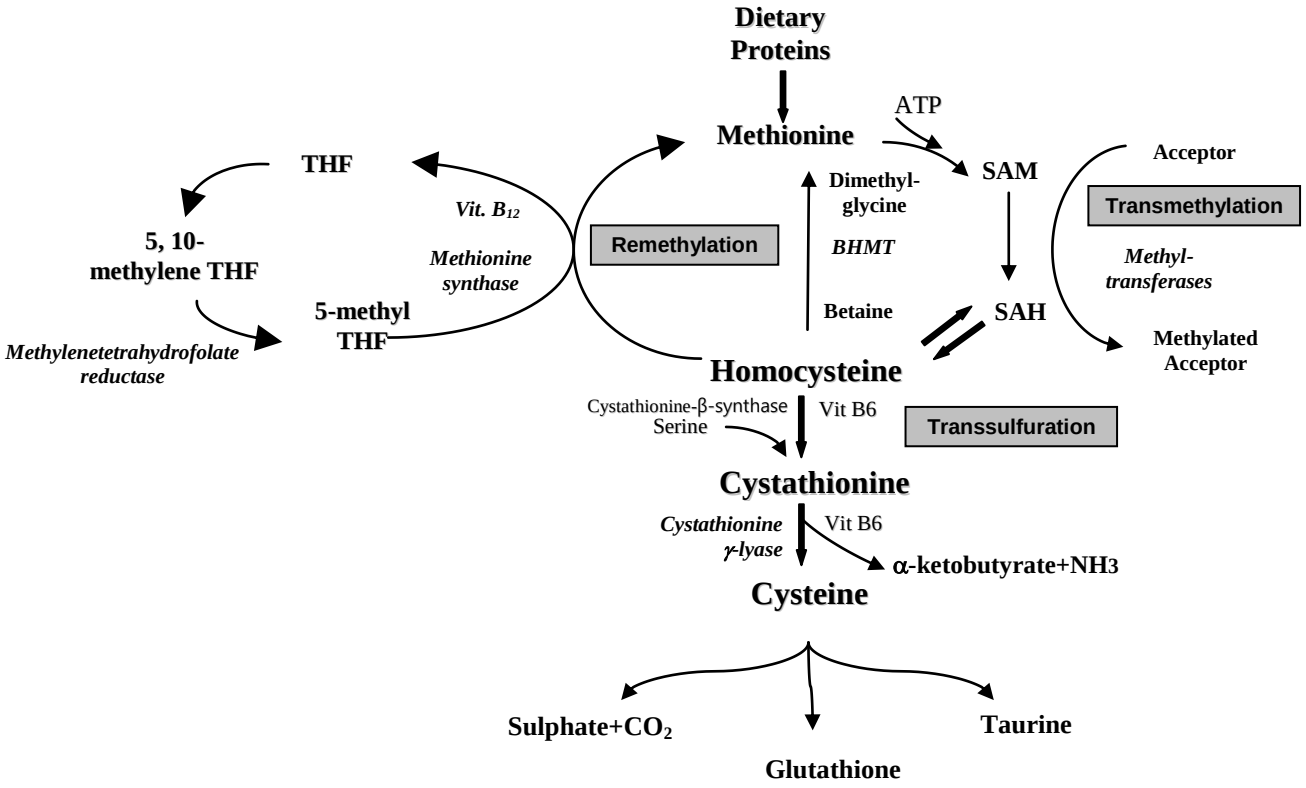

Figure 1.1.2 Metabolic pathways of sulfur-containing amino acids

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, SAM is converted to S-adenosylhomocysteine (SAH), which is reversibly hydrolyzed to homocysteine (Hcy) and adenosine. Then, Hcy is preserved back to methionine via the action of either methionine synthase or betaine: Hcy methyltransferase (BHMT). The reactions in the transsulfuration pathway irreversibly catabolize Hcy into cysteine which is the limiting amino acid for glutathione biosynthesis. (Based on ideas from Selhub et al., 1999; Stipanuk et al., 2004)

1.1.1.1 Transmethylation Pathway

Methionine is converted to Hcy by the transmethylation pathway. A major portion of methionine, which is originally derived from dietary sources and from intracellular turnover of protein, enters into the methionine cycle (**Figure 1.1.2**). This reaction is catalized by the enzyme methionine adenosyltransferase that mediates the transfer of the adenosyl moiety of ATP to the sulfur atom of methionine forming SAM. The isoenzymes methionine adenosyltransferase I (a homodimer) and methionine adenosyltransferase III (a homotetramer) are products of a gene expressed in the liver. When methionine concentrations are high, liver seems to be the only tissue in the body continuing to produce SAM carried out via methionine adenosyltransferase III. The isoenzyme methionine adenosyltransferase II is a product of the kidney and is expressed in all tissues. A branch point in the transmethylation pathway occurs with the formation of SAM (**Figure 1.1.2**). Methionine condenses with ATP yielding the phosphorylated SAM (**Figure 1.1.2**) (Mudd *et al.*, 1965). This reaction is catalyzed by S-adenosylmethionine synthase. The next reaction is catalyzed by a large number of transmethylases by which the methyl group of SAM is transferred to the large number of methyl acceptors. These include DNA, RNA and protein methylation, creatinine synthesis, and phosphatidylcholine synthesis (Finkelstein, 1990). As a result, SAM is converted to SAH which is then reversibly hydrolyzed to Hcy and adenosine by S-adenosylhomocysteine hydrolase (Finkelstein, 1974).

1.1.1.2 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 (**Figure 1.1.2**). The ubiquitous methionine cycle is completed by the remethylation of Hcy back to methionine. The enzyme that catalyzes this reaction is methionine synthase (MS), which converts Hcy to methionine using 5-methyltetrahydrofolate (5-MTHF) as the methyl-group donor and methylcobalamin, a coenzyme form of vitamin B_{12}, as catalyst. The other important product of the reaction catalyzed by MS is tetrahydrofolate (THF), the active substrate form of folic acid mediating one-carbon metabolism. Therefore, folic acid and vitamin B_{12} play important roles in Hcy metabolism, indicating that a deficiency of either can lead to hyperhomocysteinemia. Most nucleated cells express vitamin B_{12}-dependent MS. The 5-MTHF, necessary to drive Hcy remethylation through vitamin B_{12}-dependent MS, is provided by the "folate cycle" shown in **Figure 1.1.2**. The THF reacts with serine to produce glycine and 5, 10- methylenetetrahydrofolate (methylene-THF) catalyzed by vitamin B_6-dependent serine: glycine hydroxymethyltransferase (SGHMT). The 5, 10-methylene-THF is reduced to 5-MTHF by the vitamin B_2-dependent enzyme methylenetetrahydrofolate reductase (MTHFR) using NADPH as a co-substrate.

A second remethylation enzyme with very limited tissue distribution uses betaine (trimethylglycine), which is a product of choline oxidation, as the methyl donor. In liver and kidney, Hcy can be remethylated to methionine by betaine: Hcy methyltransferase (BHMT), which is a zinc metalloprotein with a hexameric structure. BHMT mRNA was detected in liver and kidney but not in pancreas, brain, lung, heart, and spleen in human tissues by Northern analysis (Sunden *et al.*, 1997). BHMT has a more limited tissue distribution than methionine synthase; in the rat there is significant BHMT activity only in the liver and kidneys though in humans BHMT expression occurs in the liver, kidneys, and eye lenses (Breksa & Garrow, 1999;

Evans *et al.*, 2002). The methyl group donor of the reaction catalyzed by BHMT is betaine which is an intermediate in choline catabolism (Skiba *et al.*, 1982).

1.1.1.3 Transsulfuration Pathway

In a limited number of human tissues, Hcy formed in the methionine cycle enters the transsulfuration pathway where it is converted to cysteine, which can undergo complete catabolism to inorganic sulfate, other sulfur-containing metabolites such as taurine, or glutathionine. However, the capacity of the transsulfuration pathway to metabolize Hcy is limited when the pathway is unable to handle the increase in intracellular Hcy due to the impairment of remethylation pathway. Transsulfuration is initiated by the vitamin B_6-dependent enzyme cystathionine-β-synthase (CBS), a type-II (β) family enzyme of the pyridoxal-5-phosphate (PLP)-containing enzyme and it is unique in being dependent on two cofactors: PLP and heme. With PLP, which is the co-factor form of vitamin B_6, the enzyme catalyzes a β-elimination reaction between serine and Hcy to produce cystathionine. CBS is a homotetramer and requires heme for catalytic activity in addition to PLP (Kery *et al.*, 1994). When the heme iron was reduced to the ferrous state, CBS enzyme activity decreased about 2-fold (Taoka *et al.*, 1998; Kery *et al.*, 1994). The activity of CBS is regulated by SAM that serves as a positive allosteric effector. In the second step of the transsulfuration pathway, cystathionine is converted to cysteine, α-ketobutyrate, and ammonia (NH_3) (**Figure 1.1.2**) by the PLP-dependent enzyme cystathionine-γ-lyase (CGL). Both CBS and CGL are responsible for the endogenous synthesis of hydrogen sulfide (H_2S). CBS catalyzes the synthesis of H_2S from cysteine and Hcy by a β-replacement reaction whereas the CGL catalyzes an α, β-disulfide eliminiation reaction resulting in the production of pyruvate and thiocysteine that is

nonenzymetically decomposed to H_2S and cysteine. It has been demonstrated that both cysteine and Hcy can serve individually or together as substrates for H_2S production catalyzed by CGL (Aitken & Kirsch, 2005; Chiku *et al.*, 2009; Chen *et al.*, 2004; Stipanuk & Beck, 1982). H_2S has emerged as an important biologically active compound due to its cytotoxic effect and physiological role in health and disease. However, apart form its deleterious effets, recent studies have demonstrated that H_2S also serves as an essential mediator at physiological concentrations due to its cytoprotective effect against myocardial ischemia/reperfusion injury in animal models. It has been demonstrated that a decrease in H_2S production leads to various diseases such as atherosclerosis, Alzheimer disease, and portal hypertension. A reduction of endogenous H_2S production is involved in kidney ischemia/reperfusion injury (Xu *et al.*, 2009; Elrod *et al.*, 2007; Xia *et al.*, 2009).

The transsulfuration pathway ends with the formation of cysteine, which is incorporated into protein and glutathione. Moreover, cysteine is the distal precursor of taurine that is a source of inorganic sulfate in the body (**Figure 1.1.2**). Cysteine has different metabolic fates depending on the tissue. These include its incorporation into proteins or glutathionine and synthesis of taurine (Stipanuk, 2004). Only a few organs such as liver, kidney, pancreas, and small intestine possess the capability to synthesize cysteine from methionine or Hcy (Mudd *et al.*, 1965; Finkelstein, 1990). Hence, the synthesis of cysteine from methionine and Hcy is limited to these tissues. Other tissues depend on an exogenous supply through the diet or export from any of those organs for their cysteine requirement. Therefore, transsulfuration is essential as a source of cysteine in addition to its role as a mode of elimination of excess Hcy. A deregulation in these pathways can lead to the accumulation of Hcy, causing hyperhomocysteinemia and associated diseases.

1.1.2 Sources of Homocysteine (Hcy)

Hcy is derived primarily from the methionine in dietary protein. Foods contain only trace amounts of Hcy reflecting the notion that Hcy is maintained at low concentrations. The abundance of methionine varies depending on the source of food proteins (**Table 1.1.1**). Food protein derived from animal sources has a higher methionine content than food protein derived from plant sources (Guttormsen *et al.*, 1994). Ingestion of a meal rich in protein (about 50 g) can elevate plasma Hcy whereas meals containing smaller amount of protein (about 15 g) do not affect plasma Hcy levels (Guttormsen *et al.*, 1994). The oral methionine-loading test was used clinically to evaluate Hcy metabolism in subjects to simulate the ingestion of a meal rich in animal protein (Silberberg *et al.*, 1997). For a 70 kg individual, 7 g of methionine (0.1 g/kg) was administered which was equivalent to ingesting 200-300 g of animal-derived protein. Normally there is a 2-3 fold increase in total plasma Hcy in 6-8 hours after methionine loading and then it declines to basal levels within 24 hours (Silberberg *et al.*, 1997). Therefore, individuals with impaired Hcy metabolism, which is thought to be associated with the transsulfuraton pathway, may have significantly higher increases in the concentration of total plasma Hcy and it may take longer for levels to normalize.

Source		Methionine (g/100g of protein)
Animal	**Meat**	**2.7**
	Milk	**2.9**
	Eggs	**3.2**
Plant	**Vegetables**	**1.2**
	Fruits	**0.9**
	Cereals	**1.8**

Table 1.1.1 Methionine Content of Food Protein (Based on ideas from Rutherfurd et al., 1998; Thapon et al., 1994)

1.1.3 Regulation of Homocysteine Metabolism

1.1.3.1 Dietary Factors

In general, dietary intake of essential micronutrients determines the efficiency of Hcy metabolism. At least four B complex vitamins, which are folic acid, vitamin B_2, B_6, and B_{12}, are essential. First, folic acid is the B complex vitamin that serves as a substrate for one-carbon methyl group metabolism in the methionine cycle and in other folate-dependent pathways requiring one-carbon groups such as thymidylate and purine ring biosynthesis. Among the four B complex vitamins, low folate seems to be the strongest determinant of increased plasma Hcy. Second, vitamin B_2 is converted to the co-factor flavin adenine dinucleotide (FAD) for MTHFR in the remethylation pathway. Third, vitamin B_6 is converted to the co-factor PLP for SGHMT in the remethylation pathway and for CBS and cystathionase in the transsulfuration pathway. Finally, vitamin B_{12} is converted to the co-factor methylcobalamin for MS.

1.1.3.2 S-adenosylmethionine (SAM)

The remethylation and transsulfuration pathways are regulated by SAM. When methionine is abundant in the diet, the intracellular concentration of SAM increases and more Hcy enters into the transsulfuration pathway. Since SAM is a positive allosteric effector of CBS, it can stimulate transsulfuration pathway activity whereas SAM is a negative allosteric effector which is accomplished at the level of MTHFR and BHMT. Therefore, inhibition of MTHFR by SAM limits the amount of 5-MTHF produced, hence lowering the amount of substrate for vitamin B_{12}-dependent MS. In addition, betaine-dependent remethylation of Hcy by BHMT in the liver and kidney can also be regulated by SAM which acts as a negative effector on the enzyme.

1.1.4 Biochemistry of Homocysteine

Most of the Hcy in serum is oxidized to a disulfide form and this occurs within a few minutes to hours after free Hcy enters into the circulation. The major forms of Hcy in circulation are listed in **Table 1.1.2**. The concentration of free reduced Hcy in healthy individuals is usually less than 2 % of total plasma Hcy. In contrast, homocystinurics have much higher concentrations of free reduced Hcy that can account for up to 5 % to 20 % of total plasma Hcy. When Hcy oxidizes with itself, the product is called "homocystine". On the other hand, when Hcy oxidizes with cysteine, the product is called "homocysteine-cysteine mixed disulfide". Hcy can also oxidize with sulfhydryl groups on proteins to form "protein-bound homocysteine mixed disulfides". The disulfide bond is a common feature of all oxidized forms of Hcy in the circulation. The forms of homocystine and homocysteine-cysteine mixed disulfide comprise approximately 5 % to 10 % of total plasma Hcy. Protein-bound Hcy makes up the largest fraction of total plasma Hcy comprising 80 % to 90 % of total Hcy in circulation carried by disulfide linkage on serum proteins. In humans, albumin is the major carrier of Hcy in the circulation. In addition, Hcy thiolactone, which is the stable 5-membered ring condensation product of Hcy, is a physiological form of Hcy in human plasma. However, it is unlikely that Hcy thiolactone can accumulate in the circulation due to the presence of esterase-like activities, which are both soluble and vessel-wall-bound enzymes catalyzing its hydrolysis to Hcy.

- **Oxidized forms:**

  Homocystine (5-10%)

  Mixed disulfides:

  Protein-bound homocysteine (80-90%)

  Cysteine-homocysteine (5-10%)

- **Reduced form:**

  Homocysteine (less than 2%)

Table 1.1.2 The major forms of homocysteine (Hcy) in circulation (Based on ideas from Selhub et al., 1999; Stipanuk et al., 2004)

It is likely that Hcy is exported from cells in a reduced state. In normal subjects, a small amount of Hcy is exported to the circulation where it undergoes oxidation with itself and other sulfhydryl-containing compounds in the presence of molecular oxygen to form a disulfide product and hydrogen peroxide.

1.1.5 Factors Causing Hyperhomocysteinemia

1.1.5.1 Genetic Factors

There are genetic factors contributing to hyperhomocysteinemia. The most common genetic factor leading to increased Hcy levels is the presence of a theromolabile variant of methylenetetrahydrofolate reductase (MTHFR) (677C→T mutation). This mutation reduces the activity of MTHFR by about 70%. About 5 % to 14 % of the general population is homozygous for this mutation (Frosst *et al.*, 1995). According to a recent meta-analysis, the homozygous MTHFR defect is associated with a higher risk for degenerative vascular disorders (Klerk *et al.*, 2002; Refsum *et al.*, 2004a). A heterozygous MTHFR defect leads to a 25% elevation of Hcy levels in the circulation (Refsum *et al.*, 2004a). Homocystinuria due to a CBS defect is also one of the most common causes of hyperhomocysteinemia (Refsum *et al.*, 2004a). Therefore, inadequate CBS activity may lead to Hcy accumulation and export into the circulation causing hyperhomocysteinemia.

1.1.5.2 Dietary Factors

Dietary factors such as high-methionine intake, low folate intake, and deficiencies of vitamin B_6 and B_{12} are associated with elevated plasma Hcy levels (Schneede *et al.*, 2000; Welch & Loscalzo, 1998). An excessive intake of protein-rich

food sources such as meat can lead to a transient elevation of Hcy level in the blood. However, vegetarians may also have higher plasma Hcy levels than non-vegetarians due to the lack of vitamin B_{12} in their diet (Schneede *et al.*, 2000; Geisel *et al.*, 2005). Low levels of dietary folic acid intake are also related to high plasma Hcy levels.

Vitamin deficiencies are one of the most common causes of hyperhomocysteinemia in the general population (Schneede *et al.*, 2000). Deficiency can occur due to the lack of vitamin supplementation, reduced absorption in the gastrointestinal tract, during periods of increased requirement such as pregnancy and from interactions with certain drugs. Therefore, elderly people, pregnant women, people following vegetarian diets, individuals suffering from malabsorption diseases such as irritable bowel syndrome, and patients with renal failure are at elevated risk for vitamin deficiency-mediated hyperhomocysteinemia (Schneede *et al.*, 2000; Castro *et al.*, 2006). Folate deficiency is one of the most common vitamin deficiencies and vitamin B_{12} deficiency also occurs frequently in elderly people (Herrmann *et al.*, 2005).

1.1.5.3 Lifestyle Factors

Studies have shown that the levels of Hcy are higher in smokers than in non-smokers. According to the Hordaland Homocysteine Study, there was a strong dose-response relationship between the number of cigarette and Hcy levels, which was independent of age and gender. Smoking increases the mean of Hcy levels and causes a shift of the whole Hcy distribution curve to higher levels (Nygard *et al.*, 1995; Nygard *et al.*, 1998). It is similar to that observed in populations with low folate intake, suggesting an influence of smoking on folate function (Nygard *et al.*, 1998). Smokers also generally have reduced intake and blood levels of vitamins involved in

Hcy metabolism including vitamins B_6 and B_{12}. Moreover, tobacco smoke contains abundant free radicals that can induce oxidative stress, which, in turn, may affect the redox status (Piyathilake *et al.*, 1994; Vermaak *et al.*, 1990).

Heavy coffee consumption is another strong lifestyle factor causing hyperhomocysteinemia. A does-response relation was observed in individuals drinking coffee more than six cups each day. In that study, the mean Hcy level was 2-3 μM/L higher than in coffee abstainers, suggesting that the caffeine might be related to its influence on the cardiovascular system and/or kidney function and its interference with essential vitamins in Hcy metabolism (Nygard *et al.*, 1997b; Holycross & Jackson, 1992).

1.1.5.4 Pathophysiological Factors

Plasma total Hcy is elevated in patients with kidney disease, which is the major interest in between Hcy and the kidney. In patients with renal failure, the increase in plasma total Hcy is inversely correlated with the glomerular filtration rate (GFR). Of course, increased plasma total Hcy concentrations that occur with increasing age also correlate with decreased GFR. A decreased GFR is not the only factor of elevated plasma total Hcy; decreased B vitamin complex including folate, vitamin B_{12}, and B_6 status can contribute to the phenomenon (Bostom & Lathrop, 1997). Moreover, elevated plasma total Hcy levels, both fasting and postmethionine load, have been found in renal transplant recipients and treatment with the immunosuppreive drug such as cyclosporine also contributes to the elevated plasma total Hcy levels (Arnadottir *et al.*, 1996). In patients with chronic renal failure, it is obvious that a decreased rate of Hcy removal is a key event. Supplementation of folic acid in patients with renal failure was able to reduce the fasting plasma total Hcy by

about 25 % (Guttormsen *et al.*, 1997). However, folic acid supplementation did not affect the elimination of Hcy from plasma after oral Hcy loading, indicating two defects in Hcy metabolism in renal disease: (1) a defect in Hcy metabolism, which contributes to hyperhomocysteinemia and can be corrected by dietary folate and (2) a massive decrease in total Hcy clearance from plasma, which is not corrected by dietary folate.

The kidney plays an important role in amino acid metabolism. For instance, amino acid reabsorption by renal tubules recovers about 70 g of filtered amino acids per day in a 70 kg man (Brosnan, 1987; Tizianello *et al.*, 1980). The major site of amino acid reabsorption is the proximal tubules. The kidney also plays a major role in the removal of certain amino acids from the circulation and addition of others. For instance, the kidney plays major roles in the metabolism of glycine, serine, citrulline, arginine, and glutamine. The kidney removes glycine from the circulation and converts it to serine released into the renal vein. This event occurs in the proximal tubular cells, which are endowed with both the glycine cleavage enzyme and serine hydroxymethyltransferase (Brosnan, 1987; Tizianello *et al.*, 1980). Indeed, the kidney produces more serine than can be accounted for by the uptake of glycine. Renal serine product decreases in patients with renal disease and is accompanied by a decreased plasma serine concentration. This may have implications for the decreased rate of disposal of Hcy in patients with renal disease since serine depletion could limit the transsulfuration pathway. Moreover, serine is the ultimate supplier of the one-carbon groups that produce 5-MTHF. Therefore, it is possible that serine depletion may also limit remethylation (Brosnan, 1987; Tizianello *et al.*, 1980).

In healthy humans, protein-bound Hcy accounts for more than 80 % of total Hcy, with homocystine, the homocysteine-cysteine mixed disulfide, and free reduced

homocysteine accounting for the remainder. In contrast, in the rat only about 30 % of plasma total Hcy is protein-bound. It is important to note that protein-bound Hcy is not filtered by the glomerulus. However, homocysteine, homocystine, and the homocysteine-cysteine mixed disulfide are filtered by the glomerulus. In both humans and rats, urinary excretion is only about 1 % of the filtered load.

1.1.6 Hyperhomocysteinemia and Diseases

1.1.6.1 Hyperhomocysteinemia as a Risk Factor for Kidney Disease

Hcy is one of the independent risk factors for atherosclerosis and atherothrombosis (Clarke *et al.*, 1991; Lawrence de Koning *et al.*, 2003). McCully first discovered that the vascular pathology of hyperhomocysteinemia was associated with arteriosclerosis (McCully, 1969). Recent studies have also demonstrated that hyperhomocysteinemia is associated with hepatic steatosis, Alzheimer's disease, osteoporosis, and kidney disease (Clarke *et al.*, 1991; Gulsen *et al.*, 2005; Seshadri *et al.*, 2002; McLean *et al.*, 2004). More specifically, in this section hyperhomocysteinemia as a risk factor for kidney disease will be reviewed.

Hyperhomocysteinemia is a common clinical finding in patients with kidney disease. Under normal conditions, approximately 80 % to 90 % of plasma Hcy is protein-bound. Therefore, unbound Hcy exists as homocysteine-cysteine mixed disulfide, homocysteine-homocysteine dimer (homocystine), or as free homocysteine. In kidney failure, the levels of both free and protein-bound Hcy fractions increase (Hultberg *et al.*, 1995). Hyperhomocysteinemia can result from either increased production or decreased removal of Hcy. A number of factors such as inborn errors in methionine metabolic enzymes, deficiency of various B vitamins playing important roles as co-factors in the methionine cycle, and renal failure are responsible for the

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). Moreover, inborn errors in CBS cause the most severe hyperhomocysteinemia and homocysteinuria (Pogribna *et al.*, 2001). The deficiency of folate, vitamin B_6 and vitamin B_{12} also accounts for hyperhomocysteinemia in the general population (Cuskelly *et al.*, 2001; Guttormsen *et al.*, 1996).

It is generally thought that a reduction in kidney function would lead to an increased plasma Hcy concentration. It has been observed that a healthy rat kidney removes about 20 % of plasma Hcy in a single pass of blood through the kidney (Bostom *et al.*, 1995). Hcy, a molecular mass of 135 Da, is within the filtration range of normal glomeruli (House *et al.*, 2000). Similar to other amino acids, the filtered Hcy is reabsorbed (Refsum *et al.*, 1985; Ueland, 1995; Bostom *et al.*, 1995; Guttormsen *et al.*, 1993) and only about 1-2 % of Hcy in the glomerular filtrate is excreted (Refsum *et al.*, 1998; Svardal & Ueland, 1987). Therefore, it is well known that kidney dysfunction is a common factor causing hyperhomocysteinemia. However, recent studies have shown that Hcy, at pathological concentrations, may cause adverse effects on kidney function (Ninomiya *et al.*, 2004; Hwang *et al.*, 2008; Zhang *et al.*, 2004; Li *et al.*, 2002; Kumagai *et al.*, 2002). Epidemiological investigation has identified a positive association between an elevation of Hcy level in the blood and the development of chronic kidney disease in the general population (Ninomiya *et al.*, 2004).

1.1.6.2 Hyperhomocysteinemia as a Risk Factor for Cardiovascular Diseases

Hcy is one of the independent risk factors for cardiovascular diseases (Wald *et al.*, 2002; Welch & Loscalzo, 1998). Hyperhomocysteinemia was observed in 28 % of patients with peripheral vascular diseases and in 30 % of patients with coronary vascular diseases, indicating a correlation between plasma Hcy level and atherosclerosis (Clarke *et al.*, 1991). In another study, hyperhomocysteinemia was found to accelerate lesion formation in mice that developed spontaneous atherosclerotic lesions in apolipoprotein E knock-out mice (Hofmann *et al.*, 2001; Wang *et al.*, 2003). Large clinical trials also showed an association of hyperhomocysteinemia with cardiovascular diseases (Bonaa *et al.*, 2006; Lange *et al.*, 2004; Stott *et al.*, 2005).

Several mechanisms of hyperhomocysteinemia-related atherosclerosis have been suggested. These proposed mechanisms include endothelial dysfunction, vascular smooth muscle cell prolifertation, endoplasmic reticulum stress, disruption of lipid metabolism, oxidative stress, and stimulation of chemokine expression (Au-Yeung *et al.*, 2004; O *et al.*, 1998; Wang *et al.*, 2000; Wang *et al.*, 2002; Werstuck *et al.*, 2001; Woo *et al.*, 2005). Endothelial dysfunction is one of the main focuses of studying hyperhomocysteinemia-related atherosclerosis characterized by a shift of the actions of the endothelium leading to reduced vasodilation, a proinflammatory state, and pro-thrombic properties (Endemann & Schiffrin, 2004). Hcy-induced oxidative stress resulting in decreased nitric oxide bioavailability contributed to endothelial dysfunction (Weiss *et al.*, 2003). Our recent study clearly showed an increase in chemokines and adhesion molecules in the aortic endothelium in a diet-induced hyperhomocysteinemia model (Wang *et al.*, 2002). Chemokines and adhesion molecules can trigger the migration of inflammatory cells into vascular beds

promoting the formation of atherosclerotic lesions (Glass & Witztum, 2001). Hcy has been shown to affect lipid metabolism, which in turn contributes to the development of atherosclerosis. Our laboratory also observed that Hcy could increase the production and secretion of cholesterol in human hepatocytes (O *et al.*, 1998). We also found an increase in serum cholesterol levels in diet-induced hyperhomocysteinemic rats (Woo *et al.*, 2005). A negative correlation of plasma Hcy level and high-density lipoprotein (HDL) level was reported in patients with coronary artery disease suggesting that Hcy inhibited the reverse transport of cholesterol which might contribute to Hcy-induced atherosclerosis (Liao *et al.*, 2006).

1.1.6.3 Hyperhomocysteinemia as a Risk Factor for Other Diseases

Recent studies have observed that Hcy is an independent risk factor for osteoporosis and cognitive deterioration (Seshadri *et al.*, 2002; van Meurs *et al.*, 2004). It was found that Hcy-induced oxidative stress could increase bone resorption by stimulation of osteoclast formation and activity (Koh *et al.*, 2006). Hcy could also enhance apoptosis of bone marrow stromal cells leading to decreased bone formation (Kim *et al.*, 2006).

Neurological studies also suggested that an increase in brain amyloid β-peptide levels might be one of the mechanisms of hyperhomocysteinemia as a risk factor for Alzheimer's disease (Pacheco-Quinto *et al.*, 2006). It was also demonstrated that Hcy could induce neurotoxicity by increasing the production of reactive oxygen species (ROS) (White *et al.*, 2001). Even though various studies on the mechanisms of hyperhomocysteinemia-associated diseases have suggested that Hcy-induced oxidative stress may play a critical role in its pathology, the mechanisms by which

Hcy induces oxidative stress still remain to be elucidated (Kim *et al.*, 2006; Koh *et al.*, 2006).

1.2 Oxidative Stress and Kidney Injury

1.2.1 Role of Oxidative Stress in the Kidney

The term "oxidative stress" describes a condition associated with increased levels of reactive oxygen species (ROS). The generation of ROS is an inevitable consequence of aerobic life. ROS are a family of molecules derived from oxygen and are produced as intermediates in reduction-oxidation (redox) reactions. ROS are characterized by their high chemical reactivity and ability to act as oxidants in redox reactions. ROS are reactive chemical entities comprising two major groups, which are "free radicals" and "non-free radicals". Free radicals are species with one or more unpaired electrons such as the superoxide anion radical (O_2^-), hydroxyl radical ($^{\cdot}OH$), and lipid radicals ($LO^{\cdot}$, LOO) whereas non-radical derivatives such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hypochlorous acid (HOCl) can act as oxidizing agents that affect biological processes. The unpaired electron imparts high reactivity and renders the radical unstable whereas non-radical derivatives are less reactive and more stable with a longer half-life than free radicals (Droge, 2002; Fridovich, 1997; Johnson & Giulivi, 2005). There is a balance between ROS generation and the activity of enzymatic antioxidants such as superoxide dismutase (SOD), catalase, and glutathione peroxidase as well as non-enzymatic antioxidants such as glutathione, α-tocopherol, ascorbate, and thioredoxin that scavenge or reduce ROS concentrations.

The formation of ROS begins with a one-electron reduction of molecular oxygen to superoxide anion by several oxidases including xanthine oxidase, cyclooxygenase, lipoxygenase, mitochondrial respiration, cytochrome P450, uncoupled nitric oxide synthases (NOS), and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (**Figure 1.2.1**). It has been observed that the cell's

endogenous defense against high levels of superoxide anion is dismutation of superoxide anion by SOD, which forms hydrogen peroxide. Hydrogen peroxide, which is much less reactive than the superoxide anion, is metabolized by catalase to form oxygen and water. Moreover, hydrogen peroxide can be further reduced by iron-containing molecules to form the highly reactive hydroxyl radical. In the presence of nitric oxide, superoxide anion reacts more rapidly with nitric oxide than with SOD to form peroxynitrite, which is one of the ROS species. Therefore, in biological systems, redox imbalance, which is caused by an overall net increase in ROS production and/or a decrease in antioxidant capacity, leads to oxidative stress. Due to free radical-induced oxidation and damage to biomolecules such as lipids, proteins, and DNA leading to membrane oxidation, membrane instability, irreversible protein modifications, and DNA instability, oxidative stress is considered harmful.

ROS-induced cellular damage has been thought to be associated with the pathogenesis of various diseases including atherosclerosis, restenosis, Alzheimers, rheumatoid arthritis, ischemia-reperfusion injury, diabetes, and kidney diseases. This is due to excess production of oxidants, decreased nitric oxide bioavailability, and decreased antioxidant capacity (Tain & Baylis, 2006; Touyz, 2003; Vaziri & Rodriguez-Iturbe, 2006). ROS comprise various molecules that have divergent effects on cellular function such as regulation of cell growth and differentiation, modulation of extracellular matrix production and breakdown, and stimulation of many kinases and proinflammatory genes. Moreover, many of those actions have been associated with pathological changes (Harrison *et al.*, 2006; Mueller *et al.*, 2005; Touyz, 2005).

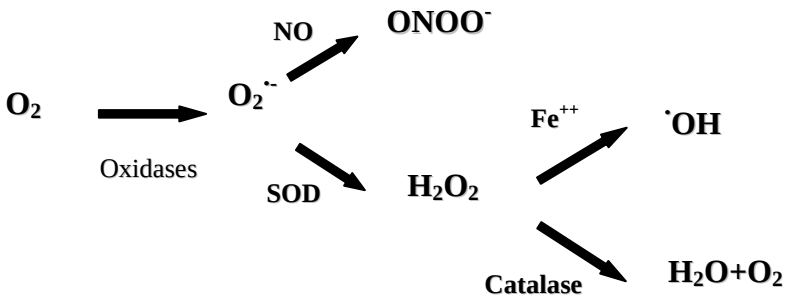

Figure 1.2.1 Pathways for generation of ROS

Single electron reduction of molecular oxygen, catalyzed by several oxidases such as xanthine oxidase, cyclooxygenase, lipoxygenase, mitochondrial respiration, cytochrome P450, uncoupled nitric oxide synthases (NOS), and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase produces superoxide anion. Reaction of superoxide anion with nitric oxide yields the reactive species peroxynitrite. Alternatively, dismutation of superoxide via superoxide dismutase (SOD) yields hydrogen peroxide which can be converted into hydroxyl radical by metal ion-dependent reactions such as the Fenton reaction. (Based on ideas from Bedard et al., 2007; Vaziri et al., 2003)

Abbreviaitons: O_2; oxygen, NO; nitric oxide, H_2O_2; hydrogen peroxide, $^{\bullet}OH$; hydroxyl radical, and $ONOO^-$; peroxynitrite.

The normal physiological environment is a balanced redox environment. However, during pathological conditions when large amounts of ROS are formed, the pro-oxidative state overrides the antioxidant capability. There are various sources of ROS in the human body. Among those several major enzymatic sources of ROS including xanthine oxidase, uncoupled NOS, and NADPH oxidase will be discussed. First of all, xanthine oxidase catalyzes the oxidation of hypoxanthine and xanthine to form superoxide anion. In spontaneously hypertensive rats, elevated levels of endothelial xanthine oxidase and increased ROS production were associated with increased arteriolar tone (DeLano *et al.*, 2006; Suzuki *et al.*, 1998). In transgenic rats with overexpression of renin and angiotensinogen, endothelial dysfunction was associated with increased xanthine oxidase activity (Mervaala *et al.*, 2001). Xanthine oxidase activity was increased in the kidney of the hypertensive rat model (Laakso *et al.*, 1998). Long-term inhibition of xanthine oxidase with allopurinol, a known xanthine oxidase inhibitor, reduced renal xanthine oxidase activity in spontaneously hypertensive rats (Laakso *et al.*, 1998). Secondly, NOS can also contribute to ROS production. All three NOS isoforms, inducible nitric oxide synthase (iNOS), endothelial nitic oxide synthase (eNOS), and neuronal nitric oxide synthase (nNOS), have been observed to be susceptible to the uncoupling leading to the formation of superoxide anion (Andrew & Mayer, 1999; Vasquez-Vivar *et al.*, 1998). It has also been demonstrated that eNOS uncoupling is due to enhanced superoxide anion production. NADPH oxidase has been observed to cause eNOS uncoupling (Widder *et al.*, 2007). Thirdly, NADPH oxidase is a multi-subunit enzyme catalyzing superoxide anion production by the one-electron reduction of superoxide anion using NADPH. During the past decade, NADPH oxidase has been studied in-depth due to its unique characteristic that its primary function is to generate ROS. Among ROS,

superoxide anion is the major product in most cases. The NADPH oxidase is a multi-subunit enzyme complex that was originally identified in phagocytic leukocytes. The backbone of this enzyme is the cell membrane-bound cytochrome b_{588} and cytosolic regulatory subunits.

In patients with glomerular diseases, increased oxidative stress and impaired antioxidative defense mechanisms have been observed (Turi *et al.,* 1997). ROS produced in the kidney by macrophages and leukocytes are important mediators in kidney disease. It has been suggested that there is an association between abnormal lipid metabolism and progression of glomerular and tubulointerstitial renal diseases. Atherosclerosis and glomerulosclerosis may share common lipoprotein-mediated pathogenic mechanisms. Oxidized low-density lipoproteins (LDL) may contribute to atherosclerosis and reduced relaxation in response to vasodilators. Oxidized-LDL induced vasoconstriction in the isolated perfused rat kidney is a result of inactivation of nitric oxide by ROS (Rahman *et al.*, 1999). Free radical scavengers provided protection against oxidized-LDL-induced renal vasoconstriction (Rahman *et al.*, 1999). Infusion of H_2O_2 into the renal artery resulted in a 50-fold increase in urinary protein excretion without any change in arterial pressure (Yoshioka *et al.*, 1991).

A relationship between oxidative stress and endothelium-dependent vasodilation in patients with chronic renal failure was also observed (Annuk *et al.*, 2001). A lower oxidized glutathione/reduced glutathione (GSSG/GSH) ratio was found in patients with chronic renal failure as compared to healthy subjects (Annuk *et al.*, 2001). Endothelium-dependent vasodilation was positively correlated with total antioxidative activity and reduced glutathione (GSH), indicating that impaired endothelium vasodilation function and oxidative stress might be associated in patients with chronic renal failure (Annuk *et al.*, 2001).

Oxidative stress is also one of the major causes of diabetic nephropathy (Ha & Kim, 1995; Salahudeen *et al.*, 1997). Patients with diabetic nephropathy showed higher oxidative stress than patients without renal complications (Kakkar *et al.*, 1997; Borcea *et al.*, 1999). It has been suggested that advanced glycation end products (AGEs) and oxidative stress may play a critical role in the pathogenesis of diabetic nephropathy (Salahudeen *et al.*, 1997). Short term infusion of AGE albumin into normal mice resulted in the appearance of malondialdehyde (MDA) determinants in the vessel wall (Yan *et al.*, 1994). Lower plasma oxygen radical absorbance capacity was found in patients with nephritic syndrome as compared to controls (Dogra *et al.*, 2001). Increased oxidative stress and subsequent activation of transcription factors such as nuclear factor-kappa B (NF-κB) have been shown to be associated with diabetic nephropathy in patients with diabetes mellitus type 1 and type 2 (Hofmann *et al.*, 1999).

1.2.2 Mechanisms of Renal Oxidative Stress

Many risk factors causing kidney injury and cardiovascular diseases share a common feature of generating intracellular ROS causing oxidative stress. Superoxide anion, a redox signaling molecule that plays a critical role in physiologic and pathologic processes, is a highly reactive oxygen free radical that can interact with nitric oxide to form another highly reactive free radical named peroxynitrite that, in turn, causes extensive protein tyrosine nitration and mediates iron-catalyzed lipid peroxidation. NADPH-dependent oxidase is primarily responsible for intracellular superoxide anion generation. It is a major source of superoxide anion generation in the kidney (Bokoch & Knaus, 2003; Shiose *et al.*, 2001; Griendling *et al.*, 2000; Gill & Wilcox, 2006). NADPH oxidase is made up of a membrane-bound component,

termed cytochrome b_{558} (comprised of gp91^{phox} and p22^{phox} subunits) and cytosolic components (p47^{phox}, p67^{phox}, p40^{phox} and Rac 1/2 subunits). Under normal conditions, a small amount of superoxide anions generated inside cells can be scavenged by the cellular antioxidant defense mechanism. However, when there is an overproduction and insufficient removal of superoxide anions, oxidative stress occurs. The gp91^{phox} is a catalytic subunit of the enzyme. Several homologues of gp91^{phox} have been identified and are termed NADPH oxidase (NOX) proteins. Among the NOX proteins, NOX4 was first characterized as a kidney NADPH oxidase and was also known as Renox, which had 39% identity to NOX2 (an analogue of gp91^{phox}). It has been shown that NOX4 is a major source of superoxide anions produced in the kidney and plays an important role in the development of kidney disease (Geiszt *et al.*, 2000; Shiose *et al.*, 2001). A recent study from our laboratory has shown that hyperhomocysteinemia can increase inducible nitric oxide synthase (iNOS) expression in the kidney, leading to increased nitric oxide production in rat kidneys (Zhang *et al.*, 2004). As a consequence, nitrotyrosine / nitrated proteins are formed in the kidney tissue (Zhang *et al.*, 2004). It is plausible that the generation of superoxide anion might also be elevated in the kidney during hyperhomocysteinemia, which will be discussed in the next section.

1.2.3 Renal Oxidative Stress in Hyperhomocysteinemia

It is increasingly recognized that oxidative stress is an important factor in the development and progression of kidney disease. NADPH oxidase is expressed in phagocytic cells as well as in non-phagocytic cells. Excessive activation of NADPH oxidase can lead to overproduction of superoxide anion, which is linked to tissue injury due to oxidative stress. It has been observed that NADPH oxidase is activated

in the kidney during hyperhomocysteinemia in animal models, leading to increased lipid peroxidation in the kidney. Several studies have shown that Hcy, at elevated levels, induces inflammatory responses and apoptosis in kidney cells and animal models (Shastry *et al.*, 2007; Yi *et al.*, 2007; Yi *et al.*, 2006; Yang & Zou, 2003). Hcy treatment was shown to increase collagen production and cell proliferation in rat mesangial cells (Yang & Zou, 2003). Such a stimulatory effect by Hcy was blocked by inhibition of NADPH oxidase activation. Moreover, one recent study demonstrated that deletion of the $gp91^{phox}$ gene in mice protects hyperhomocysteinemia-induced renal injury (Zhang *et al.*, 2010a). The authors speculated that NADPH oxidase-mediated superoxide anion production might represent a specific early mechanism mediating Hcy-induced renal injury (Shastry *et al.*, 2007; Yi *et al.*, 2007; Yi *et al.*, 2006; Yang & Zou, 2003; Zhang *et al.*, 2010a). In the rat model of hyperhomocysteinemia induced by folate free diet, glomerulosclerosis occurred which was characterized by enhanced oxidative stress, mesangial expansion, podocyte dysfunction, and fibrosis. When these rats with hyperhomocysteinemia were treated with apocynin, a known inhibitor of NADPH oxidase, the glomerular injury was significantly attenuated (Yi *et al.*, 2007; Yi *et al.*, 2006). Activation of NF-κB, induction of iNOS, and chemokine expression were found in the kidney of hyperhomocysteinemic rats (Hwang *et al.*, 2008; Zhang *et al.*, 2004). Therefore, it is evident that NADPH oxidase may play an important role in Hcy-mediated kidney injury.

In the kidney, superoxide anion produced via NADPH oxidase can be detected in fibroblasts, endothelial cells, vascular smooth muscle cells, podocytes, mesangial cells, and tubular cells (Gill & Wilcox, 2006; Glueck *et al.*, 1995; Zou & Banerjee, 2005; Griendling *et al.*, 2000). This superoxide anion producing enzyme in

the kidney has similar characteristics to an NADPH oxidase which is identified in phagocytes. It consists of a membrane-bound flavocytochrome b_{588}, the cytosolic subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small GTPase Rac1/2. The membrane-bound flavocytochrome b_{588} is a complex of two membrane subunits, a flavin and heme-binding glycoprotein $gp91^{phox}$ and a smaller subunit $p22^{phox}$. A family of $gp91^{phox}$, termed the nonphagocytic NOX proteins, has been identified including NOX1, NOX2 ($gp91^{phox}$), NOX3, NOX4, NOX5, DUOX1, and DUOX2. In the kidney, NADPH oxidase has been demonstrated to primarily mediate the production of superoxide anion (Gill & Wilcox, 2006; Glueck *et al.*, 1995; Zou & Banerjee, 2005; Griendling *et al.*, 2000). Therefore, it is possible that increased expression of NADPH oxidase may represent one of the important mechanisms initiating renal injury during hyperhomocysteinemia.

Recent studies have demonstrated that NADPH oxidase is involved in the progressive glomerular injury associated with hyperhomocysteinemia (Shastry *et al.*, 2007; Yi *et al.*, 2007; Yi *et al.*, 2006; Yang & Zou, 2003; Zhang *et al.*, 2010a). In cultured rat mesangial cells, Hcy stimulated the mRNA levels of tissue inhibitor of metalloproteinase-1 and led to accumulation of collagen type I, which was accompanied by enhanced cell proliferation and NADPH oxidase activity (Yang & Zou, 2003). These Hcy-induced biochemical changes were effectively blocked by hydroxyl-tetramethypiperidineoxyl, a suproxide dismutase mimetic. Moreover, blockade of the NADPH oxidase subunit $p22^{phox}$ by its antisense oligodeoxynucleotide eliminated the increase in NADPH oxidase activity induced by Hcy (Yang & Zou, 2003). Therefore, it can be concluded that Hcy-induced oxidative stress, cell dysfunction and extracellular matrix metabolism in renal cells are associated with enhanced NADPH oxidase acitivty.

Hcy was reported to cause mesangial cell apoptosis via ROS generation and p38-mitogen-activated protein kinase activation (Shastry *et al.*, 2007). In addition, one recent study showed that Hcy-induced upregulation and translocation of redox factor-1 was due to ROS production via NADPH oxidase. Increased redox factor-1 enhanced NF-κB activity and monocyte chemoattractant protein-1 (MCP-1) secretion in monocytes/macrophages which accelerated the development of atherosclerosis (Dai *et al.*, 2006). Taken together, these studies show that oxidative stress and/or changes in redox status in the kidney due to activation of NADPH oxidase may contribute to renal injury such as glomerularsclerosis during hyperhomocysteinemia.

1.3 Inflammatory Responses in the Kidney

Kidney macrophage accumulation has been suggested as a critical factor in the development of kidney diseases. The expression of the monocyte chemoattractant protein-1 (MCP-1) chemokine as a major promoter of inflammation has been demonstrated in various kidney diseases. In this section, a potent chemokine MCP-1 will be discussed as an important marker of inflammation in the kidney disease.

1.3.1 Monocyte Chemoattractant Protein-1 (MCP-1/CCL2)

MCP-1/CCL2 is a potent chemokine that stimulates the migration of leukocytes including monocytes into the intima of the arterial wall and other inflammed tissues. MCP-1/CCL2 is a member of the C-C chemokine family and a potent chemotactic factor for monocytes. Chemokines are small heparin-binding proteins that constitute a large family of peptides (60-100 amino acids) structurally related to cytokines whose main function is to regulate cell trafficking (Rollins, 1997; Wu *et al.*, 1977). Chemokines can be classified into four subfamilies on the basis of the number and location of the cysteine residues at the N-terminus of the molecule and are named CXC, CC, CX_3C, and C in agreement with the systematic nomenclature (Rollins, 1997; Wu *et al.*, 1977). Chemokines are secreted in response to signals such as proinflammatory cytokines where they play an important role in selectively recruiting monocytes, neutrophils, and lymphocytes. Once they are induced, the directed migration of cells, which express the appropriate chemokine receptors, occurs along a chemical ligand gradient. This allows cells to move toward the site with high local concentrations of chemokines (Rollins, 1997; Wu *et al.*, 1977; Callewaere *et al.*, 2007). The structure of chemokines comprises three distinct domains, which are a highly flexible N-terminal domain constrained by disulfide

bonding between the N-terminal cysteine, a long loop leading into three antiparallel β-pleated sheets, and an α-helix overlying the sheets. The N-terminal region is important for receptor binding and activation (Clark-Lewis *et al.*, 1991). Chemokines induce chemotaxis through the activation of G-protein-coupled receptors, which relate to adhesion molecules and glycosaminoglycans (Clark-Lewis *et al.*, 1991). Chemokines bind to specific cell surface transmembrane receptors coupled with heterotrimeric G proteins whose activation leads to the activation of intracellular signaling cascades prompting cell migration.

In terms of functionality, chemokines can be grouped into two main subfamilies, which are inflammatory and homeostatic chemokines. Inflammatory chemokines control the recruitment of leukocytes in inflammation and tissue injury, whereas homeostatic chemokines do housekeeping functions such as navigating leukocytes to and within secondary lymphoid organs as well as in the bone marrow and the thymus during hematopoiesis (Cochran *et al.*, 1983). CCL2, the best characterized human homolog, was first purified from human cell lines on the basis of its monocyte chemoattractant properties and was the first discovered human C-C chemokine.

Human MCP-1/CCL2 is a 76 amino acid protein of 13 kDa encoded by a gene located on chromosome 17 (Cochran *et al.*, 1983; Van Coillie *et al.*, 1999). MCP-1/CCL2 belongs to a family composed of at least four members which are MCP-1, -2, -3, and -4. MCP-1/CCL2 is produced by a variety of cell types either constitutively or after induction by cytokines, growth factors, and oxidative stress. Mutational analysis of MCP-1/CCL2 resulted in the identification of two regions of the primary structure which were critical for biological activity (Beall *et al.*, 1996). The first region consists of the sequence from Thr-10 to Thr-13, whereas the second

region consists of residues 34 and 35. MCP-1/CCL2 is produced by many types of cells such as endothelial, fibroblasts, epithelial, smooth muscle, monocytes, and mesangial cells (Ebisawa *et al.*, 1994). Monocytes/macrophages are found to be the major source of MCP-1/CCL2 which regulates the migration and infiltration of monocytes. MCP-1/CCL2 is among the most studied member of the chemokine family and has been known to be a potential intervention point for the treatment of a variety of diseases including multiple sclerosis, atherosclerosis, and insulin resistant diabetes mellitus.

1.3.2 Monocyte Chemoattractant Protein-1 Receptors (CCRs)

Many of the C-C chemokine receptors (CCRs) have been cloned based on conserved motifs of the earlier identified interleukin-8 receptors. Many of the genes encoding these CCR proteins contain about 360 amino acids that are closely linked in chromosome 3p21-22 (Wells *et al.*, 1996). All chemokine receptors identified are G-protein-coupled receptors which belong to the rhodopsin or serpentine receptor family. CCRs are composed of a short extracellular N-terminus, seven hydrophobic transmembrane domains each connected by three extracellular and three intracellular loops, and a C-terminal intracellular region. Most of the CCRs which bind one or more MCPs are shared by other C-C chemokines (Wells *et al.*, 1996). MCP-1/CCL2 mediates its effects through its receptor CCR2. However, unlike MCP-1/CCL2, CCR2 expression is relatively restricted to certain types of cells. There are two alternatively spliced forms of CCR2, CCR2A and CCR2B which differ only in their C-terminal tails.

1.3.3 Transcription Factor Nuclear Factor-Kappa B (NF-κB)

Since transcription factors are the nuclear components and ultimate targets modulated by upstream signaling events, they are central to signal transduction. Due to their ability to interact with very specific DNA sequences unique to each transcription factor in the regulatory regions of target genes, they modulate not only the magnitude of gene expression but also the specificity of the signal. It has been observed that redox-sensitive modulation of transcription factor activity can occur via direct oxidative modification of the transcription factor by intracellular ROS or via post-translational modifications such as phosphorylation and dephosphorylation by upstream redox-regulated intracellular signaling cascades. Nuclear factor-kappa B (NF-κB) is an inducible transcription factor complex composed of homodimeric or heterodimeric complexes of the Rel family of transcriptional activators. NF-κB was initially identified as a nuclear factor bound to the enhancer of the immunoglobulin κ light chain gene of B lymphocytes. Now it is clear that NF-κB is present in virtually every cell type (Hayden & Ghosh, 2004).

In the resting cells, NF-κB resides in the cytoplasm in an inactive form that is associated with an inhibitory protein (IκB). Although several isoforms of NF-κB such as p50/p65, $(p65)_2$ and c-rel/p65 protein complexes have been detected in various types of cells, the predominant NF-κB isoform in kidney cells is thought to be a p50/p65 heterodimer (Lynn *et al.*, 2001; Sung *et al.*, 2002; Hayden & Ghosh, 2004). The inhibitory protein IκBα is one of the best-characterized forms of IκB (Baldwin, 1996; Karin, 1999; Mercurio *et al.*, 1997; Simeonidis *et al.*, 1999). Upon stimulation, there is a rapid phosphorylation of IκBα and subsequent degradation of IκBα by the proteasome, leading to the release of NF-κB. After dissociation from IκB, the free

NF-κB can enter the nucleus, a process termed translocation. Once inside the nucleus, NF-κB binds to the κB binding motifs in the promoters or enhancers of its target genes (Karin, 1999; Mercurio *et al.*, 1997; Simeonidis *et al.*, 1999; Hayden & Ghosh, 2004). NF-κB promotes the expression of a multitude of genes involved in inflammation such as cytokines, adhesion molecules, and proliferative genes. Clinical and experimental studies have confirmed the presence of activated NF-κB in a variety of chronic and inflammatory disorders (Hayden & Ghosh, 2004). A strong body of evidence has indicated that the activation of NF-κB may be controlled by the redox status in the cell. Moreover, it was observed that NF-κB was the first eukaryotic transcription factor shown to respond directly to oxidative stress (Hayden & Ghosh, 2004). Therefore, a step in the activation mechanisms leading to degradation of IκB, the inhibitor of NF-κB, may involve ROS.

Active NF-κB is present as a homodimer or heterodimer of the five identified members of the NF-κB/Rel family in mammals. The most abundant dimer in most cell types is in subunits p50 and p65. In mammals, seven members of the IκB family have been identified which are IκBα, IκBβ, IκBγ, IκBε, Bcl-3, and the precursors of NF-κB1 (p105) and NF-κB2 (p100) (Schachter *et al.*, 2000; Simeonidis *et al.*, 1999). NF-κB can be activated by a number of physiological and non-physiological stimuli which include cytokines, mitogens, viruses, mechanical stress, a variety of chemical agents, and oxidative stress. Upon stimulation, IκBα is rapidly degraded within minutes. The degradation of IκB is achieved by a series of consecutive steps which include IκB phosphorylation at serine residues 32 and 36 by a specific kinase, recognition of phosphorylated IκB by the ubiquitin protein ligase complex, polyubiquitinylation of IκB at lysines 21 and 22, and degradation by the 26S

proteasome. All those steps are required to unmask the nuclear recognition sequences of NF-κB resulting in the translocation of active NF-κB into the nucleus through the interaction with karyopherins (Schachter *et al.*, 2000; Simeonidis *et al.*, 1999). IκB kinase (IKK) is most likely responsible for IκB phosphorylation. IKK is composed of one regulatory and two functional subunits. IKK is activated through phosphorylation by a kinase which belongs to the mitogen activated protein kinase kinase kinase family (MAPKKK). IKK appears to be the most likely point of convergence of various NF-κB activators (Schachter *et al.*, 2000; Simeonidis *et al.*, 1999).

1.3.4 Monocyte Chemoattractant Protein-1 Expression and Kidney Disease

Little MCP-1 is detectable in normal kidneys (Grandaliano *et al.*, 1997; Prodjosudjadi *et al.*, 1996; Robertson *et al.*, 2000). However, MCP-1 gene expression is greatly increased in kidneys of patients and experimental animal models with kidney diseases (Kuusniemi *et al.*, 2005; Rovin *et al.*, 1996; Sekiguchi *et al.*, 1997; Tang *et al.*, 1997; Wenzel *et al.*, 1997). Increased chemokine expression in the tissue is one of the key steps in the inflammatory response. Chemokines including MCP-1 play an important role in leukocyte infiltration and activation during the inflammatory process. Similar to its role in the pathogenesis of atherosclerosis, elevated MCP-1 production contributes significantly to the recruitment of leukocytes into the kidney during the development of glomerulosclerosis (Anders *et al.*, 2001; Kuusniemi *et al.*, 2005; Lynn *et al.*, 2001; Lynn *et al.*, 2000; Rovin *et al.*, 1996; Sekiguchi *et al.*, 1997; Tang *et al.*, 1997; Wenzel *et al.*, 1997). An increase in chemokine expression is also responsible for leukocyte recruitment into allografts, which contributes to allograft rejection in kidney transplantation (Grandaliano *et al.*, 1997; Prodjosudjadi *et al.*, 1996; Robertson *et al.*, 2000).

1.3.5 Hyperhomocysteinemia and Monocyte Chemoattractant Protein-1 Expression in the Kidney

We previously reported that Hcy stimulated MCP-1 production in vascular smooth muscle cells (Wang *et al.*, 2000), in endothelial cells (Sung *et al.*, 2001), and in macrophages (Wang *et al.*, 2001). We have postulated that Hcy-induced MCP-1 expression in vascular cells may play an important role in the development of atherosclerosis in patients with hyperhomocysteinemia (Choy *et al.*, 2004; O & Siow, 2003). However, little information is available regarding the effect of hyperhomocysteinemia on chemokine expression in the kidney. Elevated concentrations of Hcy also stimulated the expression of MCP-1 mRNA and protein in the kidney. The MPO activity, used as an index of leukocyte infiltration, was increased in the kidneys of hyperhomocysteinemic rats. The majority of patients with chronic renal disease displayed hyperhomocysteinemia (Ninomiya *et al.*, 2004; Wheeler, 1996). Epidemiological studies have revealed an inverse correlation between plasma levels of Hcy and kidney function (Ninomiya *et al.*, 2004). The activation of NF-κB is intimately involved in inflammatory reactions. NF-κB activation has been implicated in chemokine expression in the kidney and other tissues (Sung *et al.*, 2001; Wang *et al.*, 2001; Wang *et al.*, 2000). This transcription factor can be activated by diverse pathogenic signals. In general, a rapid phosphorylation and degradation of its inhibitor protein IκBα lead to nuclear translocation of NF-κB which then binds to the promoter region of the target genes including MCP-1 and regulates gene expression.

1.4 Folic Acid Supplementation

Folic acid is widely used for the treatment of megaloblastic anemia. The links between folic acid intake and birth outcome and chronic disease risks have been explored since the early 1990s. Daily supplementation of folic acid significantly reduces the risk of neural tube defects (NTDs). Hence, the evidence related to folic acid and NTD risk reduction led to the implementation of global public health policies, including mandatory folic acid fortification in North America.

1.4.1 Discovery and Structure of Folate

Folate is a generic term for the water-soluble B-complex vitamin existing in various chemical structures with similar biological activity. The name "folic acid" is derived from the Latin *folium*, which means "leaf". It was demonstrated that yeast extract was effective against the tropical macrocytic anemia observed during the late stages of pregnancy in India (Wills, 1931). Later, more researchers contributed to the isolation of this vitamin and the elucidation of its structure (Angier *et al.*, 1946).

Folic acid, the most oxidized and stable form of folate, rarely occurs naturally in food. However, it is the form used in vitamin supplements and in fortified food products.

Folic acid consists of three components which include a pteridine residue linked to a para-aminobenzoic acid which forms pteroic acid, and glutamic acid (Angier *et al.*, 1946) (**Figure 1.4.1**). It consists of a family of compounds that differ in various ways including the oxidation state of the molecule, the length of the glutamate side chain, and the specific one carbon units attached to the molecule. When one or more glutamic acid residues bond together, pteroylpolyglutamates, the most common occurring folates, are formed and these represent the main cellular forms of folates in plants and animals. The oxidation state of the pteridine structure varies from the fully

oxidized form referred to as folic acid to various levels of reduction such as dihydrofolate, tetrahydrofolate, 5, 10-methylenetetrahydrofolate, and 5-methyltetrahydrofolate (5-MTHF) (Chippel & Scrimgeour, 1970).

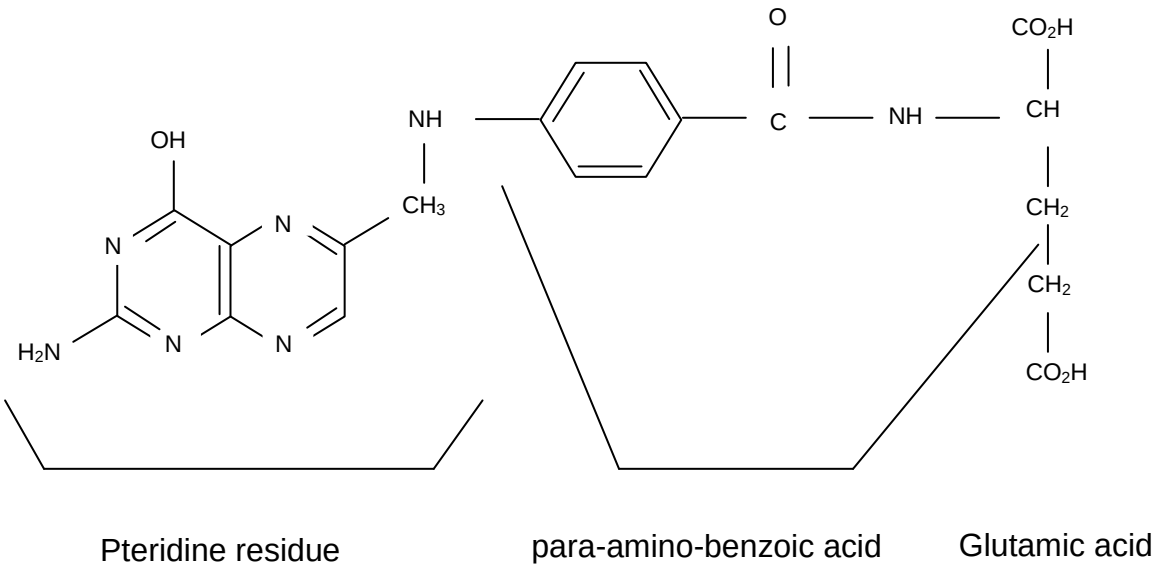

Figure 1.4.1 Folic Acid Structure

Folic acid consisits of a pteridine ring linked to para-amino-benzoic acid joined at the other end to glutamic acid. Folic acid structure can vary by reduction of the pteridine moiety to form dihydrofolic acid and tetrahydrofolic acid, elongation of the glutamate chain, and substitution of one-carbon units to the polyglutamated form of the tetrahydrofolic acid. (Based on ideas from Lucock et al., 2000; Stanger et al., 2002)

1.4.2 Dietary Sources of Folate

1.4.2.1 Naturally Occurring Folate in Food

Humans cannot synthesize folate and, therefore, must depend on a variety of dietary sources. Folate that occurs naturally in the diet will be referred to as "food folate" in this section. Food folate mainly exists as 5-MTHF and formyltetrahydrofolate. The predominant food folate, 5-MTHF, is readily oxidized to 5-methyl-5, 6-dihydrofolate (Donaldson & Keresztesy, 1962; Lucock *et al.*, 1995). Food folate is concentrated in select foods including orange juice, strawberries, citrus fruits, dark green leafy vegetables, peanuts, dried beans such as black beans and kidney beans, bread, potatoes, and dairy products. Meat, in general, is not a good source of folate, with the exception of liver and kidney.

1.4.2.2 Folic Acid in Fortified and Enriched Food Products

Folic acid is an added ingredient in a large number of food products including meal replacement, infant formulas, breakfast cereals, nutritional bars, and snack foods. In 1998, all enriched cereal grain products such as bread, flour, pasta, and breakfast cereal and mixed food items which contain these grains were required by the Food and Drug Administration to be fortified with folic acid for the purpose of reducing the risk of NTDs in the United States. The mandatory fortification of folic acid has been implemented in other countries, too, including Chile and Canada. Folic acid fortification has had a critical impact on folate status in the United States and Canada. According to the National Health and Nutrition Examination Survey (NHANES) from 1988-1994 to 1999-2000, the median serum folate concentration increased more than two-fold from 12.5 to 32.2 nmol/L and the median red blood cell folate concentration increased from 392 to 625 nmol/L in the United States (Pfeiffer *et al.*, 2005). In

Canada, the mean red blood cell folate concentration rose from 527 nmol/L during the pre-fortification to 741 nmol/L during the post-fortification in women of reproductive age (Ray *et al.*, 2002).

1.4.3 Folate Requirement

The Dietary Reference Intake (DRI) process introduced a new term, the dietary folate equivalent (DFE), as an expression of food folate values that correct for the bioavailability of folate from natural sources. The DFE assigns a bioavailability value of 50 % to all food folates relative to crystalline folic acid, irrespective of the nature of the food. The estimated Recommended Daily Allowance (RDA) requirements for dietary folate equivalents vary by life stage, with those over the age of 14 requiring 400 μg DFE per day (Bailey, 2000; Bailey, 1998). Requirements for women who are pregnant or lactating are increased to 600 μg DFE per day, to account for the increased metabolic demands arising from fetal development and milk production (Bailey, 2000; Bailey, 1998).

1.4.4 Metabolism of Folate

Dietary folate predominately occurs in a polyglutamate form which has to be hydrolyzed to the monoglutamate form before intestinal epithelial cell uptake can occur. This process takes place primarily in the jejunum (Lucock *et al.*, 1995). The enzyme responsible for the deconjugation of polyglutamylfolate is folate conjugase (also referred to as folypoly-γ-glutamate carboxypeptidase or pteroylpolyglutamate hydrolase) located primarily in the jejunal brush-border membrane (Lucock *et al.*, 1995; Chandler *et al.*, 1986). Folate conjugase functions as an exopeptidase releasing terminal glutamates sequentially with optimal activity at pH 6.5 to 7.0. The next step

is transport of monoglutamyl folate across the intestinal mucosa with maximum activity at a pH of 5 to 6 (Herbert *et al.*, 1962; Lucock *et al.*, 1989; Chandler *et al.*, 1986).

Although the transport process for internalizing folate is not completely understood, in general there appears to be two types of carrier-mediated mechanisms involved which are a folate transporter and a folate receptor. These folate membrane transport systems are essential for folate absorption into the epithelial cells of the small intestine, for reabsorption by a similar epithelial layer in the proximal renal tubules, and for internalization through the plasma membranes of both the developing embryo and the adult organism (Sirotnak & Tolner, 1999; Moscow *et al.*, 1995). In the intestine, the folate transporter is encoded by the reduced folate carrier gene expressed in most tissues (Sirotnak & Tolner, 1999; Moscow *et al.*, 1995; Said *et al.*, 1996). The reduced folate carrier is capable of mediating bidirectional flux (Sirotnak & Tolner, 1999; Moscow *et al.*, 1995). The other type is the receptor-mediated processes, in which folates are bound with high affinity at the membrane surface to folate receptor-like proteins that mediate unidirectional flux following internalization of the receptor-folate complex (Sirotnak & Tolner, 1999). It has been observed that the folate receptor is encoded by at least three genes with most tissues expressing the α-form. It has been demonstrated that when high doses of folic acid are given, intestinal uptake takes place by a nonsaturable mechanism which involves a diffusion-like process (Selhub *et al.*, 1983). Recently, a new folate transport system has been identified, which is known as proton-coupled folate transporter (PCFT). The PCFT previously recognized as heme carrier protein-1 with folate transport activity at low pH. It has been demonstrated that PCFT has a high-affinity for folic acid at low pH (Qiu *et al.*, 2006; Ashokkumar *et al.*, 2007; Yuasa *et al.*, 2009). Moreover,

examination of patients with familial folate malabsorption syndrome had a mutation in the gene encoding the PCFT (Qiu *et al.*, 2006).

Even though the degree of metabolism depends on the folate dose, once internalized, folate is metabolized to 5-MTHF with unmetabolized folic acid appearing in the portal circulation (Kelly *et al.*, 1997). When transported through the liver, folic acid is converted to 5-MTHF. However, large oral doses of folic acid result in a significant increase in urinary excretion of unmetabolized folic acid. Plasma folate, which is primarily the 5-MTHF form, is largely bound to albumin, a low-affinity folate-binding protein accounting for about 50 % of circulating bound folate (Kiil *et al.*, 1979). A smaller proportion of plasma folate is bound to a high-affinity folate binder, a soluble form of a membrane-associated folate transporter (Antony, 1996). Cellular folate uptake is mediated by both reduced folate carriers and folate receptors. Reversible internalization of folate is facilitated by reduced folate carrier proteins whereas folate receptors mediate only unidirectional transport of cellular folate (Antony, 1996).

Folate polyglutamation by folylpolyglutamate synthetase is required to be retained intracellularly (Antony, 1996; Shane, 1989). Thereafter, the polyglutamate form of folate has to be reconverted to the monoglutamate form. This process requires γ-glutamyl hydrolase to be released back into circulation (Shane, 1989). It has been observed that tissue storage of folate is limited to the amounts required for metabolic function, which is estimated to be about 15 to 30 mg (Hoppner & Lampi, 1980; Whitehead, 1973). Within the cells, folates are bound by enzymes catalyzing folate-dependent reactions and by other folate proteins (Whitehead, 1973).

Only a very small percentage of folate is excreted; when it is primarily excreted in the urine in the form of breakdown products (Caudill *et al.*, 1998). The

percentage of ingested food folate excreted as intact urinary folate is estimated to be only about 1-2 %, indicating that folate is catabolized before urinary excretion. The majority of plasma folate not associated with proteins is freely filtered in the glomerulus and is reabsorbed in the proximal renal tubules. A large quantity of folate is secreted daily into bile. However, it is reutilized via enterohepatic recirculation (Krumdieck *et al.*, 1978; Stea *et al.*, 1978).

1.4.5 Functions of Folate

The folate coenzymes are involved in various reactions that involve 1) deoxyribonucleic acid (DNA) synthesis depending on a folate as a co-enzyme for pyrimidine nucleotide biosynthesis (methylation of deoxyuridylic acid to thymidylic acid) and therefore is required for normal cell division; 2) purine synthesis (formaton of glycinamide ribonucleotide and 5-amino-4-imidazole carboxamide ribonucleotide); 3) generation of formate to replenish the formate pool; and 4) amino acid interconversions including the catabolism of histidine to glutamic acid, interconversion of serine and glycine, and conversion of Hcy to methionine, which will be more specifically discussed later in this section. Folate-mediated transfer of single-carbon units from serine provides a major source of substrate in single-carbon metabolism. The conversion of Hcy to methionine serves as a major source of methionine for the synthesis of SAM, which is an important methylating agent (Wagner, 1996). Folate inadequacy limits one-carbon transfer reactions, hence limiting the ability of folate to serve as a substrate for the transfer of single carbon atoms in the metabolism of pyrimidines and purines, formate, and amino acids including glycine, serine, and the sulfur amino acids methionine and Hcy (House *et*

al., 1999; Fox & Stover, 2008; Sokoro *et al.*, 2008). Therefore, one carbon metabolism is synonymous with folate-dependent enzymatic reactions.

1.4.5.1 Serine-Glycine Interconversion

Serine hydroxymethyltransferase catalyzes the transfer of formaldehyde from serine to tetrahydrofolate to form 5, 10-methylenetetrahydrofolate and glycine in a reversible reaction (Davis *et al.*, 2004; Schalinske & Steele, 1989). Serine can be derived through glycolysis from 3-phosphoglycerate and much of the glycine requirement is provided by serine by the action of serine hydroxymethyltransferase (Davis *et al.*, 2004; Schalinske & Steele, 1989). Both serine and glycine are non-essential amino acids. Serine hydroxymethyltransferase containing pyridoxal 5'-phosphate is present in two different isoforms which carry out the interconversion of serine and glycine either in the mitochondria or in the cytoplasm (Davis *et al.*, 2004; Schalinske & Steele, 1989). The conversion of serine to glycine via serine hydroxymethyltransferase generates 5, 10-methylenetetrahydrofolate, the primary source of methyl groups for methionine, dTMP, and purine synthesis (Davis *et al.*, 2004; Schalinske & Steele, 1989).

1.4.5.2 Formation of 5-methyltetrahydrofolate (5-MTHF)

The reduction of 5, 10-methylenetetrahydrofolate to 5-MTHF by 5, 10-methylenetetrahydrofolate reductase is a major cycle of one carbon metabolism. This reaction is dependent on FAD as a transfer agent for reducing equivalents from NADPH to the 5, 10-methylenetetrahydrofolate substrate to form 5-MTHF (Matthews *et al.*, 1982). The binding of SAM to a specific regulatory domain of

methylenetetrahydrofolate reductase results in allosteric inhibition that can be reversed by SAH (Matthews *et al.*, 1982).

1.4.5.3 Homocysteine (Hcy) Remethylation

Methionine synthase, which transfers a methyl group from 5-MTHF to the vitamin B_{12} coenzyme and to Hcy thus forming methionine and regenerating tetrahydrofolate, is a key enzyme required for the remethylation process (Herbert & Zalusky, 1962). It has been observed that the methionine synthase reaction is the only reaction where the methyl group of 5-MTHF can be metabolized in mammalian tissues (Herbert & Zalusky, 1962). Therefore, the role of this enzyme is critically important for both the folate cycle and for the production of methionine required for SAM-dependent transmethylation reactions. The methionine synthase reaction is able to reutilize Hcy as a carrier of methyl groups primarily derived from serine. 5-MTHF is the primary form of folate supplied by the diet to the liver. It enters the folate pool by conversion to tetrahydrofolate catalyzed by methionine synthase. This enzymatic step appears to be the obligatory step for assimilation of exogenous folate since 5-MTHF is a poor substrate for folate polyglutamate synthesis required for cellular folate retention (Herbert & Zalusky, 1962).

To explain why a vitamin B_{12} deficiency results in a secondary folate deficiency, the methyl-trap hypothesis has been proposed. The basis of this hypothesis is that a vitamin B_{12} deficiency inactivates methionine synthase, trapping folate as 5-MTHF since the MTHFR reaction is irreversible. The methyl-trap hypothesis provides the rationale for why either a vitamin B_{12} or a folate deficiency may result in megaloblastic anemia due to insufficient tetrahydrofolate to form 5, 10-methylenetetrahydrofolate required for normal DNA synthesis and cell division.

An alternative Hcy remethylation cycle is the betaine-dependent remethylation catalyzed by betaine: Hcy methyltransferase (BHMT). The BHMT remethylation of Hcy is an irreversible reaction, in which choline is first oxidized to betaine and then demethylated to glycine. BHMT catalyzes the first demethylation step in which a methyl group of betaine is transferred to Hcy, producing dimethlglycine and methionine. Unlike the vitamin B_{12}-dependent remethylation of Hcy catalyzed by methionine synthase, which is widely distributed in tissues throughout the body, betaine-dependent remethylation occurs in the liver, kidney, and eye lens due to the limited tissue distribution of BHMT.

1.4.5.4 Formation of S-adenosylmethionine (SAM)

Methionine is an essential amino acid that must be provided by the diet. Since methionine is usually one of the limiting amino acids in dietary protein, it has to be efficiently assimilated and recycled. In addition to protein synthesis, the other major function of methionine is the synthesis of SAM, which is the major methyl donor in mammalian systems (Ogawa *et al.*, 1998). The transfer of the adenosyl component of ATP to methionine is dependent on methionine S-adenosyltransferase to form SAM. When dietary methionine intake is adequate, SAM reduces the production of 5-MTHF by allosteric inhibition of methylenetetrahydrofolate reductase and hence the supply of methionine. In contrast, when dietary methionine is limited, SAM concentration decreases resulting in decreased inhibition of methylenetetrahydrofolate reductase allowing for an increased production of 5-MTHF (Ogawa *et al.*, 1998). Therefore, this leads to an increase in Hcy remethylation to produce methionine for protein synthesis and methylation. SAM is the methyl donor in more than 100 transmethylation reactions including methylation of DNA, RNA, and membrane phospholipids. A large

variety of methyltransferase enzymes transfer the methyl groups from SAM that in the process is converted to SAH (Konishi & Fujioka, 1988; Ogawa *et al.*, 1998).

1.4.5.5 Conversion of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH)

The conversion of SAM to SAH is a one-way process subjected to competitive inhibition by SAH. It has been observed that SAH has a higher affinity than SAM for the active site of the methyltransferase (Melnyk *et al.*, 2000). Therefore, an increase of SAH and decreased SAM/SAH ratio is associated with methyltransferase inhibition. SAH is hydrolyzed to Hcy and adenosine by SAH hydrolase via a reversible reaction (Melnyk *et al.*, 2000).

1.4.5.6 Transsulfuration Pathway

The hydrolysis of SAH leads to the formation of Hcy that can be metabolized to cysteine in the transsulfuration pathway or remethylated back to methionine by the methionine synthase reaction. In the transsulfuration pathway, Hcy is first coupled with serine to form cystathionine in a pyridoxine-dependent reaction by CBS (Finkelstein & Martin, 1984b). SAM functions as a switch between the methionine cycle and the transsulfuration pathway. When SAM concentration is low, Hcy remethylation is unimpaired. In contrast, high concentrations of SAM inhibit 5, 10-methylenetetrahydrofolate and BHMT. However, transsulfuration is enhanced by SAM stimulating CBS activity and accelerating the elimination of Hcy through the transsulfuration pathway (Finkelstein & Martin, 1984b; Finkelstein & Martin, 1984a; Daubner & Matthews, 1982).

1.4.5.7 Nucleotide Biosynthesis

The rate-limiting step in DNA synthesis is the conversion of the pyrimidine deoxyuridylate (dUMP) to deoxythymidylate (dTMP), which is dependent on thymidylate synthase and 5, 10-methylenetetrahydrofolate as a source of one carbon groups (Wakabayashi *et al.*, 1994). The one carbon unit is initially at the formaldehyde oxidation level and is reduced to the methanol level during the reaction by the reducing power of the tetrahyropyrazine ring (Wakabayashi *et al.*, 1994). The product of this reaction is dihydrofolate, which is inactive as a coenzyme and must be reduced back to the active tetrahydrofolate coenzyme form by dihydrofolate reductase (Wakabayashi *et al.*, 1994). Both thymidylate synthase and dihydrofolate reductase can be used in a coordinated manner for efficient folate metabolism. It has been observed that during the synthesis of the purine ring, 10-formyltetrahydrofolate is used in two reactions in which glycinamide ribonucleotide transformylase and aminocarboxamide ribotide transformylase add formyl groups at positions C-8 and C-2, respectively (Wakabayashi *et al.*, 1994).

1.4.5.8 Histidine Catabolism

In the final steps of histidine catabolism, the transfer of the formimino group of formiminoglutamate to tetrahydrofolate is catalyzed by formiminotransferase. The formimino moiety is converted to 5, 10-methenyltetrahydrofolate in a formimidoltetrahydrofolate cyclodeaminase reaction (Wagner, 1996).

1.4.6 Factors Affecting Folate Metabolism

1.4.6.1 Single Nucleotide Polymorphisms of Methylenetetrahydrofolate reductase (MTHFR)

It has been reported that single nucleotide polymorphisms (SNPs) affect the enzymes and transport proteins which are required for normal folate metabolism and are linked to elevations in plasma Hcy (Finnell *et al.*, 2003; Molloy, 2004; Gellekink *et al.*, 2005). A polymorphism of particular importance to folate metabolism is the 677C→T base substitution in the gene encoding the enzyme MTHFR which catalyzes the reduction of 5, 10-methylenetetrahydrofolate to 5-MTHF which is the methyl donor for Hcy remethylation to methionine (Finnell *et al.*, 2003; Molloy, 2004; Gellekink *et al.*, 2005). It has been demonstrated that homozygosity for the 677C→T polymorphism (TT genotype) is associated with a 70 % lower MTHFR enzyme activity (Finnell *et al.*, 2003; Molloy, 2004; Gellekink *et al.*, 2005). The prevalence of the MTHFR 677C→T polymorphism in the overall population is about 12 % for the TT genotype and about 50 % for the heterozygous (CT genotype) variant. It has been reported the MTHFR 677C→T polymorphism is associated with significantly increased plasma Hcy concentrations and reduced global DNA methylation when folate status is low (Botto & Yang, 2000; Friso *et al.*, 2002). When low folate diets were consumed, both serum and red blood cell folate concentrations were lower and Hcy concentrations were higher in women with the TT genotype compared with the CC genotype (Friso *et al.*, 2002; Shelnutt *et al.*, 2003; Guinotte *et al.*, 2003). In response to the low folate diets, a larger percentage of women with the TT genotype had low folate status compared with the CC genotype (Shelnutt *et al.*, 2003; Guinotte *et al.*, 2003).

The MTHFR 677C→T polymorphism combined with low folate status has also been observed to reduce global DNA methylation in epidemiological studies (Friso *et al.*, 2002; Stern *et al.*, 2000; Shelnutt *et al.*, 2004). In a European population, significantly less DNA methylation was observed in subjects with the TT genotype compared with the CC genotype providing evidence that the DNA methylation response to folate intake may be modified by the MTHFR 677C→T polymorphism (Friso *et al.*, 2002).

1.4.6.2 Alcohol

It has been observed that alcohol interferes with absorption, hepatic uptake, and renal reabsorption of folate. Therefore, when consumed chronically in large amounts, alcohol consumption may contribute to folate deficiency. The ability of alcohol to interfere with folate absorption has been linked to the negative effect of alcohol on the function of two proteins which regulate folate absorption, which are folypoly-γ-glutamyl carboxy peptidase and reduced folate carriers. Studies demonstrated increased urinary folate excretion in chronic alcoholic patients and alcohol-fed rats (Halsted *et al.*, 2002; Naughton *et al.*, 1989; Reisenauer *et al.*, 1989; Villanueva *et al.*, 2001). Another study also showed that a decreased hepatic uptake or retention of folic acid and reduced renal tubular reabsorption were observed in alcohol-fed monkeys (Tamura & Halsted, 1983).

1.4.6.3 Drugs

Drugs such as methotrexate and 5-fluorouracil which interfere with folate metabolism are key components of chemotherapy for both neoplastic and non-neoplastic diseases. Methotrexate has been used as a major chemotherapeutic agent

with antifolate activity to treat a large variety of both neoplastic and non-neoplastic diseases (van Ede *et al.,* 2002). Treatment of rapidly growing malignant cells with methotrexate traps folate as dihydrofolate. Since dihydrofolate is non-functional as a coenzyme, patients receiving methotrexate may show significant increase in plasma Hcy concentrations associated with the chronic use of methotrexate. Another major chemotherapeutic antifolate drug, 5-fluorouracil, inhibits the activity of thymidylate synthase. It functions as a covalent inhibitor of thymidylate synthase, which blocks DNA synthesis and reduces cell proliferation (van Ede *et al.*, 2002).

1.4.7 The Role of Folate in Diseases

Folic acid supplementation is used to prevent fetal neural defects during pregnancy and folate deficiency can lead to megaloblastic anemia (1991; Butterworth, 1993; Kamei *et al.*, 1993). For folate attention was turned to a growing evidence base that linked folate to the reduction of developmental disorders and chronic degenerative diseases. Of the disorders related to development, the potential for folate to reduce the risk of both occurrence and recurrence of NTDs has been well established. NTDs, including spina bifida, have been linked to maternal folate supply for over 40 years. With respect to disease states, the one chronic disease for which a potential link exists with folate supply is cardiovascular disease. Cardiovascular disease due to atherosclerosis is the leading cause of death worldwide. Atherosclerosis is an inflammatory process characterized by thickening, hardening, and plaque formation in the arteries. Plaque rupture and the consequent thrombosis may lead to sudden blockage of the arteries causing stroke and heart attack. Various factors associated with the development of coronary artery disease have been identified. The traditional risk factors include hypercholesterolemia, hypertension, smoking, gender,

diabetes mellitus, and family history. Hyperhomocysteinemia has emerged as an independent risk factor for cardiovascular and cerebral vascular disease (Clarke *et al.*, 1991; Welch & Loscalzo, 1998; Refsum *et al.*, 1998; Duell & Malinow, 1997).

1.4.8 Mechanism of Action of Folate

1.4.8.1 Homocysteine (Hcy)-dependent Effect of Folate

Folic acid supplementation is regarded as a promising approach in reducing blood Hcy levels. The current treatment for patients with hyperhomocysteinemia is supplementation of folic acid alone or in combination with vitamin B_6 or vitamin B_{12} (Chambers *et al.*, 2000). Due to the ability of folic acid to lower plasma Hcy levels during hyperhomocysteinemia, its role in cardiovascular disease has been increasingly studied. Folate deficiency due to a genetic defect or malnutrition is associated with hyperhomocysteinemia.

The active metabolite of folic acid, 5-MTHF, facilitates remethylation of Hcy to methionine by donating a methyl group to Hcy. This reaction is catalyzed by methionine synthase with vitamin B_{12} as a cofactor. Studies in animal models and clinical trials have demonstrated that folic acid depletion can cause hyperhomocysteinemia. It has been postulated that the beneficial effect of folic acid on cardiovascular disease is mediated by its Hcy-lowering effect. Folic acid supplementation alone can reduce plasma Hcy levels, which is thought to be one of the key mechanisms underlying the beneficial effects of folic acid. The Hcy-lowering effect of folic acid is attributed to its ability to enhance the remethylation of Hcy in cells and therefore reduce the intracellular level of Hcy. This, in turn, lowers the efflux of Hcy into the blood (Marinou *et al.*, 2005).

1.4.8.2 Homocysteine (Hcy)-independent Effect of Folate

More and more studies have demonstrated that the benefits of folic acid supplementation are independent of Hcy-lowering effect. An intake of 5 mg folic acid per day for 6 weeks was shown to improve flow-mediated dilatation of the brachial artery and such an improvement was not correlated with a reduction of plasma Hcy levels (Verhaar *et al.*, 1999). Folic acid may improve endothelial function through an endothelial nitric oxide pathway (Antoniades *et al.*, 2006). A recent study analyzing the data from 16,958 participants in 12 randomized controlled trials also showed no reduction of cardiovascular risk by folic acid administration in patients with a history of end stage renal disease or vascular disease including stroke, myocardial infarction, and coronary heart disease (Bazzano *et al.*, 2006). The severity of the pre-existing cardiovascular diseases or renal diseases in these patients might have contributed to the lack of risk reduction observed by folic acid supplementation. The controversy may be clarified by individual studies with large sample sizes, longer monitoring, and earlier folic acid intervention outcomes. Supplementation of folic acid was shown to prevent dietary-induced hyperhomocysteinemia in rats. Consequently, Hcy-stimulated chemokine expression in the aortic endothelium could be abolished (Wang *et al.*, 2002).

Even though lowering Hcy levels may contribute to improved vascular function, more evidence has suggested that some benefits of folic acid supplementation are independent of its Hcy-lowering effect. One of the mechanisms underlying the acute beneficial effect of folic acid on endothelial function may be mediated by a reduction of intracellular superoxide anion production (Doshi *et al.*, 2001). Many risk factors causing atherosclerosis share a common feature of generating intracellular oxidative stress (Dayal *et al.*, 2002; Upchurch *et al.*, 1997;

Kanani *et al.*, 1999). Superoxide anion is one of the potent ROS and its overproduction can lead to cell injury. NADPH-dependent oxidase is the major source of superoxide anions produced in macrophages (Van Heerebeek *et al.*, 2002). NADPH oxidase activity is significantly elevated in atherosclerotic lesions leading to increased superoxide anion production (Kalinina *et al.*, 2002; Sorescu *et al.*, 2002; Robinson *et al.*, 2004). Increased superoxide anion generation via NADPH oxidase not only impairs endothelial function but also stimulates the expression of inflammatory factors in vascular cells (Au-Yeung *et al.*, 2004; Edirimanne *et al.*, 2007). Increased superoxide anion production was shown to be responsible for Hcy-induced NF-κB activation and subsequently MCP-1 expression in vascular smooth muscle cells and monocyte-derived macrophages (Au-Yeung *et al.*, 2004; Au-Yeung *et al.*, 2006). Our study demonstrated that supplementation of folic acid to rats fed a high-methionine diet significantly reduced plasma Hcy levels as well as abolished MCP-1 expression and adhesion molecules in the aortic endothelium (Wang *et al.*, 2002). Further investigation demonstrated that folic acid treatment directly antagonized Hcy-induced NADPH oxidase activation by inhibiting the phosphorylation of the $p47^{phox}$ and $p67^{phox}$ NADPH oxidase subunits (Au-Yeung *et al.*, 2004; Au-Yeung *et al.*, 2006).

Another proposed mechanism of the beneficial effect of folic acid supplementation may derive from its effect in restoration of eNOS-mediated nitric oxide production (Verhaar *et al.*, 1998; Wilmink *et al.*, 2000). It has been known that eNOS-mediated nitric oxide production may play an important role in regulating vessel tone (Verhaar *et al.*, 1998; Wilmink *et al.*, 2000). Under physiological conditions, eNOS catalyzes the formation of nitric oxide by incorporating molecular oxygen into the substrate L-arginine, a reaction that requires NADPH, the allosteric

activator calmodulin, and several co-factors such as tetrahydrobiopterin (BH_4) (Beckman & Koppenol, 1996). On the other hand, under pathophysiological conditions, eNOS can switch from mainly nitric oxide synthesis to superoxide anion production, namely NOS uncoupling (Stroes *et al.*, 1998). Due to the uncoupling of NOS, reduced nitric oxide synthesis and increased ROS production contribute to cardiovascular diseases (Ohara *et al.*, 1993; Ohara *et al.*, 1995; Seiler *et al.*, 1993; Egashira *et al.*, 1995; Harrison & Ohara, 1995; Williams *et al.*, 1996). Under oxidative stress BH_4 is oxidized to its inactive form, BH_2, leading to eNOS uncoupling with decreased nitric oxide synthesis and increased superoxide anion production (Stroes *et al.*, 1998; Pritchard *et al.*, 1995). An active biological metabolite of folic acid, 5-MTHF, can restore BH_4 bioavailability by enhancing the binding affinity of BH_4 to eNOS and the regeneration of BH_4 from BH_2 or by direct interaction with eNOS (Verhaar *et al.*, 1998; Wilmink *et al.*, 2000). The protective effect of folic acid treatment against myocardial dysfunction during ischemia and post reperfusion injury in animal models has been demonstrated (Moens *et al.*, 2008). It has been suggested that the beneficial effects of folic acid might be through preservation of high-energy phosphate levels in the heart by increasing purine synthesis, reduced superoxide anion production, and increased nitric oxide synthesis (Moens *et al.*, 2008).

II. Hypothesis and Objectives

In many studies, hyperhomocysteinemia has been induced in genetically modified animal models such as heterozygous or homozygous CBS deficient mice. CBS is a critical enzyme in the transsulfuration pathway. Elimination of CBS activity may result in decreased production of biologically important downstream products such as cysteine and glutathione. With this model, it is difficult to evaluate the specific effects elicited by hyperhomocysteinemia alone. Therefore, in the present study, a diet-induced hyperhomocysteinemia model was developed to examine the resulting physiological abnormalities and the underlying mechanisms in the kidney. Along with the results of other studies, the present study may provide a more comprehensive mechanism of Hcy-induced renal disorders. Our laboratory employs a well-established diet-induced hyperhomocysteinemia model which yields high blood Hcy levels. Hyperhomocysteinemia is a common clinical finding in patients with chronic kidney disease and occurs almost uniformly in patients with end-stage renal disease. Although impaired kidney function is one of the common factors causing hyperhomocysteinemia, the adverse effects of Hcy on kidney function has not been well documented. Therefore, we hypothesized that (1) diet-induced hyperhomocysteinemia would result in renal injury via oxidative stress; (2) hyperhomocysteinemia-induced oxidative stress would stimulate MCP-1 expression via transcriptional regulation; and (3) folic acid supplementation could offer a renal protective effect during hyperhomocysteinemia.

The general objective of the present study was to investigate the biochemical and molecular mechanisms of Hcy-induced renal injury.

The specific aims were:

(1) To investigate the role of oxidative stress in Hcy-induced kidney injury;

(2) To investigate Hcy-induced oxidative stress on MCP-1 expression via transcriptional regulation in the kidney; and

(3) To investigate whether folic acid supplementation was effective in protecting the kidney during hyperhomocysteinemia.

III. Study 1: Regulation of Oxidative Stress in the Kidney

(Manuscript 1: Folic acid supplementation inhibits NADPH oxidase-mediated superoxide anion production in the kidney)

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3.1 Abstract

Hyperhomocysteinemia, a condition of elevated blood homocysteine (Hcy) levels, is a metabolic disease. It is a common clinical finding in patients with chronic kidney diseases and occurs almost uniformly in patients with end-stage renal disease. Hyperhomocysteinemia is also a risk factor for cardiovascular disease. Our recent studies indicate that hyperhomocysteinemia can lead to renal injury by inducing oxidative stress. Oxidative stress is one of the important mechanisms contributing to Hcy-induced tissue injury. Folic acid supplementation is regarded as a promising approach for prevention and treatment of cardiovascular disease associated with hyperhomocysteinemia due to its Hcy lowering effect. However, its effect on the kidney is not clear. The aim of this study was to examine the effect of folic acid supplementation on Hcy-induced superoxide anion production via NADPH oxidase in the kidney during hyperhomocysteinemia. Hyperhomocysteinemia was induced in male Sprague-Dawley rats fed a high-methionine diet for 12 wk with or without folic acid supplementation. A group of rats fed a regular diet was used as control. There was a significant increase in levels of superoxide anions and lipid peroxides in kidneys isolated from hyperhomocysteinemic rats. Activation of NADPH oxidase was responsible for hyperhomocysteinemia-induced oxidative stress in the kidney. Folic acid supplementation effectively antagonized hyperhomocysteinemia-induced oxidative stress via its Hcy lowering and Hcy-independent effect. *In vitro* study also showed that 5-methyltetrahydrofolate, an active form of folate, effectively reduced Hcy-induced superoxide anion production via NADPH oxidase. Xanthine oxidase activity was increased and superoxide dismutase (SOD) activity was decreased in the kidney of hyperhomocysteinemic rats, which might also contribute to an elevation of superoxide anion levels in the kidney. Folic acid supplementation attenuated xanthine

oxidase activity and restored SOD activity in the kidney of hyperhomocysteinemic rats. These results suggest that folic acid supplementation may offer a renal protective effect against oxidative stress.

3.2 Introduction

Hyperhomocysteinemia is a metabolic disorder that is characterized by an elevation of homocysteine (Hcy) level in the circulation. It is a common clinical finding in patients with chronic kidney disease and occurs almost uniformly in patients with end-stage renal disease (ESRD). Although kidney dysfunction is a common factor causing hyperhomocysteinemia, the adverse effect of Hcy on kidney function is not well documented. Recent epidemiological investigations have identified a positive association between an elevation of Hcy level in the blood and the development of chronic kidney disease in the general population (Ninomiya *et al.*, 2004). Hcy is a sulfhydryl-containing amino acid formed during the metabolism of methionine to cysteine. Mechanisms of Hcy-induced cardiovascular diseases have been studied extensively in the recent decade. Hcy, at pathological concentrations, elicits inflammatory responses and impairs endothelial function via activation of transcription factors such as nuclear factor kappa-B (NF-κB), inducing oxidative stress and chemokine expression causing monocyte accumulation in the vascular endothelium. Recent studies indicate that Hcy, at pathological levels, can cause kidney injury (Ninomiya *et al.*, 2004; Hwang *et al.*, 2008; Zhang *et al.*, 2004; Li *et al.*, 2002; Kumagai *et al.*, 2002). In contrast to the cardiovascular system, the mechanisms by which hyperhomocysteinemia may exert adverse effects on the kidney are not well identified. Hyperhomocysteinemia is now thought to be an independent risk factor both in the progression of kidney disease and in the development of cardiovascular complications related to ESRD.

Many risk factors causing kidney injury and cardiovascular diseases share a common feature of generating intracellular reactive oxygen species (ROS) causing oxidative stress. Superoxide anion, a redox signaling molecule that plays a critical

role in physiologic and pathologic processes, is a highly reactive oxygen free radical that can interact with nitric oxide to form another highly reactive free radical named peroxynitrite that, in turn, causes extensive protein tyrosine nitration and mediates iron-catalyzed lipid peroxidation. Nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase is primarily responsible for intracellular superoxide anion generation. It is a major source of superoxide anion generation in the kidney (Bokoch & Knaus, 2003; Shiose *et al.*, 2001; Griendling *et al.*, 2000; Gill & Wilcox, 2006). NADPH oxidase comprises membrane-bound components, termed cytochrome b_{558} ($gp91^{phox}$ and $p22^{phox}$ subunits) and cytosolic components ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac 1/2 subunits). Under normal conditions, small amount of superoxide anions generated inside cells can be scavenged by the cellular antioxidant defense mechanism. However, when there is an overproduction and insufficient removal of superoxide anions, oxidative stress occurs. The $gp91^{phox}$ is a catalytic subunit of the enzyme. Several homologues of $gp91^{phox}$ have been identified and are termed NADPH oxidase (NOX) proteins. Among the NOX proteins, NOX4 was first characterized as a kidney NADPH oxidase and is also known as Renox which has 39% identity to NOX2 (analogue of $gp91^{phox}$). It has been shown that NOX4 is a major source of superoxide anions produced in the kidney and plays an important role in the development of kidney disease (Geiszt *et al.*, 2000; Shiose *et al.*, 2001). Our recent study has shown that hyperhomocysteinemia can increase inducible nitric oxide synthase (iNOS) expression in the kidney leading to an increased nitric oxide production in rat kidneys (Zhang *et al.*, 2004). As a consequence, nitrotyrosine / nitrated proteins are formed in the kidney tissue (Zhang *et al.*, 2004). It is plausible that the generation of superoxide anion might also be elevated in the kidney during hyperhomocysteinemia.

Folic acid is a synthetic form of the naturally occurring folate that is a water soluble B vitamin. The active metabolite of folic acid is 5-methyltetrahydrofolate (5-MTHF). Folate plays an important role in regulating Hcy metabolism. Hcy can be metabolized via two major pathways, namely, the remethylation pathway and the transsulfuration pathway. In the remethylation pathway, Hcy can be converted to methionine catalyzed by methionine synthase with folate as a co-substrate. In the transsulfuration pathway, Hcy is irreversibly converted to cystathionine by cystathionine-β- synthase. Factors that interrupt the steps in Hcy metabolic pathways can cause an increase in cellular Hcy levels and lead to its elevation in the blood (Refsum *et al.*, 1998; Kang *et al.*, 1992). Although folate reduces the concentration of plasma Hcy and has a protective potential against cardiovascular diseases, recent studies have suggested that folate may exert protective effects independent of Hcy lowering. The aim of the present study was to investigate the effect of folic acid on Hcy-induced superoxide anion production via NADPH oxidase activation and underlying mechanism of such an effect in the kidney of hyperhomocysteinemic rats and human kidney proximal tubular cells.

3.3 Materials and Methods

3.3.1 Animal model

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) aged 8 wk were divided into 6 groups and maintained for 12 wk on the following diets: (1) regular diet (Control), consisting of Lab Diet® Rat Diet 5012 (PMI® Nutrition International, St. Louis, MO); (2) high-methionine diet, consisting of regular diet plus 1.7% (wt/wt) methionine (Met); (3) high-methionine plus folic acid diet, consisting of regular diet plus 1.7% (wt/wt) methionine and 0.25% (wt/wt) folic acid (Met+Folic acid); (4) in one set of experiments, high-methionine-fed rats were injected with 4 mg/kg of apocynin, an inhibitor of NADPH oxidase (Met+Apocynin) (Calbiochem, San Diego, Calif.) intraperitoneally once a day for 7 days before euthanasia; (5) a regular diet plus 0.25% (wt/wt) folic acid (Control+Folic acid); and (6) apocynin (4 mg/kg) was given to rats fed a regular diet (Control+Apocynin) for 7 days intraperitoneally prior to euthanasia. After being on the experimental diets for 12 wk, rats were euthanized by injection of a high dose of sodium pentobarbital intraperitoneally. The portion of the rat kidney used for assays including PCR consisted of cortex and medullar. Results from our previous studies demonstrated that hyperhomocysteinemia could be induced in rats by feeding a high-methionine diet (Woo *et al.*, 2008; Hwang *et al.*, 2008; Wu *et al.*, 2009). Total Hcy (tHcy) concentrations in the serum were measured with the IMx Hcy assay, which was based on fluorescence polarization immunoassay technology (Abbott Diagnostics, Abbott Park, IL). All procedures were performed in accordance with the Guide for the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and approved by the University of Manitoba Protocol Management and Review Committee.

3.3.2 Cell culture

Human kidney cortex proximal tubular cells (HK-2, CRL-2190) were purchased from the American Type Culture Collection (ATCC). According to the Instruction by ATCC, cells were cultured in keratinocyte-serum free medium (GIBCO-BRL 17005-042) with 5 ng/mL recombinant epidermal growth factor and 0.05 mg/ml bovine pituitary extract.

3.3.3 Determination of lipid peroxidation

The degree of lipid peroxidation in the kidney tissue including both cortex and medulla was determined by measuring malondialdehyde (MDA) levels with thiobarbituric acid reactive substances (TBARS) (Bjorkegren *et al.*, 2002; Sung *et al.*, 2002). Briefly, a portion of the kidney was homogenized in 0.1 M KCI solution containing 3 mM ethylenediaminetetraacetic acid (EDTA) followed by centrifugation at 600 g for 15 min at 4^{o}C. An aliquot of supernatant was added to the reaction mixture containing 8% SDS, 20% acetic acid, 0.8% thiobarbituric acid and water. After incubation at 95 ^{o}C for 1h, the amount of MDA formed in the reaction mixture was measured by a spectrophotometer at absorbance of 532 nm. MDA was used as the standard, and results were expressed as a percentage of the control group. The amount of MDA correlates to the degree of lipid peroxidation produced in the tissues.

3.3.4 Determination of NADPH oxidase activity, xanthine oxidase activity and superoxide dismutase activity (SOD)

The NADPH oxidase activity was measured by the lucigenin chemiluminescence's assay (Kashiwagi *et al.*, 1999). A portion of the kidney (cortex and medulla) or tubular cells were homogenized in a 50 mM phosphate buffer (pH 7.0,

1:10, w/v) containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 3,000 g for 10 min, an aliquot of the supernatant was incubated with lucigenin (5 µM) in a phosphate buffer (50 mM, pH 7.0) for 2 min followed by adding the substrate, 100 µM NADPH (Kashiwagi *et al.*, 1999). In one set of experiments, cellular fraction free of mitochondria was prepared (Frezza *et al.*, 2007). In brief, a portion of the kidney tissue including both cortex and medulla was homogenized in a 50 mM phosphate buffer (pH 7.0, 1:10, w/v) containing 1 mM EDTA and 1 mM PMSF. After centrifugation of homogenate at 600 g for 10 min at 4^{o}C, the supernatant was collected and then centrifuged at 7, 000 g for 10 min at 4^{o}C. After centrifugation, the supernatant was collected and used as mitochondria-free fraction (Frezza *et al.*, 2007) for measuring NADPH oxidase activity. Chemiluminescent signal (photon emission) was measured every 15 sec for 3 min using a luminometer (Lumer LB9507, Berthold Technologies GmbH and Co. KG, Bad Wildbad, Germany). In principle, reaction of lucigenin with superoxide anion leads to the formation of lucigenin dioxetane that decomposes to produce two molecules of N-methylacridone (Li *et al.*, 1998). One of these two N-methylacridone molecules is in an electronically excited state and emits a photon. The photon emission that reflects the amount of superoxide anion in the sample can be detected using a luminometer (Li *et al.*, 1998). A standard curve was prepared with xanthine (100 µM) and known serial concentrations of xanthine oxidase (Sigma-Aldrich, St. Louis, MO, USA) to yield the known concentrations of superoxide anion. The NADPH oxidase activity was calculated based on the amount of superoxide anion produced in the reaction mixture. A similar experiment was performed using xanthine, another source of oxidants in the kidney, as a substrate in the lucigenin chemiluminescence assay to reveal that xanthine oxidase was also one of the sources

of oxidants in the kidney of hyperhomocysteinemic rats. SOD activity was determined as previously described (Crapo *et al.*, 1978). In brief, a portion of the kidney was homogenized in a 50 mM phosphate buffer (pH 7.8, 1:8, w/v) containing 0.1 mM EDTA. SOD activity was assayed by monitoring the inhibition rate of xanthine oxidase mediated reduction of cytochrome c in the kidney homogenates. Calibrations were performed using known amounts of SOD (Sigma Aldrich, St. Louis, MO).

3.3.5 Measurement of NADPH oxidase subunit mRNA expression

Total RNAs were isolated from rat kidney (cortex and medullar) or tubular cells with TriZol reagent (Invitrogen Life Technologies, Carlsbad, CA). NADPH oxidase subunits of mRNA were measured by real-time polymerase chain reaction (PCR) analysis using the iQ5 real time PCR detection system (Bio-Rad, Hercules, CA). In brief, 2 µg of total RNA were converted to cDNA by reverse transcriptase. The reaction mixture of real-time PCR contained 0.4 µM of 5' and 3' primer and 2 µL of cDNA product in iQ-SYBR green supermix reagent (Bio-Rad). The primers (Invitrogen) used for rat NOX4 were (forward) 5'-GGGCCTAGGATTGTGTTTGA-3' and (reverse) 5'-CTGAGAAGTTCAGGGCGTTC-3', rat $p22^{phox}$ (forward) 5'-TTGTTGCAGGAGTGCTCATC-3' and (reverse) 5'-CTGCCAGCAGGTAGATCACA-3'and those used for rat GAPDH were (forward) 5'-TCAAGAAGGTGGTGAAGCAG-3' and (reverse) 5'-AGGTGGA AGAATGGGAGTTG-3'. The primers for human NOX4 were (forward) 5'-CTTCCGTT GGTTTGCAGATT-3' and (reverse) 5'-TGGGTCCACAACAGAAAACA-3', human $p22^{phox}$ (forward) 5'-GTCCCTGCATTCTGTGC TTT-3' and (reverse) 5'-GAACACCTCTGCACCCTGAT -3' and those for human GAPDH were (forward)

5’-ATCATCCCTGCCTCTACTGC-3’ and (reverse) 5’-GTCAGGTCCACCACTGACAC-3’. All primers were synthesized by Invitrogen. The relative changes in mRNA expression were determined by the fold change analysis in which the degree of change = $2^{-\Delta\Delta Ct}$, where C_t = ($C_{t\ target\ gene}$ – C_{tGAPDH}) treatment – ($C_{t\ target\ gene}$ – C_{tGAPDH}) control (Livak & Schmittgen, 2001). C_t was the cycle number at which the fluorescence signal crossed the threshold, which was determined by iQ5 Optical System software (version 2: Bio-Rad).

3.3.6 Determination of intracellular superoxide anion levels

The intracellular superoxide anion level was measured by the nitroblue tetrazolium (NBT) reduction assay (Woo *et al.*, 2003; Au-Yeung *et al.*, 2004). In brief, cells were incubated in Krebs-Henseleit buffer in the presence of 1.0 mg/mL NBT for 60 min. The formazan generated by the reduction of NBT in the presence of superoxide anion was proportional to the amount of superoxide anion generated intracellularly. At the end of the incubation period, the culture medium was removed and the cells were washed with pre-warmed (37^{o} C) Hank’s balanced salt solution. Cells were lysed with a phosphate buffer (80 mmol/L, pH 7.8) containing 5% sodium dodecyl sulphate and 0.45% gelatin. The cell lysate was centrifuged for 5 min at 13,000 g. The supernatant was used for the measurement of the absorbance at 540 nm (formazan) and 450 nm. The calculation of relative concentration of superoxide anion was based on the amount of formazan formed.

3.3.7 Transfection of cells with NOX4 and $p22^{phox}$ siRNAs

Cells were transfected with NOX4 and $p22^{phox}$ siRNA duplex oligoribonucleotides (Stealth RNAi^{TM}, Invitrogen). Tubular cells were seeded in 6-well plates and transfected with NOX4 and $p22^{phox}$ siRNAs according to the manufacturer's instruction. For a negative control, cells were transfected with Stealth RNAi^{TM} negative control (Invitrogen) consisting of a scrambled sequence that was unable to inhibit gene expression. At 48 h after transfection, cells were incubated with or without Hcy for 12 h, NOX4 and $p22^{phox}$ mRNA, the intracellular superoxide anion level was measured.

3.3.8 Histological staining

A portion of the kidney (cortex and medullar) was immersion-fixed in 10% neutral-buffered formalin overnight followed by embedding in paraffin. Sequential 5μm paraffin–embedded cross sections were prepared. Kidney sections were placed in xylene to remove the paraffin and stained with hematoxylin and eosin staining to examine histological changes in the kidney (Wu *et al.*, 2009). Images were acquired using a Zeiss Axioskop2 microscope equipped with an Axiovision digital camera. Using a camera mounted on microscope, slides were analyzed at 200X magnification. Mean glomerular volume, by measuring the maximum glomerular diameter of 20 randomly chosen glomeruli (n=4/group) per kidney, was determined. The radius was used to estimate glomerular volume using the following formula: mean glomerular volume=$\beta/K(\pi r^2)^{3/2}$. The value of the coefficients β and K is based on assumptions made for the maximum diameter of spheres (β=1.38) and the distribution bias of section location (K=1.10) (Hirose *et al.*, 1982).

3.3.9 Statistical analysis

The results were analyzed using one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. Data were presented as the means ± SEM. The level of statistical significance was set at $P < 0.05$.

3.4 Results

3.4.1 Effect of folic acid on lipid peroxidation in the kidney during hyperhomocysteinemia

Rats that were fed a high-methionine diet for 12 wk developed hyperhomocysteinemia. Those rats had a much higher level of serum tHcy than the control group (Fig. 3.1). Supplementation of folic acid to rats fed a high-methionine diet significantly lowered the serum tHcy levels (Fig. 3.1). These results suggested that feeding a high-methionine diet for 12 wk was able to induce hyperhomocysteinemia in rats. Next, the degree of lipid peroxidation in the kidney was examined by measuring tissue malondialdehyde (MDA) level, an indicator of lipid peroxidation. The MDA level was significantly elevated in kidneys of hyperhomocysteinemic rats, indicating an increased lipid peroxidation in the kidney (Fig. 3.2). Folic acid supplementation effectively reduced the MDA level in the kidney (Fig. 3.2). Administration of apocynin, an inhibitor for NADPH oxidase, to hyperhomocysteinemic rats also reduced the MDA level in kidneys of hyperhomocysteinemic rats (Fig. 3.2). Folic acid supplementation or apocynin injection to rats fed a regular diet did not affect the serum Hcy level (Fig. 3.1) and the kidney MDA level (Fig. 3.2). These results suggested that NADPH oxidase might be involved in increased free radical generation in the kidney leading to lipid peroxidation in hyperhomocysteinemic rats.

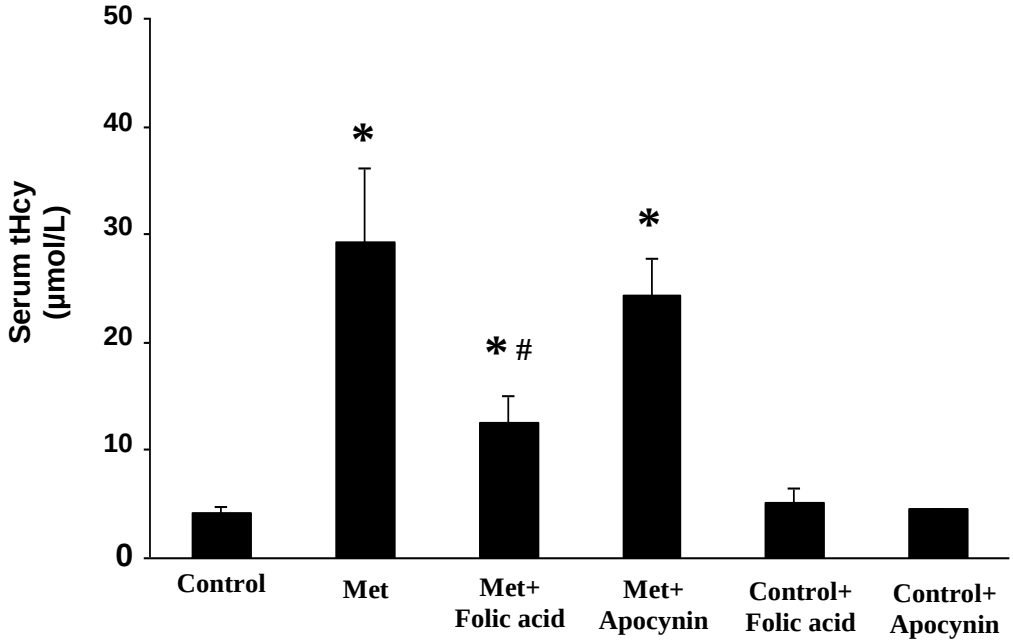

Figure 3.1 Measurement of serum total homocysteine (tHcy) levels

Rats were fed with the following diets for 12 wk: a regular diet (Control), a high methionine diet (1.7% Met), a high methionine diet plus folic acid (0.25%) (Met + Folic acid), or a regular diet (Control) plus folic acid (0.25%) (Control+Folic acid).
In one set of experiments, apocynin (4 mg/kg, daily, ip), a known inhibitor of NADPH oxidase, was given to rats fed a high-methionine diet (Met+Apocynin) or fed a regular diet (Control+Apocynin) for 7 days prior to euthanasia. Hcy levels in the serum were measured. Results were expressed as mean ± SEM (n=6). $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the high-methionine fed group.

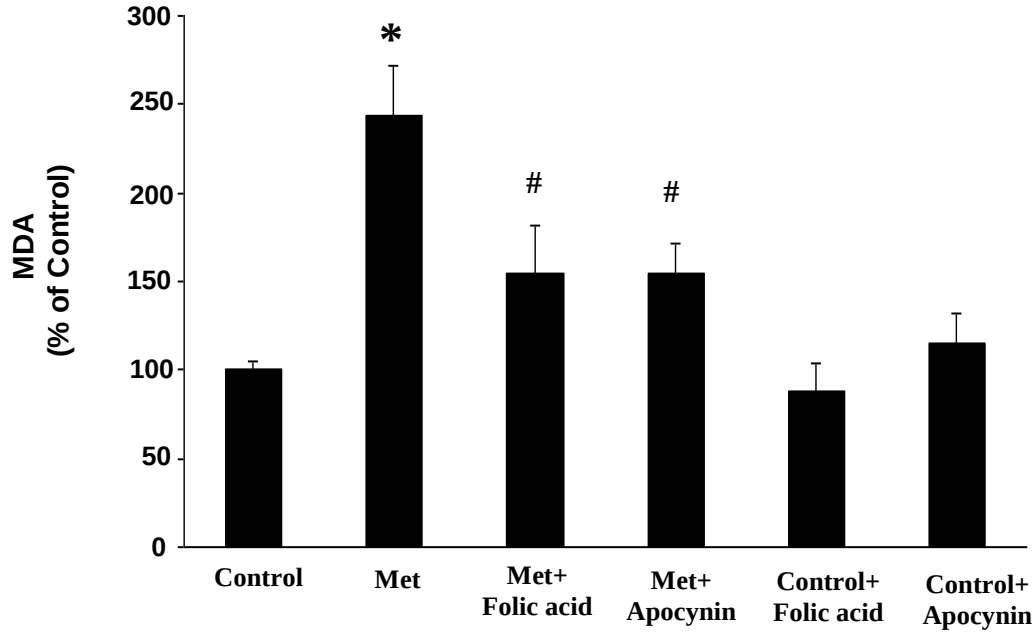

Figure 3.2 Measurement of lipid peroxidation in rat kidneys

Rats were fed with the following diets for 12 wk: a regular diet (Control), a high methionine diet (1.7% Met), a high methionine diet plus folic acid (0.25%) (Met + Folic acid), or a regular diet (Control) plus folic acid (0.25%) (Control+Folic acid). In one set of experiments, apocynin (4 mg/kg, daily, ip), a known inhibitor of NADPH oxidase, was given to rats fed a high-methionine diet (Met+Apocynin) or fed a regular diet (Control+Apocynin) for 7 days prior to euthanasia. Lipid peroxides in the kidney were determined by measuring the amount of MDA in the kidney tissue. Results were expressed as mean ± SEM (n=6). $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the high-methionine fed group.

3.4.2 Effect of folic acid on NADPH oxidase activation in the kidney during hyperhomocysteinemia

The activity of NADPH oxidase was significantly elevated in kidneys of hyperhomocysteinemic rats (Fig. 3.3). Folic acid supplementation reduced NADPH oxidase activity in kidneys of hyperhomocysteinemic rats (Fig. 3.3). Treatment of hyperhomocysteinemic rats with apocynin, NADPH oxidase inhibitor, abolished NADPH oxidase activation in the kidney (Fig. 3.3). Further analysis revealed that the mRNA levels of NADPH oxidase subunits, NOX4 and $p22^{phox}$, were significantly elevated in the same kidney tissue (Fig. 3.4 and 3.5). These results suggested that the increased expression of NOX4 and $p22^{phox}$ might contribute to Hcy-induced activation of NADPH oxidase leading to increased free radical generation in the kidney of hyperhomocysteinemic rats. Folic acid supplementation effectively reduced the mRNA level of NOX4 and $p22^{phox}$ in the kidney of hyperhomocysteinemic rats (Fig. 3.4 and 3.5). Such an inhibitory effect of folic acid might contribute to a reduction of NADPH oxidase activity in those rats (Fig. 3.3). Folic acid supplementation or apocynin injection to rats fed a regular diet did not affect the basal levels of NADPH oxidase activity, NOX4 and $p22^{phox}$ mRNA expression (Fig. 3.3-3.5). Another superoxide generating enzyme xanthine oxidase was also significantly increased in kidneys of hyperhomocysteinemic rats while folic acid supplementation had an inhibitory effect (Fig. 3.6). In addition, an antioxidant enzyme SOD activity was significantly decreased in the kidney tissue of hyperhomocysteinemic rats and folic acid supplementation partially restored SOD activity (Fig. 3.7). Mitochondria could be another source of superoxide overproduction in the kidney of hyperhomocysteinemic rats leading to oxidative stress. In one set of experiments, NADPH oxidase activity was measured in the cellular fraction that was free of

mitochondria. The activity of NADPH oxidase was significantly elevated in the mitochondria-free fraction prepared from kidneys of hyperhomocysteinemic rats (Fig. 3.8). Folic acid supplementation reduced NADPH oxidase activity in this fraction (Fig. 3.8).

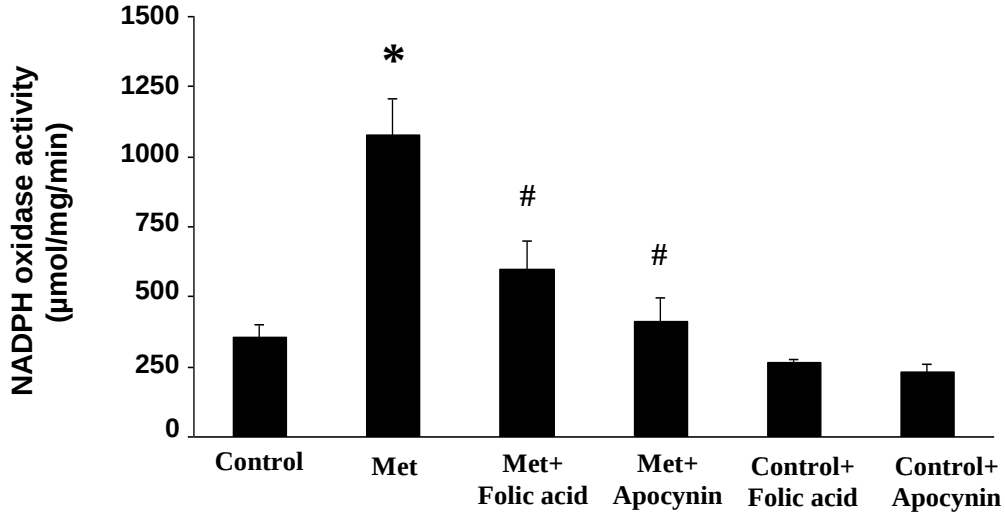

Figure 3.3 NADPH oxidase activity in rat kidneys

Rats were fed with the following diets for 12 wk: a regular diet (Control), a high methionine diet (1.7% Met), a high methionine diet plus folic acid (0.25%) (Met + Folic acid), or a regular diet (Control) plus folic acid (0.25%) (Control+Folic acid). In one set of experiments, apocynin (4 mg/kg, daily, ip), a known inhibitor of NADPH oxidase, was given to rats fed a high-methionine diet (Met+Apocynin) or fed a regular diet (Control+Apocynin) for 7 days prior to euthanasia. NADPH oxidase activity expressed as the amount of superoxide anion produced per mg of protein per min was measured by lucigenin chemiluminescence's assay. Results were expressed as mean ± SEM (n=6). $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the high-methionine fed group.

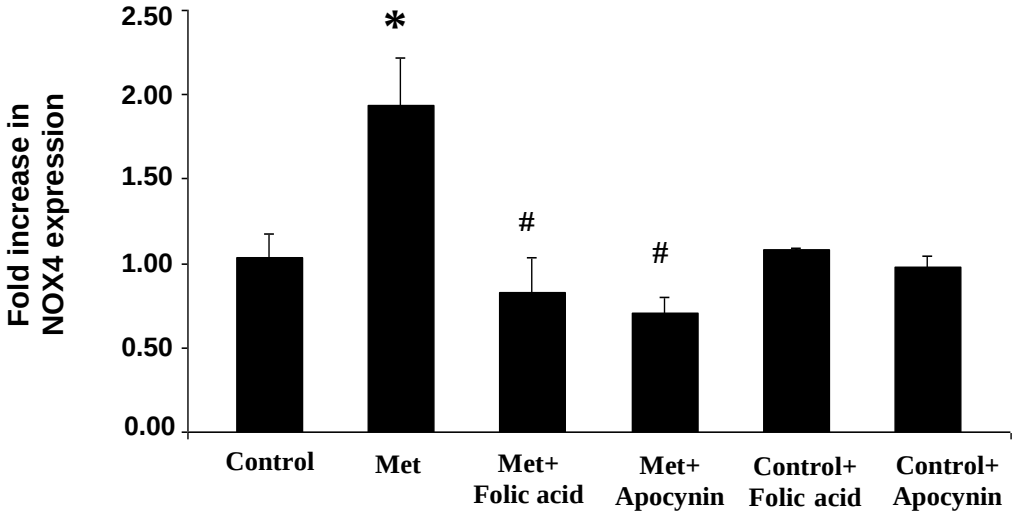

Figure 3.4 NOX4 mRNA level in rat kidneys

Rats were fed with the following diets for 12 wk: a regular diet (Control), a high methionine diet (1.7% Met), a high methionine diet plus folic acid (0.25%) (Met + Folic acid), or a regular diet (Control) plus folic acid (0.25%) (Control+Folic acid). In one set of experiments, apocynin (4 mg/kg, daily, ip), a known inhibitor of NADPH oxidase, was given to rats fed a high-methionine diet (Met+Apocynin) or fed a regular diet (Control+Apocynin) for 7 days prior to euthanasia. NOX4 mRNA level was measured using a real-time PCR detection system. Results were expressed as mean ± SEM (n=6). $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the high-methionine fed group.

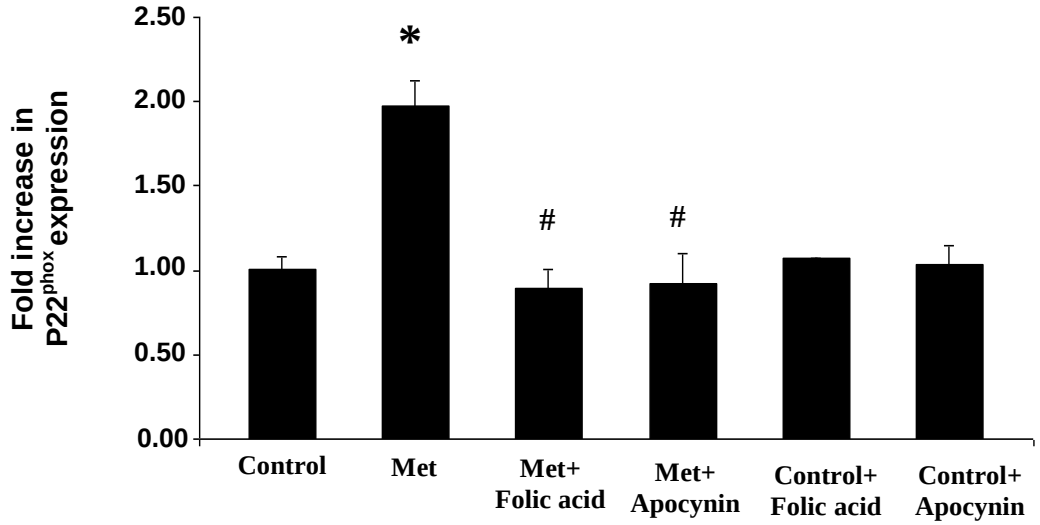

Figure 3.5 $P22^{phox}$ mRNA level in rat kidneys

Rats were fed with the following diets for 12 wk: a regular diet (Control), a high methionine diet (1.7% Met), a high methionine diet plus folic acid (0.25%) (Met + Folic acid), or a regular diet (Control) plus folic acid (0.25%) (Control+Folic acid). In one set of experiments, apocynin (4 mg/kg, daily, ip), a known inhibitor of NADPH oxidase, was given to rats fed a high-methionine diet (Met+Apocynin) or fed a regular diet (Control+Apocynin) for 7 days prior to euthanasia. The mRNA level of $p22^{phox}$ was measured using a real-time PCR detection system. Results were expressed as mean ± SEM (n=6). $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the high-methionine fed group.

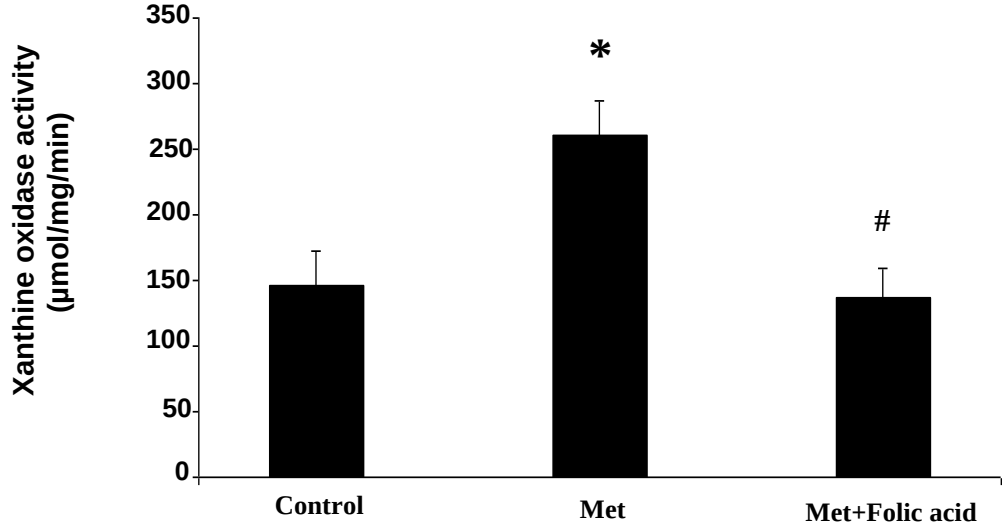

Figure 3.6 Xanthine oxidase activity in rat kidneys.

Rats were fed with the following diets for 12 wk: a regular diet (Control), a high methionine diet (1.7% Met), or a high methionine diet (1.7% Met) plus folic acid (0.25%) (Met+Folic acid). Xanthine oxidase activity was measured in the kidney tissue. Results were expressed as mean ± SEM (n=6). *P < 0.05 compared with control values. $^{\#}P$ < 0.05 compared with values of the high-methionine fed group.

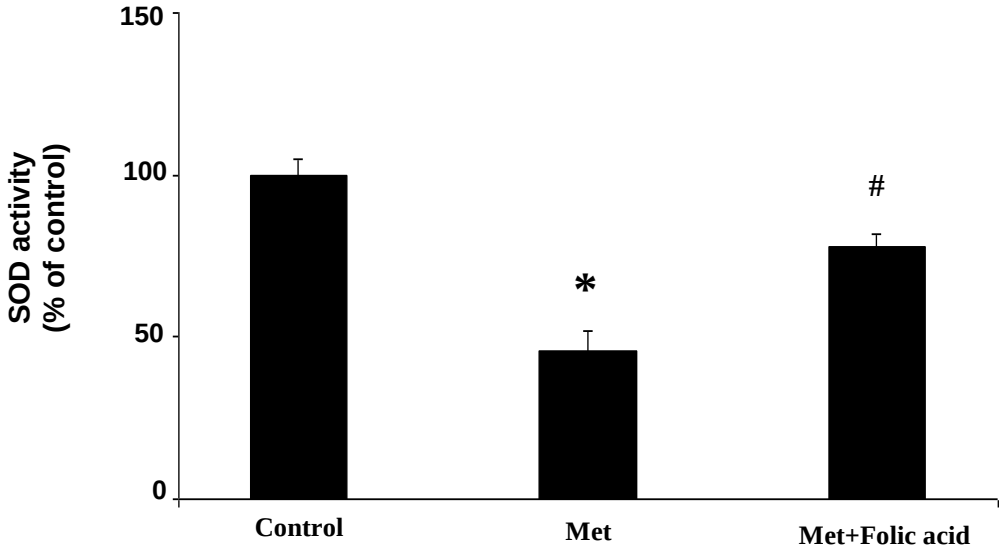

Figure 3.7 Superoxide dismutase activity (SOD) in rat kidneys

Rats were fed with the following diets for 12 wk: a regular diet (Control), a high methionine diet (1.7% Met), or a high methionine diet (1.7% Met) plus folic acid (0.25%) (Met+Folic acid). SOD activity was measured in the kidney tissue. Results were expressed as mean ± SEM (n=6). $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the high-methionine fed group.

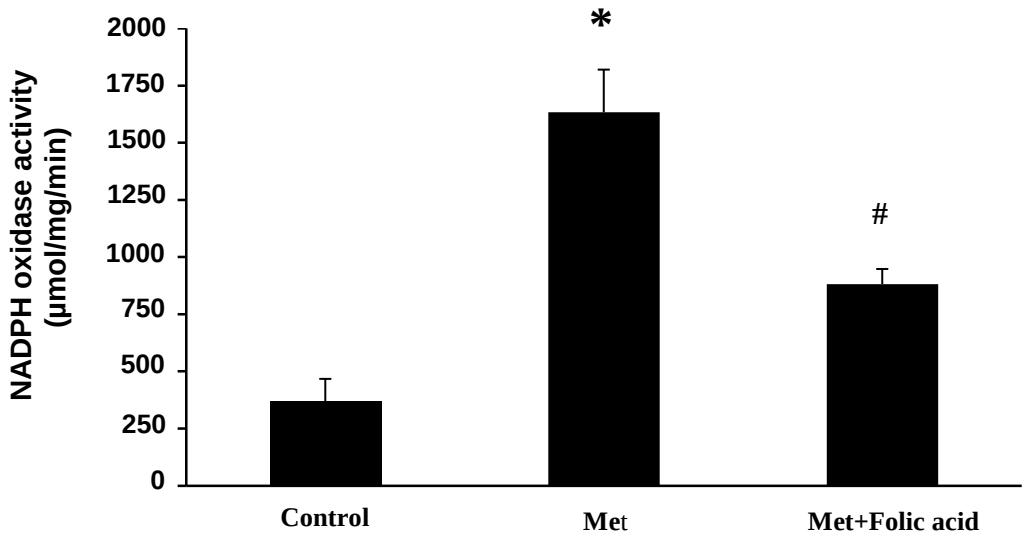

Figure 3.8 NADPH oxidase activity in rat kidneys

Rats were fed with the following diets for 12 wk: a regular diet (Control), a high methionine diet (1.7% Met), or a high methionine diet (1.7% Met) plus folic acid (0.25%) (Met+Folic acid). NADPH oxidase activity was measured in mitochondria-free fraction prepared from the kidney. Results were expressed as mean ± SE (n=6). $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the high-methionine fed group.

3.4.3 Effect of Hcy on NADPH oxidase-mediated superoxide anion generation in tubular cells

The effect of Hcy on superoxide anion generation was further examined in human kidney proximal tubular cells. Cells were incubated with Hcy (25 – 250μM) for 12 h. The intracellular level of superoxide anion was significantly increased in cells treated with Hcy at 50 – 250μM (Fig. 3.9). In the rest of cell culture experiments, Hcy at 100μM was added to the culture medium. Pretreatment of cells with NADPH oxidase inhibitor (apocynin) for 15 min followed by incubation with Hcy reduced the intracellular level of superoxide anion (Fig. 3.10). Apocynin treatment did not affect the basal level of intracellular superoxide anion (Fig. 3.10). Next, to examine whether other superoxide generating sources were also activated upon Hcy treatment, several inhibitors of superoxide producing enzymes were added to the culture medium. Pretreatment of cells with oxypurinol (an inhibitor for xanthine oxidase) also abolished Hcy-induced elevation of intracellular superoxide levels (Fig. 3.11) indicating that the xanthine oxidase might be involved in Hcy-induced superoxide production in tubular cells. On the other hand, pretreatment of cells with rotenone (an inhibitor for mitochondrial respiratory chain complex I) or cimetidine (an inhibitor for cytochrome P450) did not affect intracellular superoxide anion levels upon Hcy treatment (Fig. 3.11). The NADPH oxidase activity was significantly increased in cells incubated with Hcy (Fig. 3.12). These results suggested that activation of NADPH oxidase was responsible for Hcy-induced superoxide anion generation in tubular cells. A discrepancy between changes in the NADPH oxidase activity (2-fold increase in Hcy-treated cells) and superoxide levels (approximately 40% increase in Hcy-treated cells) might be due to a rapid metabolism of superoxide anion in cultured cells or assay sensitivity for measuring the enzyme activity and superoxide anion.

To investigate the mechanism of NADPH oxidase activation in tubular cells, the mRNA levels of NADPH oxidase subunits were measured. The expression of NOX4 and $p22^{phox}$ was significantly elevated in tubular cells incubated with Hcy (Fig. 3.13 and 3.14). To further examine the role of NOX4 and $p22^{phox}$ in Hcy-induced NADPH oxidase activation, tubular cells were transfected with NOX4 and $p22^{phox}$ siRNA, respectively. Transfection of cells with NOX4 siRNA or $p22^{phox}$ siRNA not only inhibited NOX4 (Fig. 3.15) and $p22^{phox}$ (Fig. 3.16) expression but also abolished Hcy-induced elevation of superoxide anion level in tubular cells (Fig. 3.17). In accordance with the intracellular superoxide anion levels, transfection of cells with NOX4 siRNA or $p22^{phox}$ siRNA abolished the stimulatory effect of Hcy on NADPH oxidase activity while transfection of cells scramble siRNA did not affect the NADPH oxidase activity. These results suggested that Hcy-induced activation of NADPH oxidase was mainly mediated via increased NOX4 and $p22^{phox}$ expression in tubular cells.

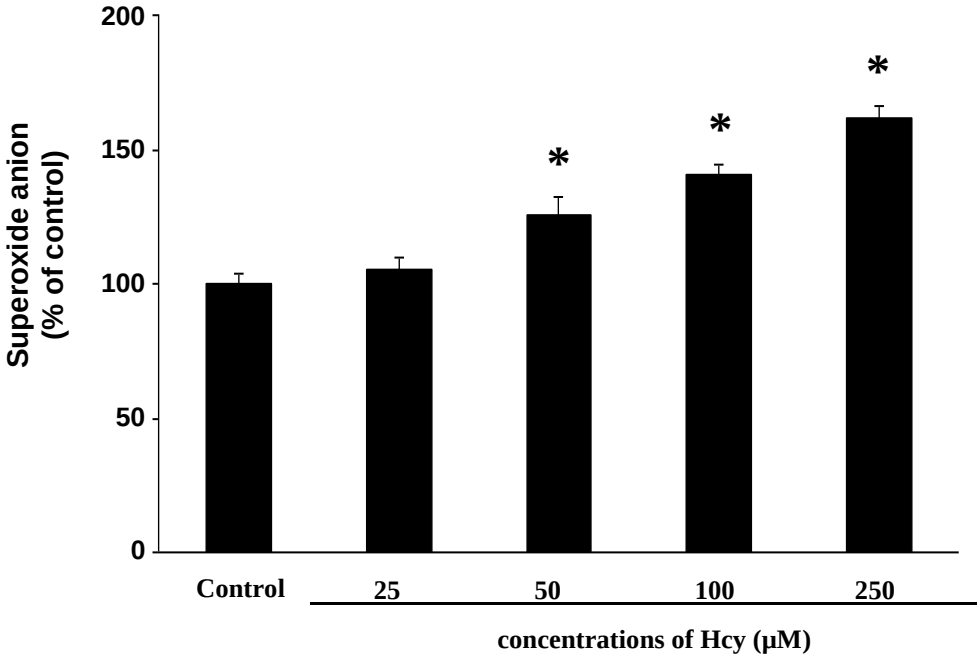

Figure 3.9 Measurement of intracellular superoxide anion in tubular cells (I)

Human kidney proximal tubular cells were incubated in the absence (Control) or presence of Hcy (25- 250 μM) for 12 h. Intracellular superoxide anion levels were measured. Results were expressed as mean ± SEM (n=6-8). **P* < 0.05 compared with control values.

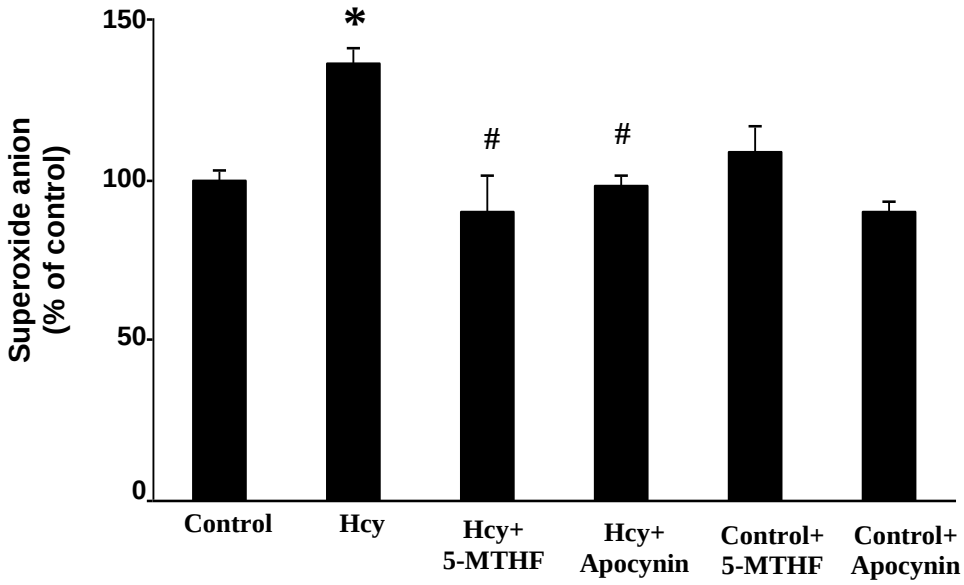

Figure 3.10 Measurement of intracellular superoxide anion in tubular cells (II)

Human kidney proximal tubular cells were incubated in the absence (Control) or presence of Hcy (100 µM) for 12 h. Cells were pre-incubated with 5-methyltetrahydrofolate (5-MTHF, 500 ng) or apocynin (300 µM), a known inhibitor of NADPH oxidase, for 15 min followed by incubation with Hcy (100 µM) for 12 h. Intracellular superoxide anion levels were measured. Results were expressed as mean ± SEM (n=6-8). *$P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the Hcy treated group.

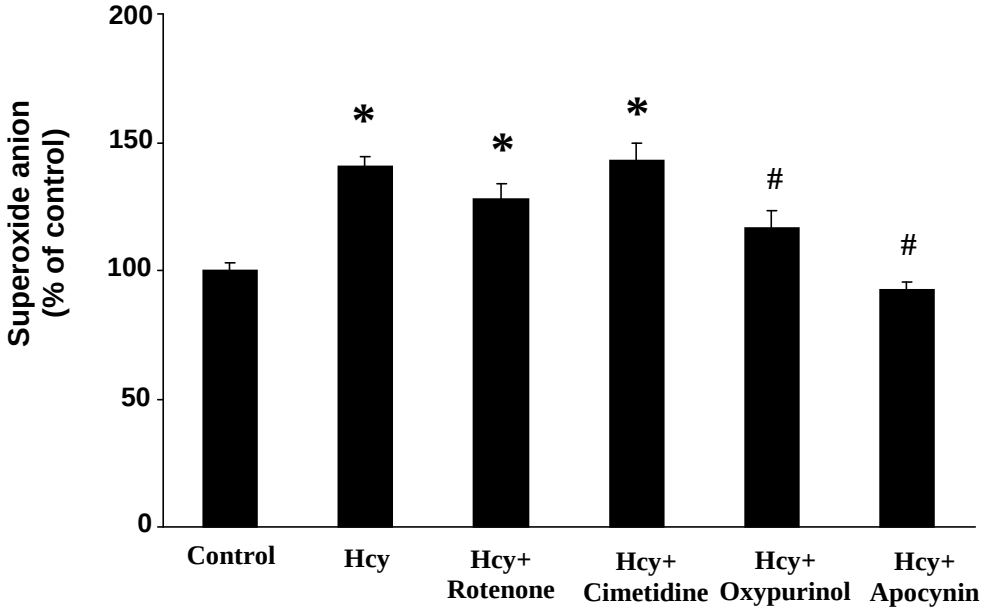

Figure 3.11 Measurement of intracellular superoxide anion in tubular cells (III)

Human kidney proximal tubular cells were incubated in the absence (Control) or presence of Hcy (100 μM) for 12 h. Cells were pre-incubated with rotenone (1μM), an inhibitor of mitochondrial respiratory chain complex I, cimetidine (100 μM), an inhibitor of cytochrome P-450, oxypurinol (10 μM), an inhibitor of xanthine oxidase, or apocynin (300 μM), an inhibitor of NADPH oxidase, for 15 min followed by incubation with Hcy (100 μM) for 12 h. Intracellular superoxide anion levels were measured. Results were expressed as mean ± SEM (n=6-8). *$P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the Hcy treated group.

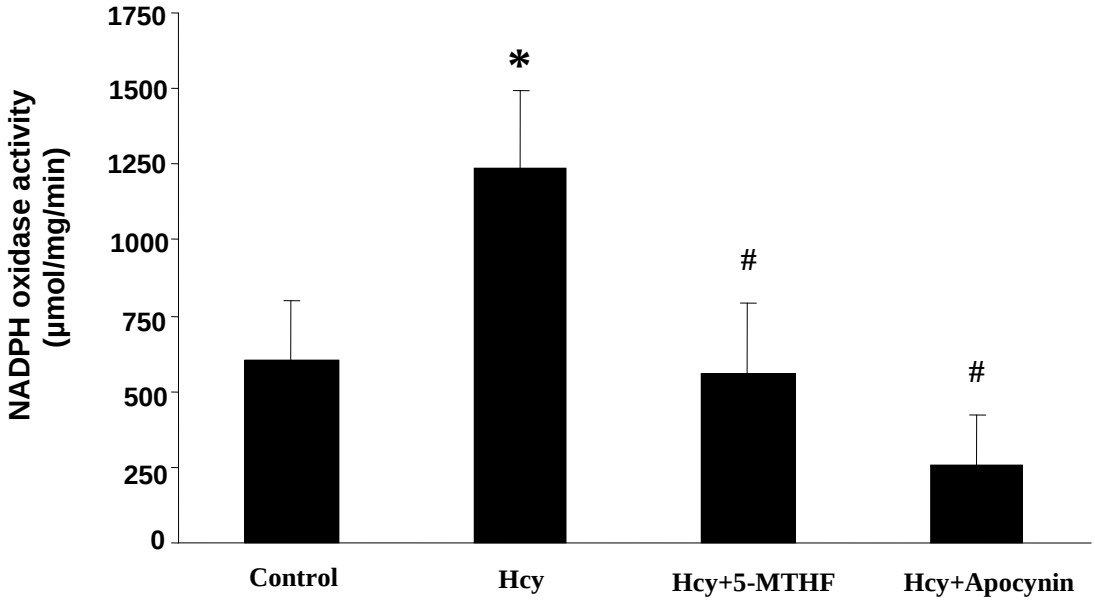

Figure 3.12 NADPH oxidase activity in tubular cells

Human kidney proximal tubular cells were incubated in the absence (Control) or presence of Hcy (100 µM) for 12 h. NADPH oxidase activity was measured by lucigenin chemiluminescence's assay. Results were expressed as mean ± SEM (n=6-8). $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the Hcy treated group.

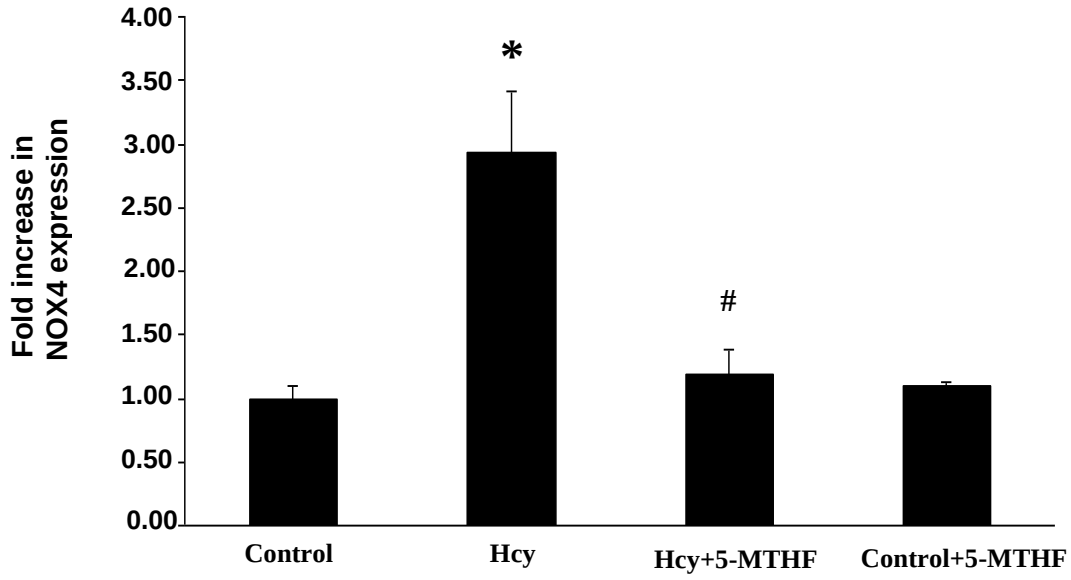

Figure 3.13 Measurement of NOX4 mRNA level in tubular cells

Human kidney proximal tubular cells were incubated in the absence (Control) or presence of Hcy (100 µM) for 12 h. In one set of experiments, cells were pre-incubated with 5-methyltetrahydrofolate (5-MTHF, 500 ng) for 15 min followed by incubation in the absence of Hcy (Control+5-MTHF) or in the presence of Hcy (100 µM) for 12 h. The mRNA level of NOX4 was measured. Results were expressed as mean ± SEM (n=4-6). $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the Hcy treated group.

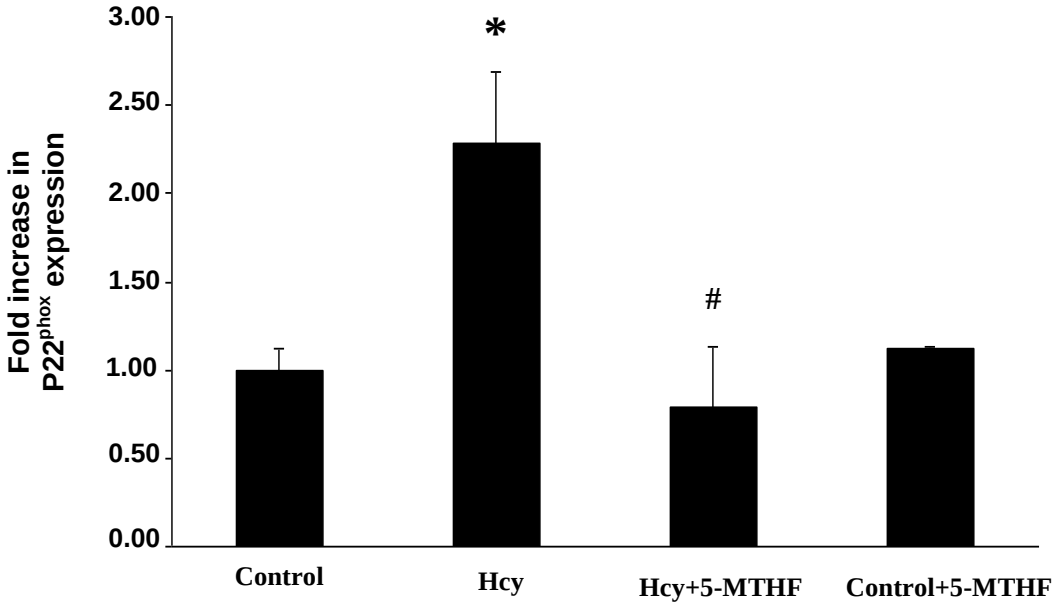

Figure 3.14 Measurement of p22^{phox} mRNA level in tubular cells

Human kidney proximal tubular cells were incubated in the absence (Control) or presence of Hcy (100 µM) for 12 h. In one set of experiments, cells were pre-incubated with 5-methyltetrahydrofolate (5-MTHF, 500 ng) for 15 min followed by incubation in the absence of Hcy (Control+5-MTHF) or in the presence of Hcy (100 µM) for 12 h. The mRNA level of p22^{phox} was measured. Results were expressed as mean ± SEM (n=4-6). $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the Hcy treated group.

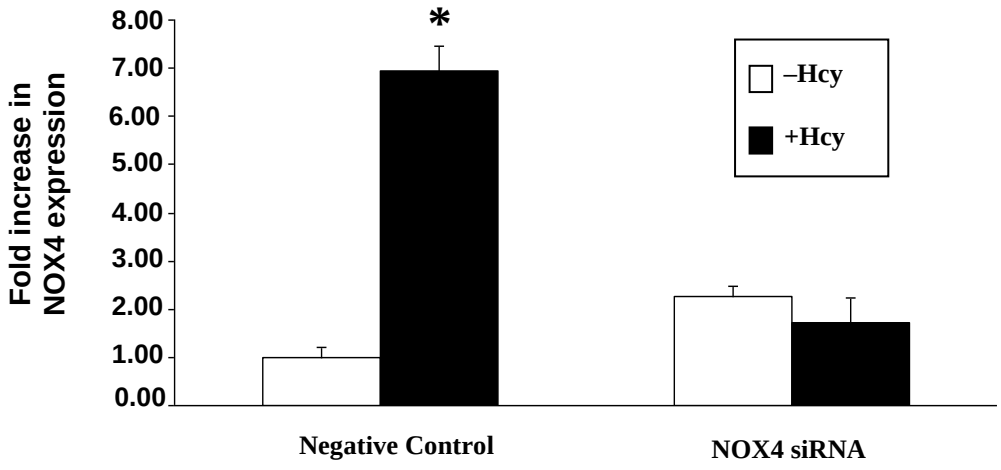

Figure 3.15 Determination of mRNA expression of NOX4 in tubular cells transfected with NOX4 siRNA

Human kidney proximal tubular cells were transfected with NOX4 siRNA or with a negative control siRNA. Transfected cells were incubated for 12 h in the absence (Control) or presence of Hcy (100 uM). The mRNA level of NOX4 was measured. Results were expressed as mean ± SEM (n=4-6). **P* < 0.05 compared with control values.

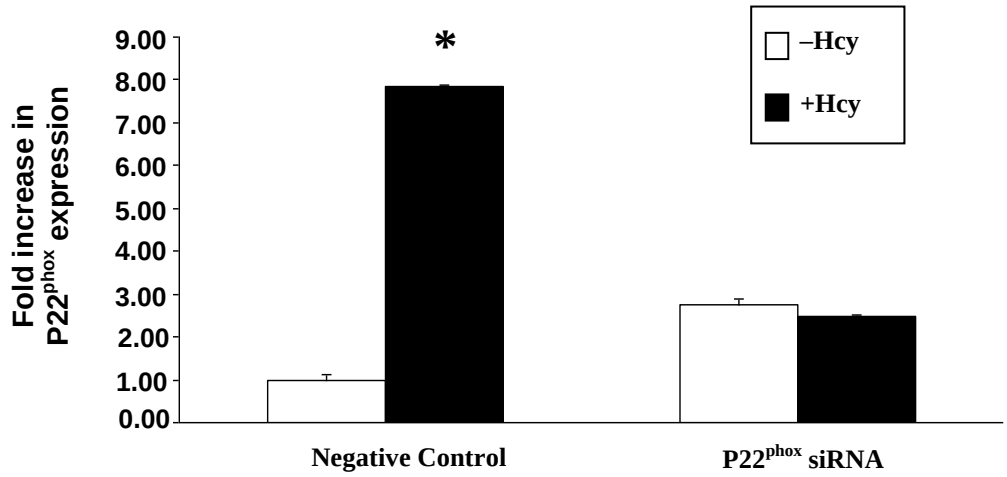

Figure 3.16 Determination of mRNA expression of $p22^{phox}$ in tubular cells transfected with $p22^{phox}$ siRNA

Human kidney proximal tubular cells were transfected with $p22^{phox}$ siRNA or with a negative control siRNA. Transfected cells were incubated for 12 h in the absence (Control) or presence of Hcy (100 uM). The mRNA level of $p22^{phox}$ was measured. Results were expressed as mean ± SEM (n=4-6). *P < 0.05 compared with control values.

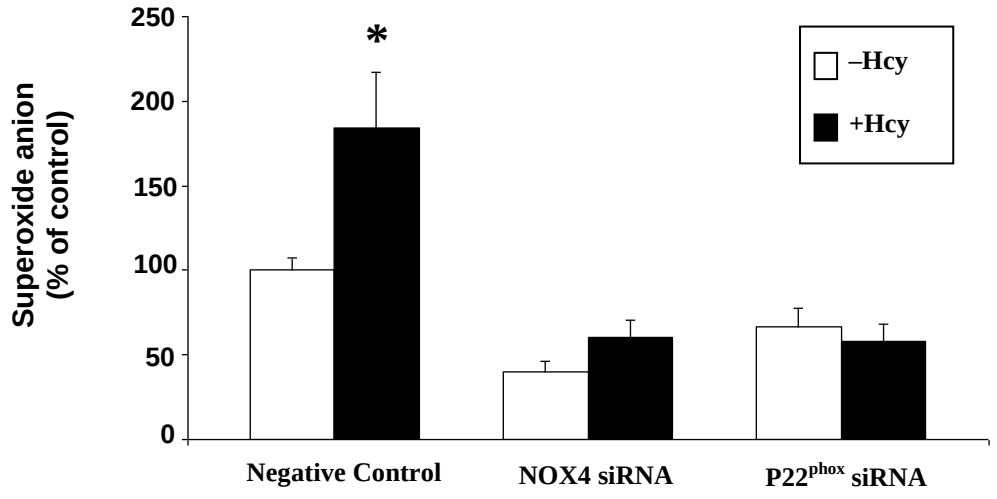

Figure 3.17 Superoxide anion production in tubular cells transfected with NOX4 and p22^{phox} siRNAs

Human kidney proximal tubular cells were transfected with NOX4 and p22^{phox} siRNAs or with a negative control siRNA. Transfected cells were incubated for 12 h in the absence (Control) or presence of Hcy (100 uM). Superoxide anion levels were determined by NBT reduction assay. The levels of superoxide anions in transfected cells without Hcy treatment were measured. Results were expressed as mean ± SEM (n=4-6). *$P < 0.05$ compared with control values.

3.4.4 Effect of folic acid on NADPH oxidase-mediated superoxide anion generation in tubular cells

The effect of folic acid on Hcy-induced superoxide anion generation was examined in tubular cells. Incubation of tubular cells with 5-MTHF, an active form of folate, abolished Hcy-induced NADPH oxidase activation (Fig. 3.12) and reduced the intracellular level of superoxide anion (Fig. 3.11). Incubation of cells with 5-MTHF also reduced the mRNA levels of NOX4 and $p22^{phox}$ (Fig. 3.13 and 3.14). Hcy can be remethylated to methionine via the enzymatic action of methionine synthase which utilizes 5-MTHF as a methyl donor. Since folate in the form of 5-MTHF can methylate Hcy to methionine, it has been used in Hcy lowering therapy. Treatment of control cells with 5-MTHF did not affect the basal level of intracellular superoxide anion (Fig. 3.10) or NOX4 and $p22^{phox}$ expression (Fig. 3.13 and 3.14).

Next, to examine whether the reduction of superoxide anion by 5-MTHF was due to its Hcy-lowering effect, lipopolysaccharide (LPS) (Sarna *et al.*, 2010) was added to tubular cells to induce NADPH oxidase activity and superoxide anion generation. LPS treatment caused a significant elevation of NADPH oxidase activity (Fig. 3.18) and intracellular superoxide anion level (Fig. 3.19). Incubation of tubular cells with 5-MTHF effectively reduced the LPS-induced elevation of NADPH oxidase activity (Fig. 3.18) and intracellular superoxide anion level (Fig. 3.19). These results suggested that the inhibitory effect of 5-MTHF on Hcy-induced superoxide anion generation in tubular cells was not merely dependent on Hcy reduction.

3.4.5 Effect of hyperhomocysteinemia and folic acid supplementation on kidney morphology

Histological staining was performed to examine the effect of hyperhomocysteinemia and folic acid supplementation on morphological changes in the kidney. The glomerular size was significantly increased in kidneys of hyperhomocysteinemic rats as indicated by larger mean glomerular volumes (Fig. 3.20 and 3.21). Folic acid supplementation reduced the glomerular size in rats fed a high-methionine diet (Fig. 3.20 and 3.21). An increase in the glomerular size might be one of the histological changes associated with the glomerular hypertrophy. Our results demonstrated that folic acid supplementation not only reduced oxidative stress but also improved the size of glomeruli in the kidney.

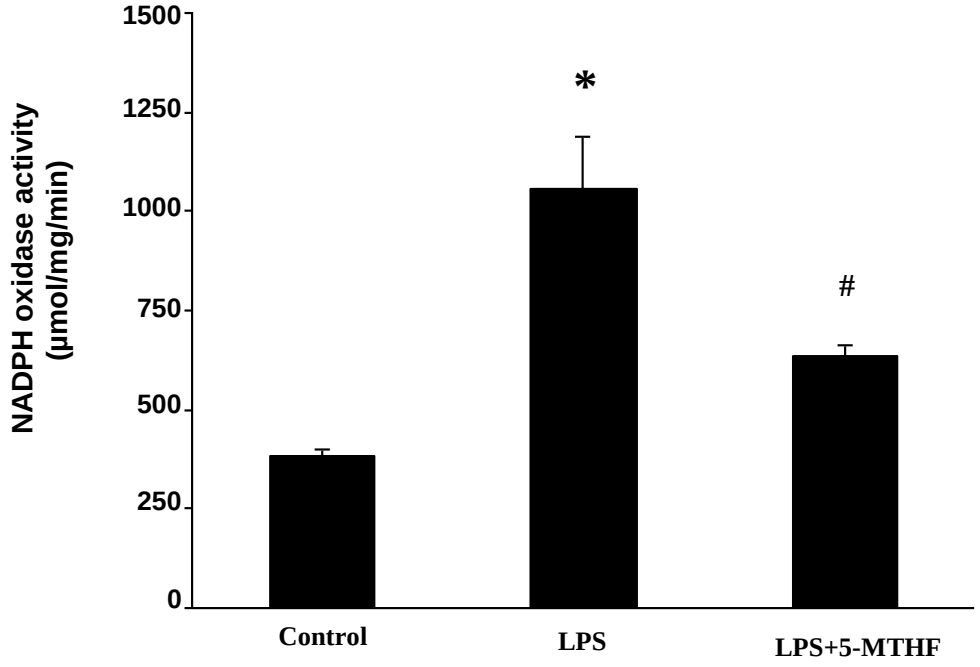

Figure 3.18 Determination of NADPH oxidase activity in lipopolysaccharide (LPS) stimulated tubular cells

Human kidney proximal tubular cells were incubated in the absence (Control) or presence of lipopolysaccharide (LPS, 10 µg) for 12 h. In one set of experiments, cells were pre-incubated with 5-methyltetrahydrofolate (5-MTHF, 500 ng) for 15 min followed by incubation with LPS (10 µg). NADPH oxidase activity was measured by lucigenin chemiluminescence's assay. Results were expressed as mean ± SEM (n=6-8). *P<0.05 compared with control values. $^{\#}P$<0.05 compared with values of the LPS group.

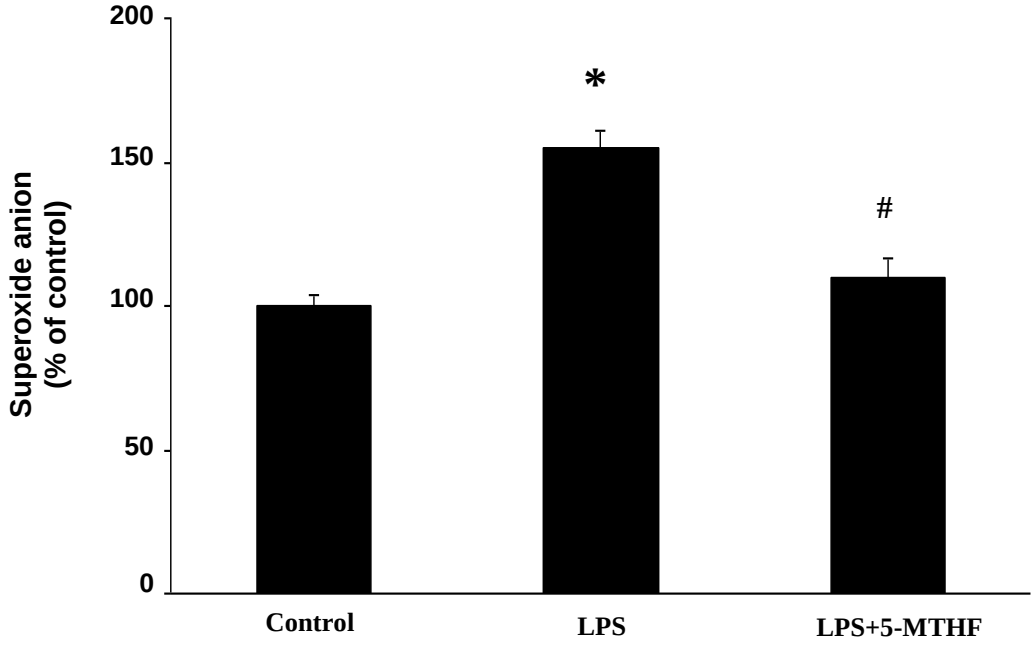

Figure 3.19 Determination of intracellular superoxide anions in lipopolysaccharide (LPS) stimulated tubular cells

Human kidney proximal tubular cells were incubated in the absence (Control) or presence of lipopolysaccharide (LPS, 10 µg) for 12 h. In one set of experiments, cells were pre-incubated with 5-methyltetrahydrofolate (5-MTHF, 500 ng) for 15 min followed by incubation with LPS (10 µg). Intracellular superoxide anion levels were determined by NBT reduction assay. Results were expressed as mean ± SEM (n=6-8). **P*<0.05 compared with control values. $^{\#}$*P*<0.05 compared with values of the LPS group.

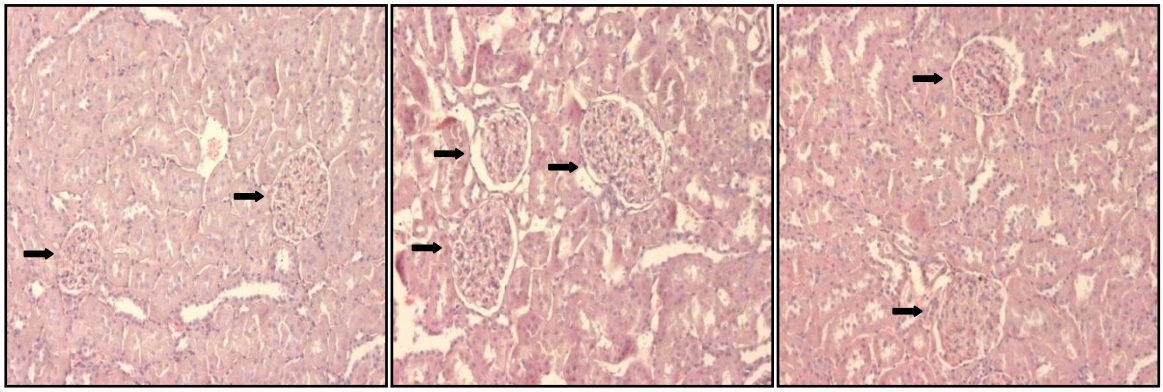

Figure 3.20 Measurement of mean glomerular volume (MGV) in rat kidneys (I)

Kidneys were collected from rats fed with the following diets for 12 wk: a regular diet (Control), a high methionine diet (1.7% Met), or a high methionine diet plus folic acid (0.25%) (Met + Folic acid). Glomerular size in rat kidneys was determined at 200X magnification (Arrows point to glomeruli). Kidney morphology was examined by hematoxylin and eosin staining.

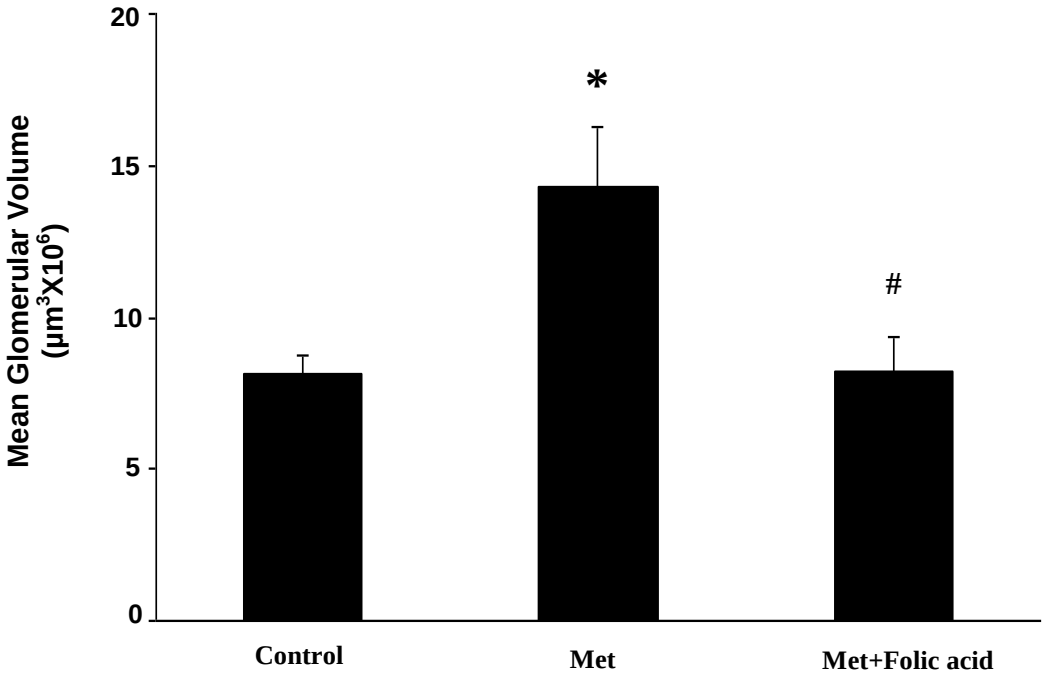

Figure 3.21 Measurement of mean glomerular volume (MGV) in rat kidneys (II)

Kidneys were collected from rats fed with the following diets for 12 wk: a regular diet (Control), a high methionine diet (1.7% Met), or a high methionine diet plus folic acid (0.25%) (Met + Folic acid). Results were expressed as mean ± SEM (n=4). *$P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the high-methionine fed group.

3.5 Discussion

The present study clearly demonstrated that NADPH oxidase plays an important role in superoxide anion generation in the kidney leading to increased lipid peroxidation during hyperhomocysteinemia. Folic acid supplementation could suppress NADPH oxidase activity by inhibiting the expression of NOX4 and $p22^{phox}$. Such an inhibitory effect alleviates oxidative stress in the kidney of hyperhomocysteinemic rats.

It is increasingly recognized that oxidative stress is an important factor in the development and progression of kidney disease. NADPH oxidase is expressed in phagocytic cells as well as in non-phagocytic cells. Excessive activation of NADPH oxidase can lead to over-production of superoxide anion, which is linked to tissue injury due to oxidative stress. Several lines of evidence from the present study suggested that NADPH oxidase was activated in the kidney of hyperhomocysteinemic rats, leading to increased renal lipid peroxidation. First, NADPH oxidase activity was markedly increased in the kidney during hyperhomocysteinemia. Such an elevation of the oxidase activity was a result of increased expression of NOX4 and $p22^{phox}$ subunits in the kidney. Secondly, the level of lipid peroxides in the kidney of hyperhomocysteinemic rats was found to be significantly elevated, indicating that oxidative stress occurred. Administration of a known NADPH oxidase inhibitor, apocynin not only reduced the NADPH oxidase activity to the basal level but also effectively inhibited lipid peroxidation in the kidneys of those rats. These results suggested that NADPH oxidase was likely involved in renal oxidative stress during hyperhomocysteinemia. Thirdly, the involvement of NADPH oxidase was further confirmed by using siRNA transfection technique. Transfection of tubular cells with NOX4 siRNA or $p22^{phox}$ siRNA effectively inhibited the expression of these subunits

as well as abolished Hcy-induced elevation of intracellular superoxide levels. Taken together, these results indicate that NADPH oxidase-dependent superoxide anion generation plays a major role in Hcy-induced oxidative stress in the kidney during hyperhomocysteinemia.

Apart from NADPH oxidase, superoxide anion might also be derived from other intracellular sources. To investigate the effect of Hcy on other superoxide anion generating sources, several inhibitors of superoxide producing enzymes were added to cultured tubular cells. Pretreatment of cells with an inhibitor for xanthine oxidase also abolished Hcy-induced elevation of intracellular superoxide levels. Furthermore, xanthine oxidase activity was found to be significantly increased in the kidney tissue of hyperhomocysteinemic rats. These results suggested that xanthine oxidase might be involved in Hcy-induced superoxide production in kidney cells. On the other hand, pretreatment of tubular cells with an inhibitor for mitochondrial respiratory chain complex I or an inhibitor for cytochrome P450 did not significantly affect intracellular superoxide anion levels upon Hcy treatment. In addition, NADPH oxidase activity was elevated in the mitochondria-free fraction prepared from the kidney of hyperhomocysteinemic rats. These results suggested that generation of superoxide anion by mitochondria might not be significantly altered in the kidney of hyperhomocysteinemic rat. However, it could not exclude the possibility that prolonged hyperhomocysteinemia (longer than 12 weeks) might affect superoxide anion generation by mitochondria in the kidney tissue. Our results also demonstrated that the activity of antioxidant enzyme SOD was markedly decreased in the kidney of hyperhomocysteinemic rats. Taken together, those results suggested that an increase in NADPH oxidase and xanthine oxidase activities combined with a reduction of SOD

activity might have contributed to increased oxidative stress in the kidney of hyperhomocysteinemic rats.

Many risk factors causing kidney injury share a common feature of generating intracellular free radicals causing oxidative stress. A recent study has shown an increase in NOX4 and $p22^{phox}$ expression in the kidney of streptozotocin-induced diabetic rats (Etoh *et al.*, 2003). It has been suggested that increased NADPH oxidase-mediated superoxide generation contributes to the development of diabetic nephropathy (Etoh *et al.*, 2003). It has also been shown that Hcy, at elevated levels, can induce inflammatory response and apoptosis in kidney cells and animal models (Yang & Zou, 2003; Shastry *et al.*, 2007; Yi *et al.*, 2006; Yi *et al.*, 2007). Hcy treatment was shown to increase collagen production and cell proliferation in rat mesangial cells (Yang & Zou, 2003). Such a stimulatory effect by Hcy could be blocked by inhibition of NADPH oxidase activation (Yang & Zou, 2003). Recently, the deletion of the $gp91^{phox}$ gene in mice was reported to protect hyperhomocysteinemia-induced kidney cell injury (Zhang *et al.*, 2010a) . In a rat model of hyperhomocysteinemia induced by folate free diet, glomerulosclerosis was characterized by enhanced oxidative stress, mesangial expansion, podocyte dysfunction, and fibrosis. When these rats with hyperhomocysteinemia were treated with apocynin, an inhibitor of NADPH oxidase, the glomerular injury was attenuated (Yi *et al.*, 2006; Yi *et al.*, 2007). In a recent study, Zhang *et al.* has demonstrated that NMDA receptor mediates NADPH oxidase dependent superoxide anion production induced by Hcy in mesangial cells, which may contribute to hyperhomocysteinemia-induced glomerulosclerosis (Zhang *et al.*, 2010b). Our recent study has demonstrated that hyperhomocysteinemia causes activation of NF-κB, induction of iNOS and chemokine expression in rat kidneys (Hwang *et al.*, 2008; Zhang *et al.*, 2004). NF-κB

is a transcription factor that plays an important role in regulating gene expression of various inflammatory factors in the kidney and is a redox-sensitive transcription factor (Hwang *et al.*, 2008; Zhang *et al.*, 2004). We have also observed that ROS can induce phosphorylation and degradation of IκB, an inhibitory protein normally associated with NF-κB, leading to NF-κB activation in endothelial cells and in macrophages (Woo *et al.*, 2003; Au-Yeung *et al.*, 2004). It is evident that NADPH oxidase plays an important role in Hcy-mediated kidney injury by producing NADPH-dependent superoxide anions. Regulation of its gene expression may offer a renal protective effect against oxidative stress related injury.

There is an inverse correlation between folate and Hcy levels in the circulation. The 5-MTHF that is formed from folic acid via the action of 5, 10-methylenetetrahydrofolate reductase is the active form of folic acid. 5-MTHF serves as a co-substrate in the remethylation pathway catalyzed by methionine synthase to convert Hcy to methionine. Therefore, folic acid supplementation is regarded as a promising approach in reducing blood Hcy levels. However, data from large prospective randomized clinical trials failed to demonstrate a beneficial effect of folic acid supplementation in reducing the mortality of cardiovascular disease (Lonn *et al.*, 2006; Bonaa *et al.*, 2006). In the HOPE-2 and NORVIT secondary prevention studies, participants were more than 55 years of age with cardiovascular disease, hypertension, myocardial infarction, and diabetes in which cardiovascular damaging effects might be irreversible. This might contribute to the observation that despite a reduction in plasma Hcy levels by folic acid supplementation in conjunction with vitamin B_6 and B_{12}, there was no significant cardiovascular benefit. However, the same studies revealed that Hcy lowering with folic acid in conjunction with vitamin B_6 and B_{12} decreased the risk of stroke. In the present study, folic acid supplementation

effectively abolished hyperhomocysteinemia-induced oxidative stress in the kidney by inhibition of NADPH oxidase activation. Such a renal protective effect might be mediated, in part, via a reduction of Hcy level by folic acid. The level of serum Hcy was significantly reduced in rats fed with a high-methionine diet supplemented with excess folic acid. However, the level of serum Hcy was still considerably higher (4-fold higher) in this group of rats as compared to the control group. Results obtained from the present study provided evidence that the inhibitory effect of folic acid on NADPH oxidase activation was not merely due to its ability to reduce the Hcy level. When tubular cells were incubated with Hcy or LPS, the intracellular levels of superoxide anions were significantly elevated. Incubation of cells with 5-MTHF effectively reduced the intracellular superoxide anions to the basal level not only in Hcy-treated cells but also in LPS-treated cells. A recent study has demonstrated that folic acid supplementation improves endothelial function in diabetic rabbits through the inhibition of intravascular oxidative stress, which is independent of Hcy lowering effect (Shukla *et al.*, 2008). In our previous study, folic acid supplementation has been shown to reduce oxidative stress and alleviate liver injury in hyperhomocysteinemic rats (Woo *et al.*, 2006a). Moreover, we have also observed that folic acid supplementation could prevent dietary-induced hyperhomocysteinemia in rats by abolishing Hcy-stimulated chemokine expression in the aortic endothelium and by inhibiting Hcy-induced NADPH oxidase activation leading to a reduction of superoxide anion generation in macrophages (Au-Yeung *et al.*, 2006; Wang *et al.*, 2002). Superoxide anion can react with nitric oxide to form peroxynitrite which is a potent oxidant and can cause lipid peroxidation. Folic acid supplementation also attenuated xanthine oxidase activity and restored SOD activity in the kidney of hyperhomocysteinemic rats. Taken together, these results suggested that folic acid

supplementation might offer a protective effect against oxidative stress via a reduction of Hcy levels as well as regulation of superoxide anion generation and metabolism.

In summary, the present study clearly demonstrates that hyperhomocysteinemia induces NADPH oxidase-mediated superoxide anion generation in the kidney leading to oxidative stress. Folic acid supplementation can effectively antagonize NADPH oxidase activation through inhibition of NOX4 and $p22^{phox}$ expression and hence abolish renal oxidative stress in hyperhomocysteinemic rats.

IV. Study 2: Hyperhomocysteinemia and Chemokine Expression in the Kidney

(Manuscript 2: Homocysteine stimulates monocyte chemoattractant protein-1 expression in the kidney via nuclear factor-kappa B activation)

Am J Physiol Renal Physiol 294 (1):F236-F244, 2008

4.1 Abstract

Hyperhomocysteinemia or an elevation of blood homocysteine (Hcy) levels is associated with cardiovascular disorders. Although kidney dysfunction is an important risk factor causing hyperhomocysteinemia, the direct effect of homocysteine (Hcy) on the kidney is not well documented. There is a positive association between an elevation of blood Hcy levels and the development of chronic kidney disease. Inflammatory response such as increased chemokine expression has been implicated as one of the mechanisms for renal disease. Monocyte chemoattractant protein-1 (MCP-1) is a potent chemokine that is involved in the inflammatory response in renal disease. Nuclear factor kappa-B (NF-κB) plays an important role in upregulation of MCP-1 expression. We investigated the effect of hyperhomocysteinemia on MCP-1 expression and the molecular mechanism underlying such an effect in rat kidneys as well as in proximal tubular cells. Hyperhomocysteinemia was induced in rats fed a high-methionine diet for 12 weeks. The MCP-1 mRNA expression and MCP-1 protein levels were significantly increased in kidneys isolated from hyperhomocysteinemic rats. The NF-κB activity was significantly increased in the same kidneys. Pretreatment of hyperhomocysteinemic rats with a NF-κB inhibitor abolished hyperhomocysteinemia-induced MCP-1 expression in the kidney. To confirm the causative role of NF-κB activation in MCP-1 expression, human kidney proximal tubular cells were transfected with decoy NF-κB oligodeoxynucleotide to inhibit NF-κB activation. Such a treatment prevented Hcy-induced MCP-1 mRNA expression in tubular cells. Our results suggest that hyperhomocysteinemia stimulates MCP-1 expression in the kidney via NF-κB activation. Such an inflammatory response may contribute to renal injury associated with hyperhomocysteinemia.

4.2 Introduction

Hyperhomocysteinemia, a condition of elevated homocysteine (Hcy) level in the blood, is an independent risk factor for cardiovascular disorders (Clarke *et al.*, 1991; McCully, 1996; Nygard *et al.*, 1997a). Impaired kidney function is one of the common factors causing hyperhomocysteinemia (Ninomiya *et al.*, 2004; Wheeler, 1996). In patients with chronic renal failure, there is a marked elevation of plasma Hcy levels and a striking increase in the risk for vascular disease (Ninomiya *et al.*, 2004). Although renal dysfunction or failure is an important factor causing hyperhomocysteinemia, the effect of Hcy, at abnormally high concentrations, on the kidney is unknown. Recent findings from an epidemiological study in the general population have revealed a positive association between an elevation of serum Hcy levels and the development of chronic kidney disease (Ninomiya *et al.*, 2004). Results from animal studies have also suggested that hyperhomocysteinemia may cause renal injury (Kumagai *et al.*, 2002; Li *et al.*, 2002; Zhang *et al.*, 2004). In contrast to Hcy-induced atherosclerosis, little information is available regarding the mechanisms by which Hcy may exert adverse effect on the kidney. We have demonstrated that hyperhomocysteinemia causes an increased nitric oxide production leading to the formation of peroxynitrite, a potent oxygen free radical, in rat kidneys (Zhang *et al.*, 2004). Further analysis reveals that activation of a transcription factor, namely, nuclear factor-kappa B (NF-κB) is involved in Hcy-induced inducible nitric oxide synthase expression in the kidney (Zhang *et al.*, 2004). Other investigators have reported that hyperhomocysteinemia causes vascular remodeling and tubulointerstitial injury in rat kidneys and glomerular damage in hypertensive rats with hyperhomocysteinemia (Li *et al.*, 2002; Kumagai *et al.*, 2002). Our recent study

suggests that elevation of Hcy levels in the kidney during ischemia-reperfusion also contributes to oxidative stress and renal injury (Prathapasinghe *et al.*, 2007).

Activation of NF-κB has been shown to be involved in the onset of glomerulosclerosis (Lynn *et al.*, 2001; Schachter *et al.*, 2000). In the resting cell, NF-κB resides in the cytoplasm in an inactive form that is associated with an inhibitory protein (IκB). Although several isoforms of NF-κB such as p50/p65, $(p65)_2$ and c-rel/p65 protein complexes have been detected in various types of cells, the predominant NF-κB isoform in kidney cells is thought to be a p50/p65 heterodimer (Lynn *et al.*, 2001; Sung *et al.*, 2002). The inhibitory protein IκBα is one of the best-characterized forms of IκB (Baldwin, 1996; Karin, 1999; Mercurio *et al.*, 1997; Simeonidis *et al.*, 1999). Upon stimulation, there is a rapid phosphorylation of IκBα and subsequent degradation of IκBα by the proteasome, leading to the release of NF-κB. After dissociation from IκB, the free NF-κB can enter the nucleus, a process termed translocation. Once inside the nucleus, NF-κB binds to the κB binding motifs in the promoters or enhancers of its target genes (Karin, 1999; Mercurio *et al.*, 1997; Simeonidis *et al.*, 1999). Activation of NF-κB has been shown in the kidney after ischemia/reperfusion injury (Sung *et al.*, 2002) as well as in the atherosclerotic lesions (Brand *et al.*, 1996; Marumo *et al.*, 1997). Once activated, NF-κB is able to upregulate gene expression of many inflammatory molecules including monocyte chemoattractant protein-1 (MCP-1). MCP-1 is a potent chemokine that stimulates the migration of leukocytes including monocytes into the intima of arterial wall and other tissues including kidney. Little MCP-1 is detectable in normal kidneys (Grandaliano *et al.*, 1997; Prodjosudjadi *et al.*, 1996; Robertson *et al.*, 2000). However, MCP-1 gene expression is greatly increased in kidneys of patients and animal models with

kidney diseases (Kuusniemi *et al.*, 2005; Rovin *et al.*, 1996; Sekiguchi *et al.*, 1997; Tang *et al.*, 1997; Wenzel *et al.*, 1997). Although hyperhomocysteinemia is a common clinical finding in patients with chronic kidney disease, the effect of Hcy on the kidney is not clear. In the present study, we investigated the effect of Hcy on MCP-1 expression in the kidney and the underlying mechanism of such an effect in hyperhomocysteinemic rats and in human proximal tubular cells.

4.3 Materials and Methods

4.3.1 Animal model

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) aged 8 weeks were divided into 3 groups and maintained for 12 weeks on the following types of diet: (1) control diet (regular diet), consisting of Lab Diet® Rat Diet 5012 (PMI® Nutrition International, St. Louis, MO); (2) high-methionine diet, consisting of regular diet plus 1.7% (wt/wt) methionine; and (3) high-cysteine diet, consisting of regular diet plus 1.2% (wt/wt) cysteine. Each group consisted of 12 rats. Results from our previous studies demonstrated that hyperhomocysteinemia could be induced in rats by feeding a high-methionine diet (Au-Yeung *et al.,* 2004; Wang *et al.,* 2002; Woo *et al.,* 2005; Zhang *et al.,* 2004). The serum total Hcy (tHcy) concentration was measured with the IMx Hcy assay, which was based on fluorescence polarization immunoassay technology (Abbott Diagnostics, Abbott Park, IL) (Mudd *et al.,* 2000). All procedures were performed in accordance with the Guide for the Care and Use of Experimental Animals published by Canadian Council on Animal Care and approved by University of Manitoba Protocol Management and Review Committee.

4.3.2 Cell culture

Human kidney cortex proximal tubular cells (HK2, CRL-2190) were purchased from the American Type Culture Collection (ATCC). Cells were cultured in keratinocyte-serum free medium (GIBCO-BRL 17005-042) with 5 ng/mL recombinant epidermal growth factor and 0.05 mg/ml bovine pituitary extract according to the ATCC instruction. THP-1, a human monocytic cells line, were

purchased from ATCC and cultured in RPMI-1640 medium (Hyclone). L-Hcy was prepared from L-Hcy thiolactone (Sigma-Aldrich, St Louis, MO) as described previously (Woo *et al.*, 2006b). In brief, L-Hcy thiolactone was hydrolyzed in NaOH (5 mol/L) to remove the thiolactone group and then neutralized with HCl. Freshly prepared L-Hcy was used in the experiments.

4.3.3 Measurement of monocyte chemoattractant protein-1 (MCP-1) mRNA and protein levels

Total RNAs were isolated from kidneys or HK-2 cells with TriZol reagent (Invitrogen Life Technologies, Carlsbad, CA). The MCP-1 mRNA was measured by a real-time polymerase chain reaction (PCR) analysis using iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). In brief, 2 μg of total RNA was converted to cDNA by reverse transcriptase. The reaction mixture of real-time PCR contained 0.4μM of 5'- and 3'- primer and 2μL cDNA product in iQ-SYBR Green supermix reagent (Bio-Rad). The primers used for rat MCP-1 were (forward) 5'-CAGAAACCAGCCAACTCTCA-3' and (reverse) 5'-AGACAGCACGTGGATGCTAC-3' (GenBank™/ Accession number: AF058786) and for rat GAPDH were (forward) 5'-TCAAGAAGGTGGTGAAGCAG -3' and (reverse) 5'- AGGTGGAAGAATGGGAGTTG-3' (GenBank™/ Accession number: NM_017008). The primers for human MCP-1 were (forward) 5'-CCGAGAGGCTGAGACTAACC-3' and (reverse) 5'-GGAATGAAGGTGGCTGCTAT-3' (GenBank™/ Accession number: NM_002982) and for human GAPDH were (forward) 5'- ATCATCCCTGCCTCTACTGG -3' and (reverse) 5'- GTCAGGTCCACCACTGACAC-3' (GenBank™/ Accession number: NM_002046). All primers were synthesized by Invitrogen. The relative change in

MCP-1 mRNA expression was determined by the fold change analysis in which fold change = $2^{-\Delta\Delta Ct}$, where Ct = $(Ct_{MCP-1} - Ct_{GAPDH})_{\text{treatment}} - (Ct_{MCP-1} - Ct_{GAPDH})_{\text{Control}}$ (Livak & Schmittgen, 2001). Ct was the cycle number at which the fluorescence signal crossed the threshold, which was determined by iQ5 Optical System Software (version 2, Bio-Rad). The MCP-1 protein in the kidney tissue was quantified by using a commercial ELISA kit (Biosource International, Camarillo, CA).

4.3.4 Immunohistochemical staining

For detection of MCP-1 protein, kidneys were excised from rats fed different types of diet. A portion of the kidney was immersion-fixed in 10% neutral-buffered formalin overnight and then embedded in paraffin. Sequential 5-μm paraffin-embedded sections were immunostained using rabbit polyclonal antibodies (1:100) as primary antibodies against rat MCP-1 (PeproTech EC Ltd., Rocky Hill, NJ). Endogenous peroxidase was blocked with 0.3% H_2O_2 for 20 min. The secondary antibodies for immunostaining were biotin-conjugated anti- rabbit immunoglobulins (1:200) (Dako Canada, Inc., Mississauga, ON). Sections were then treated with 3,3-diaminobenzidine (DAB)-H_2O_2 colorimetric substrate solution. The attached peroxidase catalyzed the H_2O_2-mediated oxidation of DAB to yield brown color. The area displayed brownish color indicating the MCP-1 protein adducts. For a negative control, non-specific rabbit IgG was used as primary antibodies.

4.3.5 Myeloperoxidase activity assay

The myeloperoxidase (MPO) activity was measured in the kidney to assess the leukocyte infiltration into the tissue as described previously (Chatterjee *et al.*, 2000; Sung *et al.*, 2002). A portion of the kidney was homogenized in 3mL of 50mM

potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltri-methylammoniun bromide. The homogenate was centrifuged at 20,000×*g* for 20 min and the supernatant was collected. The reaction mixture containing 0.6 mM 3,3',5,5'-tetramethylbenzidine, 0.03 M hydrogen peroxide, 80 mM sodium phosphate buffer (pH 5.5) and an aliquot of supernatant was incubated at 25 °C for 15 min. The reaction was stopped by the addition of 1 mL of 0.5 M sulfuric acid. The absorbance was measured at 450 nm. Peroxidase (Sigma Aldrich) was used as an external standard. MPO activity was expressed as mU enzyme activity per mg protein.

4.3.6 Western immunoblot analysis

Kidney IκBα and cyclooxygenase-2 (COX-2) protein levels were determined by a Western immunoblot analysis. For the measurement of total IκBα and phosphorylated IκBα proteins, kidney proteins (100 μg/mL) were separated by electrophoresis on a 12.5% SDS polyacrylamide gel while for COX-2, the electrophoresis was carried out on a 8% SDS polyacrylamide gel. Proteins on the gel were then transferred to a nitrocellulose membrane. The membrane was probed with rabbit anti-IκBα polyclonal antibodies or anti-phospho-IκBα (Ser32) polyclonal antibodies (New England Biolabs Inc., Beverly, MA) and with rabbit anti-COX2 polyclonal antibodies (Lab Vision Co., Fremont, CA). Horseradish peroxidase-conjugated secondary antibodies (Zymed, South San Francisco, CA) were used to develop the membranes. The IκBα, phospho-IκBα or COX-2 protein bands were visualized using enhanced chemiluminescence reagents and analyzed with a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst® version 1.1).

4.3.7 Electrophoretic mobility shift assay

Nuclear proteins were isolated and electrophoretic mobility shift assay (EMSA) was performed to determine the NF-κB/DNA binding activity (Au-Yeung *et al.*, 2004; Zhang *et al.*, 2004). In brief, nuclear proteins (10 μg) were incubated with the reaction buffer for 15 min at room temperature followed by incubation with ^{32}P-end-labelled oligonucleotide containing the consensus sequence for the κB binding site (5'-GGGGACTTTCC-3') (Promega, Madison, WI). The reaction mixture was separated in a non-denaturing polyacrylamide gel (6%) that was later exposed to X-ray film at –70^{o}C. The binding of labeled oligonucleotide to nuclear proteins was blocked by adding 100-fold excess unlabelled specific competitor oligonucleotide to the reaction mixture. This was to confirm that the binding of ^{32}P-end-labeled oligonucleotide to NF-κB was sequence-specific.

4.3.8 Decoy oligodeoxynucleotide transfection

Double stranded oligodeoxynucleotide (ODNs) (Sigma-Genosys) were prepared from complementary single-stranded phosphorothioate-bonded ODNs by melting at 95°C for 5 min followed by a cool-down phase of 3 h to room temperature (O & Siow, 2003). The sequence of the single stranded ODNs were: 5'-AGTTGAGGGGACTTTCCCAGGC-3' for wild type NF-κB and 5'-CATGTCGTCACTGCGCTCAT -3'for scrambled ODN (underlined letters denote phosphorothioate-bonded bases while bolded letters denote the consensus binding site) (Choy *et al.*, 2004). Proximal tubular cells were treated with 100nM ODN and 4μL Oligofectamine® in supplement free medium for 4 h. After the delivery of ODN into the tubular cells, the medium containing the decoy ODN was replaced with fresh

medium containing supplement. Cells were subsequently incubated in the absence or presence of Hcy for a defined time period.

4.3.9 Chemotaxis assay

Monocyte migration was determined by monocyte chemotaxis assay using a 48-well Micro Chemotaxis Chamber (Neuro Probe Inc., Gaithersburg, MD) as described in previous studies (Lynn *et al.*, 2001; Sung *et al.*, 2001). In brief, human proximal tubular cells were incubated in the presence or absence of Hcy (100 μM). After incubation, an aliquot of the medium (defined as conditioned medium) was transferred to the lower chamber of the Micro Chemotaxis Chamber. The lower and upper chambers were separated by a 5 μm pore-size polycarbonate membrane (Neuro Probe Inc., Gaitherburg, MD). An aliquot of THP-1 monocyte suspension ($2x10^6$ cells/mL) was added to the upper chamber and the monocytes were allowed to transmigrate from the upper chamber to the lower chamber for 2 h. After transmigration, the surface of the membrane facing the THP-1 cell suspension was scraped and washed three times according to the manufacturer's instructions. Cells that had migrated towards the conditioned medium were fixed and then stained with haematoxylin. The number of migrated monocytes was determined by counting the cells in five high-power fields under light microscopy. The results are expressed as a percentage of control.

4.3.10 Statistical analysis

The results were analyzed using two-tailed independent Student t-test and one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple

comparison test. Data were presented as the mean ± SEM. The level of statistical significance was set at $P < 0.05$.

4.4 Results

4.4.1 Expression of MCP-1 in the kidney

Rats that were fed a high-methionine diet for 12 weeks developed hyperhomocysteinemia (Fig. 4.1). The high-methionine diet resulted in a significant increase in the serum Hcy levels. There was no significant change in serum tHcy levels in rats fed a high-cysteine diet compared with the control group (Fig. 4.1). There was a significant increase in MCP-1 mRNA levels in kidneys isolated from rats fed a high-methionine diet (Fig. 4.2). In accordance with these results, the levels of MCP-1 protein were significantly elevated in those kidneys (Fig. 4.3). Cysteine is another sulfhydryl (SH)-containing amino acid. There was no change in MCP-1 mRNA and protein levels in rats fed a high-cysteine diet compared with the control group (Fig. 4.2 and 4.3). These results suggested that hyperhomocysteinemia-induced MCP-1 expression in the kidney was not due to a general effect produced by SH-containing amino acids. When pyrrolidine dithiocarbamate (PDTC), a known inhibitor for NF-κB activation (Sung *et al.*, 2002; Zhang *et al.*, 2004), was injected into the high-methionine fed rats, the renal level of MCP-1 protein was reduced to that of the control group (Fig. 4.3). These results suggested that increased MCP-1 expression in the kidney during hyperhomocysteinemia might be mediated via NF-κB activation. In addition, another inflammatory factor, namely, COX-2 protein, was also measured in kidneys by Western immunoblot analysis. There was no significant difference in the levels of COX-2 proteins in kidneys isolated from control, high-methionine fed and high-cysteine fed rats (Fig. 4.4).

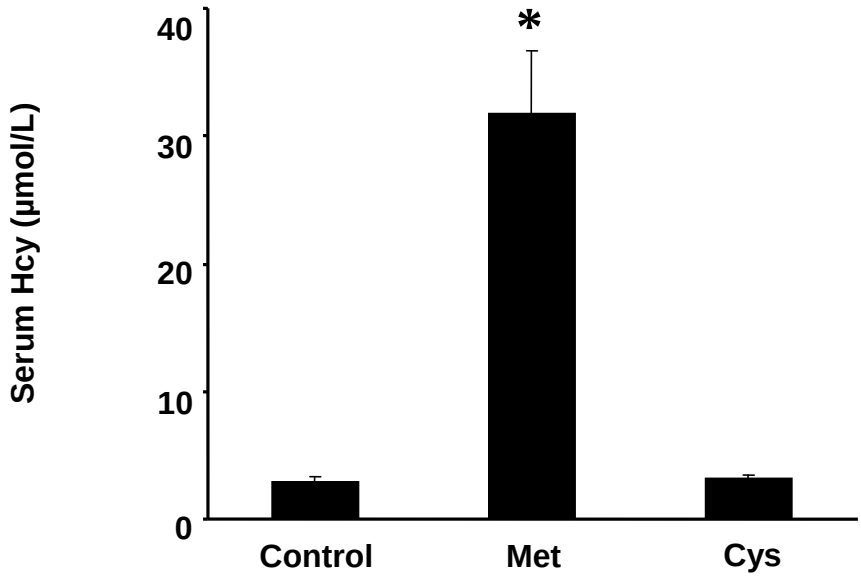

Figure 4.1 Measurement of serum total homocysteine (tHcy) levels

Rats were fed a control diet (Control), a high-methionine diet (1.7 % Met) or a high-cysteine diet (1.2 % Cys) for 12 weeks. Serum tHcy levels were measured. Results were expressed as mean ± SEM (n=12). **P*<0.05 when compared with the value obtained from the control group.

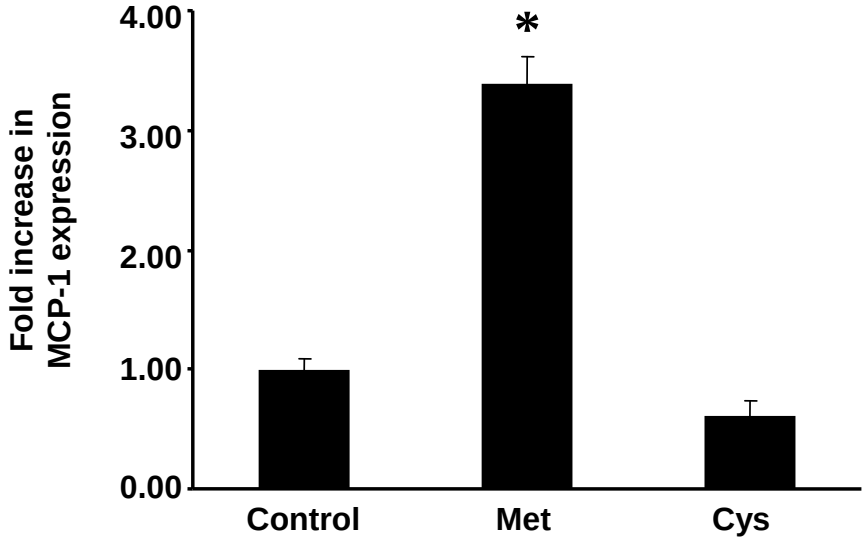

Figure 4.2 Expression of MCP-1 mRNA in rat kidneys

Rats were fed a control diet (Control), a high-methionine diet (1.7 % Met) or a high-cysteine diet (1.2 % Cys) for 12 weeks. Total RNAs were prepared from kidneys and the MCP-1 mRNA levels were measured by a real-time PCR detection system. Results were expressed as mean ± SEM (n=12). *P<0.05 when compared with the value obtained from the control group.

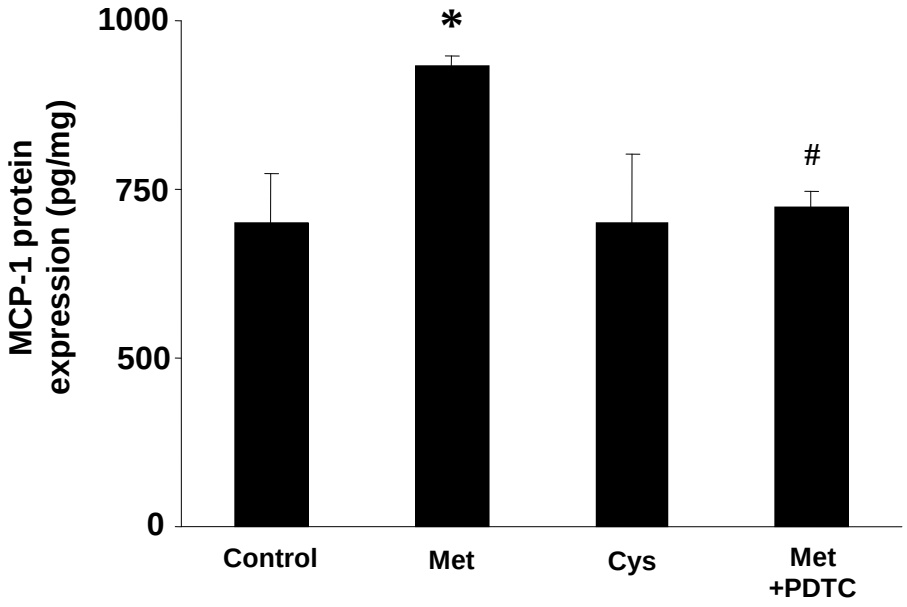

Figure 4.3 Expression of MCP-1 protein in rat kidneys

Rats were fed a control diet (Control), a high-methionine diet (1.7 % Met) or a high-cysteine diet (1.2 % Cys) for 12 weeks. The MCP-1 protein levels in kidneys were determined by ELISA. In one set of experiments, rats fed with a high-methionine diet for 12 weeks was treated with pyrrolidine dithiocarbamate (PDTC, 100 mg/kg, daily, IP), an inhibitor of NF-κB activation, for 3 days prior to euthanasia (Met + PDTC). Results were expressed as mean ± SEM (n=12). *P<0.05 when compared with the value obtained from the control group. #P<0.05 when compared with the value obtained from the methionine fed group.

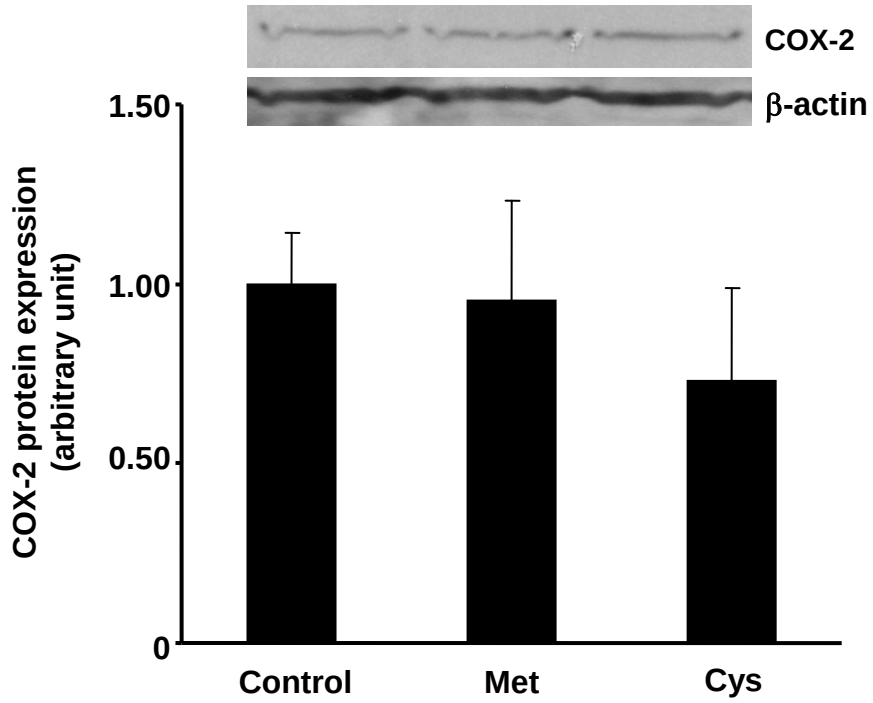

Figure 4.4 Expression of COX-2 protein in rat kidneys

Rats were fed a control diet (Control), a high-methionine diet (1.7 % Met) or a high-cysteine diet (1.2 % Cys) for 12 weeks. The COX-2 protein levels in the kidney tissue were measured by Western immunoblot analysis. β-actin was used to confirm equal amount of protein loading for each sample. Results were expressed as mean ± SEM (n=12).

4.4.2 Distribution of MCP-1 protein in the kidney

The distribution of MCP-1 protein in the kidney was examined by immunohistochemical analysis. Compared with the control group, kidneys isolated from rats fed a high-methionine diet displayed an increased staining for MCP-1 protein in the cortex and medulla (Fig. 4.5). As a negative control, the immunohistochemical staining was performed with non-specific antibodies (IgG) and no positive staining was observed (Fig. 4.5). Leukocyte infiltration was assessed by measuring the MPO activity in the kidney. The MPO activity was significantly increased in kidneys isolated from hyperhomocysteinemic rats (Fig. 4.6), indicating an elevation of leukocyte infiltration into the kidney during hyperhomocysteinemia.

4.4.3 Role of NF-κB activation in MCP-1 expression in the kidney

The NF-κB activation in the kidney was examined by EMSA. The NF-κB/DNA binding activity was significantly elevated in kidneys isolated from rats fed a high-methionine diet as compared to the control (Fig. 4.7). Western immunoblot analysis was performed to determine the levels of phosphorylated IκBα protein and total IκBα protein in the kidney. There was a significant increase in the level of phosphorylated IκBα protein (Fig. 4.8) and a significant reduction in the level of IκBα protein (Fig. 4.9) in kidneys isolated from rats fed a high-methionine diet. To further investigate the link between NF-κB activation and hyperhomocysteinemia-induced MCP-1 expression, rats fed a high-methionine diet were treated with PDTC. Such a treatment completely blocked hyperhomocysteinemia-induced MCP-1 mRNA expression (Fig. 4.11) and elevation of MCP-1 protein levels in kidneys isolated from rats fed a high-methionine diet (Fig. 4.3). These results suggested that

hyperhomocysteinemia-induced MCP-1 expression in rat kidneys was mediated via NF-κB activation.

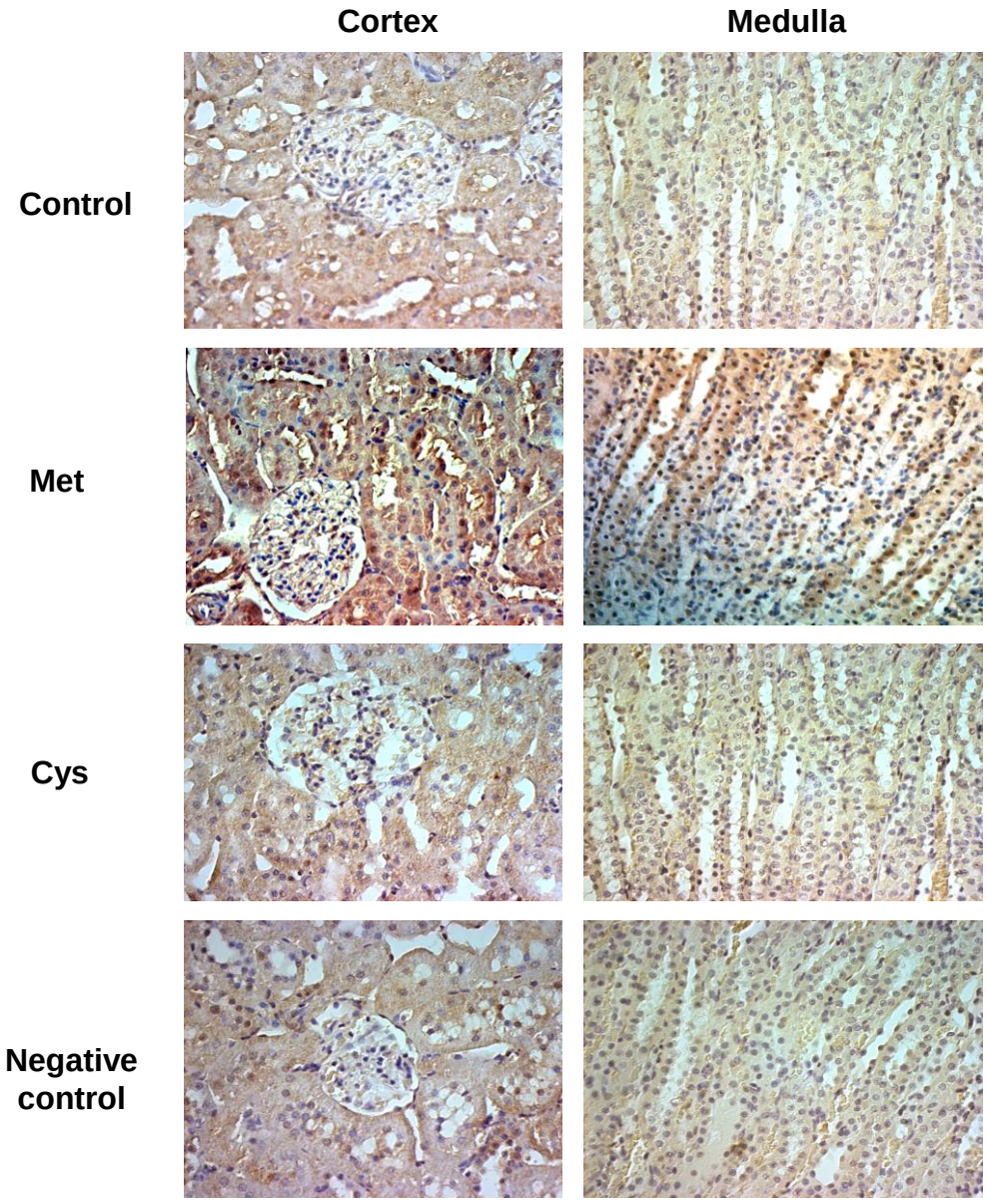

Figure 4.5 Immunohistochemical staining of MCP-1 protein in rat kidneys

Rats were fed a control diet (Control), a high-methionine diet (Met) or a high-cysteine diet (Cys) for 12 weeks. Immunohistochemical staining for MCP-1 protein was performed with anti-MCP-1 antibodies. After counterstaining with Mayer's hematoxylin, MCP-1 protein was identified under light microscope at a magnification of ×400. As a negative control, immunohistochemical staining was performed by using nonspecific rabbit IgG as primary antibodies. All staining analyses were performed in kidneys isolated from 12 rats of each group.

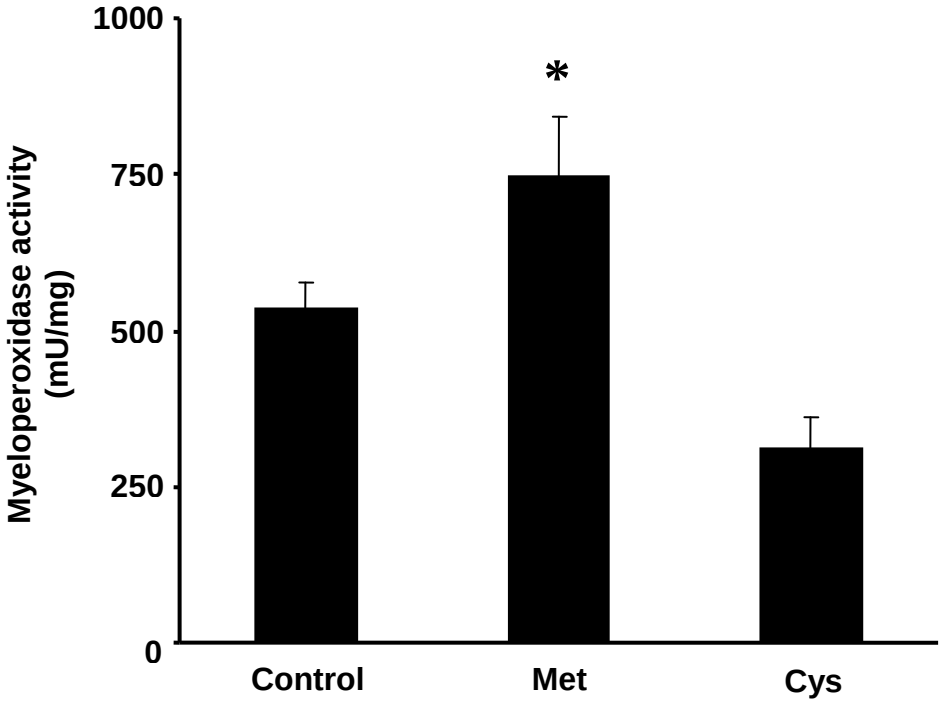

Figure 4.6 Determination of MPO activity in rat kidneys

Kidneys were isolated from rats fed a control diet (Control), a high-methionine diet (Met) or a high-cysteine diet (Cys) for 12 weeks. Myeloperoxidase (MPO) activity was determined to evaluate leukocyte infiltration. Results were expressed as mean ± SEM (n=12). **P*<0.05 when compared with the value obtained from the control group.

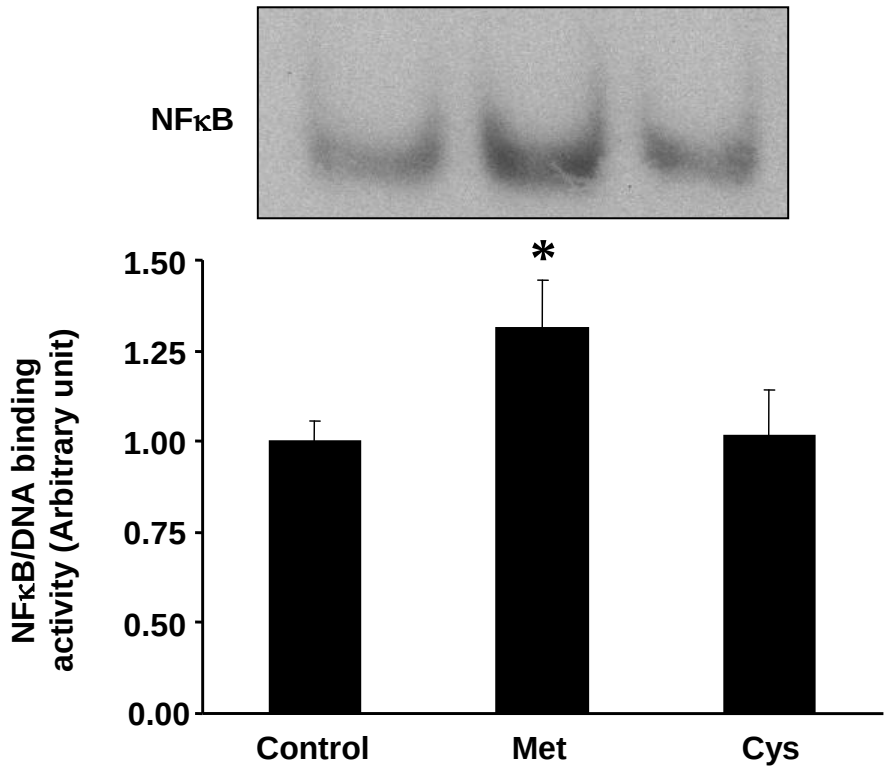

Figure 4.7 NF-κB activation in rat kidneys

Kidneys were isolated from rats fed a control diet (Control), a high-methionine diet (Met) or a high-cysteine diet (Cys) for 12 weeks. The NF-κB/DNA binding activity was determined by EMSA. The results were expressed as mean ± SEM (n=12). **P*<0.05 when compared with the value obtained from the control group.

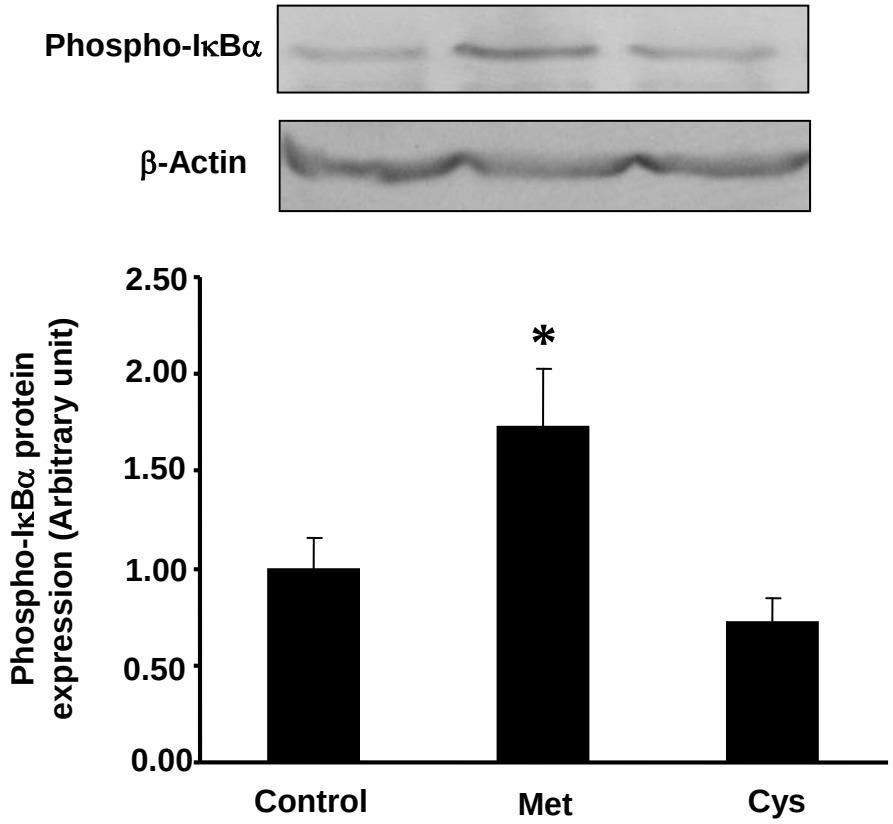

Figure 4.8 Expression of phosphor-IκBα protein in rat kidneys

Kidneys were isolated from rats fed a control diet (Control), a high-methionine diet (Met) or a high-cysteine diet (Cys) for 12 weeks. The level of phosphor-IκBα protein was measured by Western immunoblot analysis. The results were expressed as mean ± SEM (n=12). **P*<0.05 when compared with the value obtained from the control group.

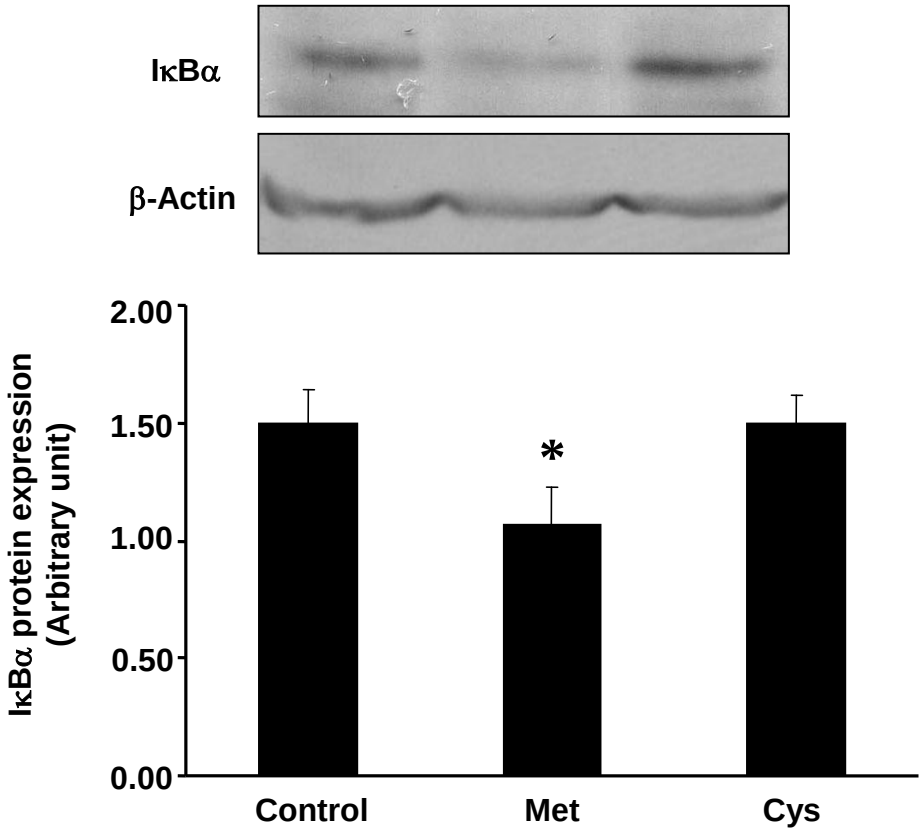

Figure 4.9 Expression of total IκBα protein in rat kidneys

Kidneys were isolated from rats fed a control diet (Control), a high-methionine diet (Met) or a high-cysteine diet (Cys) for 12 weeks. The level of total IκBα protein was measured by Western immunoblot analysis. Results were expressed as mean ± SEM (n=12). **P*<0.05 when compared with the value obtained from the control group.

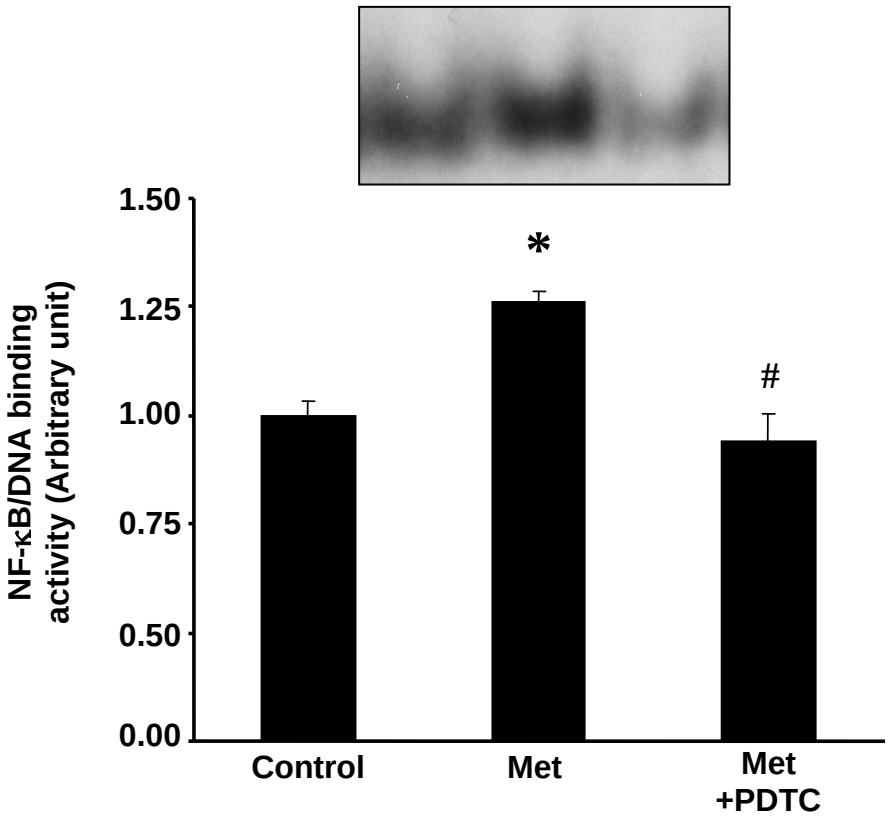

Figure 4.10 NF-κB activation in rat kidneys

Rats were fed a control diet (Control) or a high-methionine diet (Met) for 12 weeks. One group of rats fed with a high-methionine diet for 12 weeks was treated with pyrrolidine dithiocarbamate (PDTC, 100 mg/kg, daily), an inhibitor of NF-κB activation, for 3 days prior to euthanasia. The NF-κB/DNA binding activity was determined by EMSA. Results were expressed as mean ± SEM (n=12). **P*<0.05 when compared with the value obtained from the control group. #*P*<0.05 when compared with the value obtained from the methionine fed group.

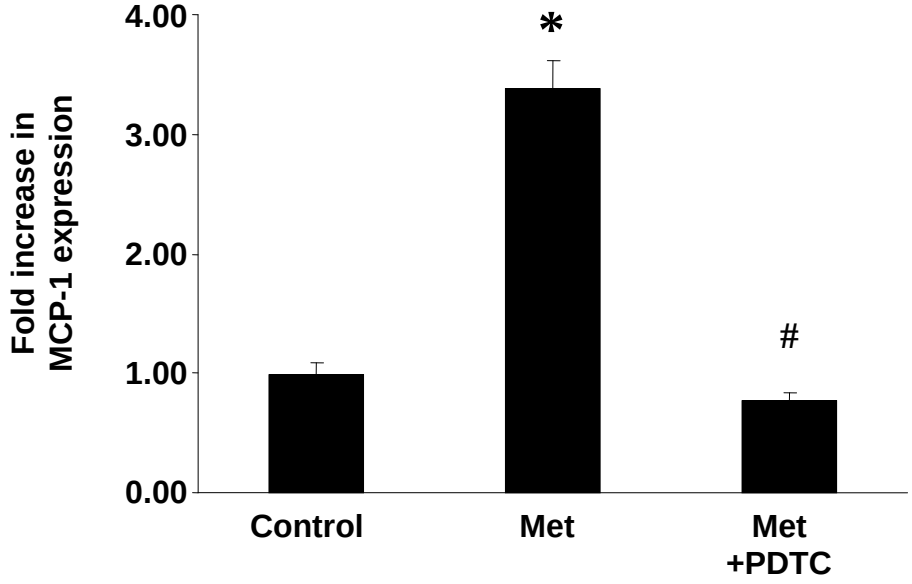

Figure 4.11 MCP-1 expression in rat kidneys

Rats were fed a control diet (Control) or a high-methionine diet (Met) for 12 weeks. One group of rats fed with a high-methionine diet for 12 weeks was treated with pyrrolidine dithiocarbamate (PDTC, 100 mg/kg, daily), an inhibitor of NF-κB activation, for 3 days prior to euthanasia. The MCP-1 mRNA expression was measured by a real-time PCR detection system. Results were expressed as mean ± SEM (n=12). **P*<0.05 when compared with the value obtained from the control group. #*P*<0.05 when compared with the value obtained from the methionine fed group.

4.4.4 Effect of Hcy on MCP-1 expression in tubular cells

The effect of Hcy on MCP-1 expression was also examined in human kidney proximal tubular cells. Treatment of tubular cells with Hcy resulted in a significant increase in cellular MCP-1 mRNA expression (Fig. 4.12) and MCP-1 protein secreted into the culture medium (Fig. 4.13). To determine whether Hcy-induced MCP-1 production could lead to monocyte chemotaxis, the culture medium was collected from the Hcy-treated tubular cells (defined as conditioned medium) and used for measuring monocyte migration in the chemotaxis assay (Lynn *et al.*, 2001; Sung *et al.*, 2001). The conditioned medium significantly enhanced monocyte migration in the chemotaxis assay (Fig. 4.14). Anti- MCP-1 antibodies abolished such a stimulatory effect (Fig. 4.14). These results suggested that Hcy-induced MCP-1 production in tubular cells could lead to monocyte chemotaxis. To further investigate the causative relationship between Hcy-induced NF-κB activation and MCP-1 expression, tubular cells were transfected with decoy NF-κB ODN to inhibit the activation of NF-κB. The interaction of decoy ODN with NF-κB prevents the binding of this transcription factor with the target genes including MCP-1. EMSA showed that transfection of tubular cells with NF-κB ODN completely prevented NF-κB/DNA binding activity (Fig. 4.15). Inhibition of NF-κB activation also abolished Hcy-induced MCP-1 mRNA expression in tubular cells (Fig. 4.16). As a control, cells were transfected with scrambled ODN, which had no effect on Hcy-induced NF-κB activation and MCP-1 mRNA expression in those cells (Fig. 4.15 and 4.16). In addition, tubular cells were also incubated with other amino acids. Cysteine treatment did not stimulate MCP-1

expression in tubular cells (Fig. 4.17). In another set of experiments, cells were incubated with methionine that was used in the diet to induce hyperhomocysteinemia in rats. Methionine treatment did not affect MCP-1 expression in tubular cells (Fig. 4.17). Furthermore, addition of S-adenosyl-methionine (SAM, Sigma Aldrich), a metabolite formed during metabolism of methionine to Hcy, to the culture medium did not affect the expression of MCP-1 mRNA in tubular cells (Fig. 4.18).

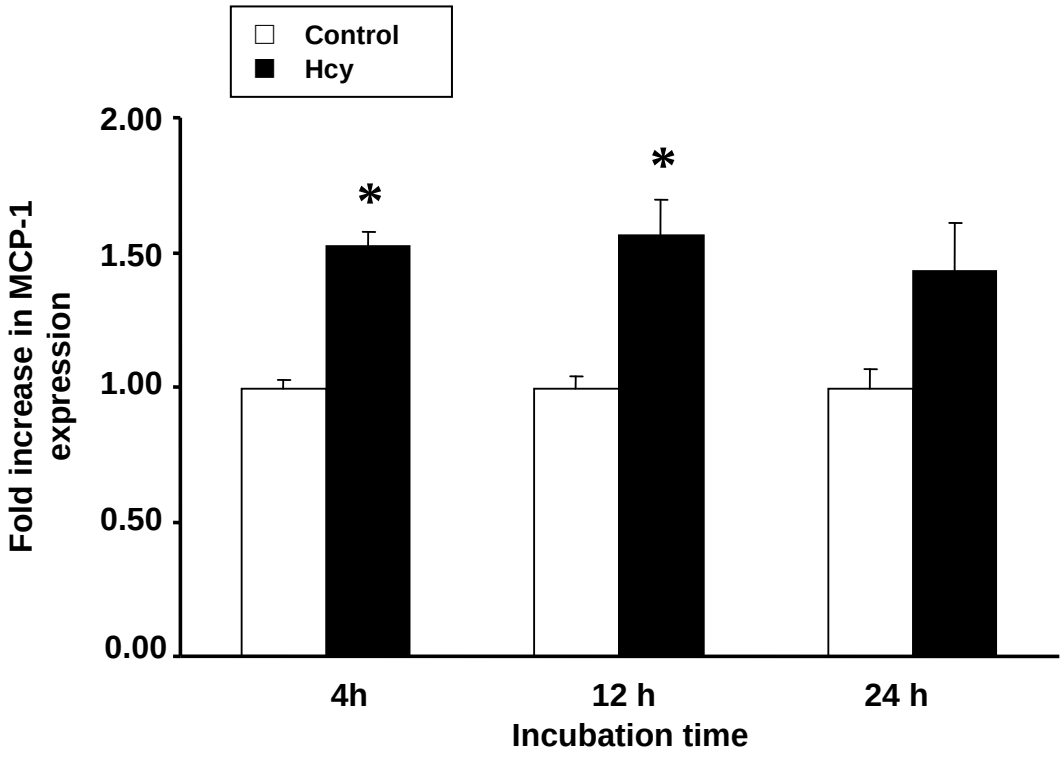

Figure 4.12 Effect of Hcy on MCP-1 gene expression in tubular cells

Human kidney proximal tubular cells were incubated in the absence or presence of Hcy (100 μM) for various time periods. The levels of MCP-1 mRNA were measured by a real-time PCR detection system. Results were expressed as mean ± SEM (n=5). **P*<0.05 when compared with the value obtained from the control group.

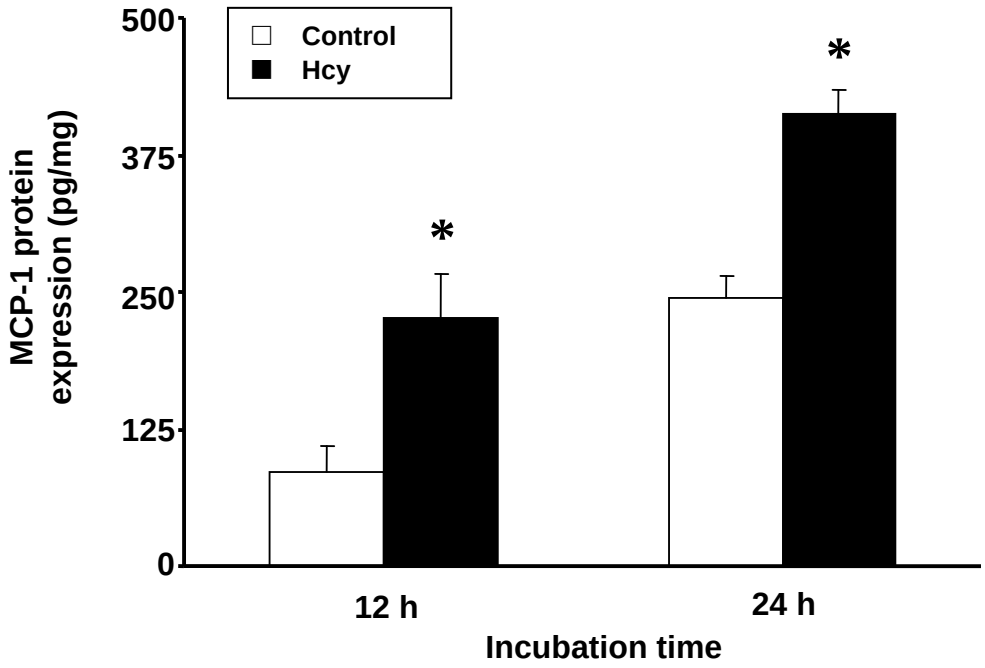

Figure 4.13 Effect of Hcy on MCP-1 protein expression in tubular cells

Human kidney proximal tubular cells were incubated in the absence or presence of Hcy (100 μM) for various time periods. The amount of MCP-1 protein secreted into the culture medium was measured by ELISA. Results were expressed as mean ± SEM (n=5). *P<0.05 when compared with the value obtained from the control group.

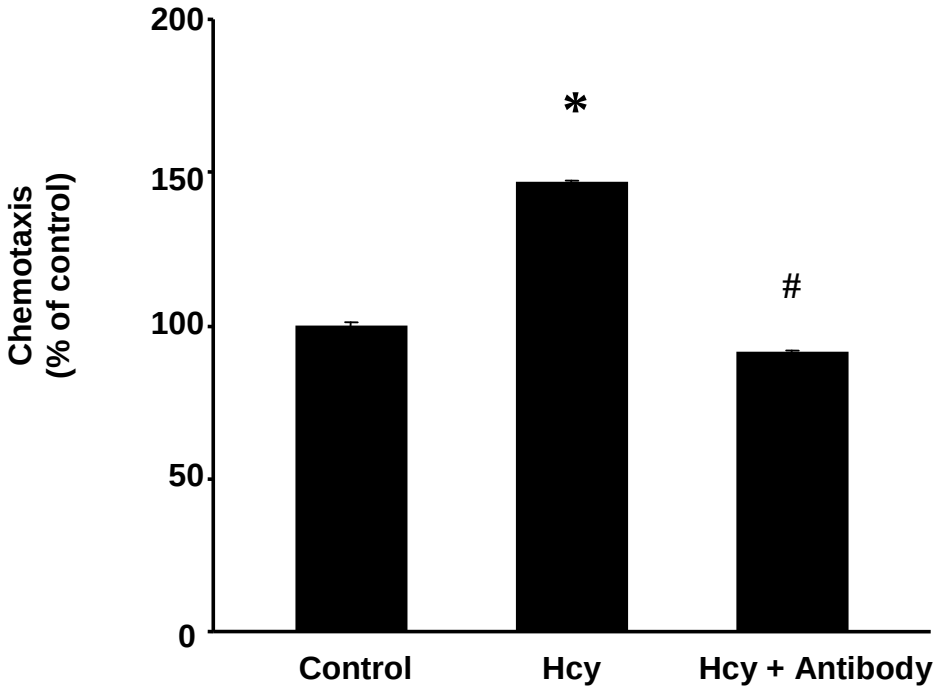

Figure 4.14 Effect of Hcy on monocyte chemotaxis

Human kidney proximal tubular cells were incubated in the absence or presence of Hcy (100 μM) for various time periods. For monocyte chemotaxis assay, the culture medium was collected from cells incubated in the absence (Control) or presence of Hcy. In one set of the experiment, anti-MCP-1 antibodies (0.5 μg/mL) were added to the conditioned medium for 10 min prior to the monocyte chemotaxis assay. Results were expressed as mean ± SEM (n=5). *P<0.05 when compared with the value obtained from the control group. #P<0.05 when compared with the value obtained from Hcy-treated cells.

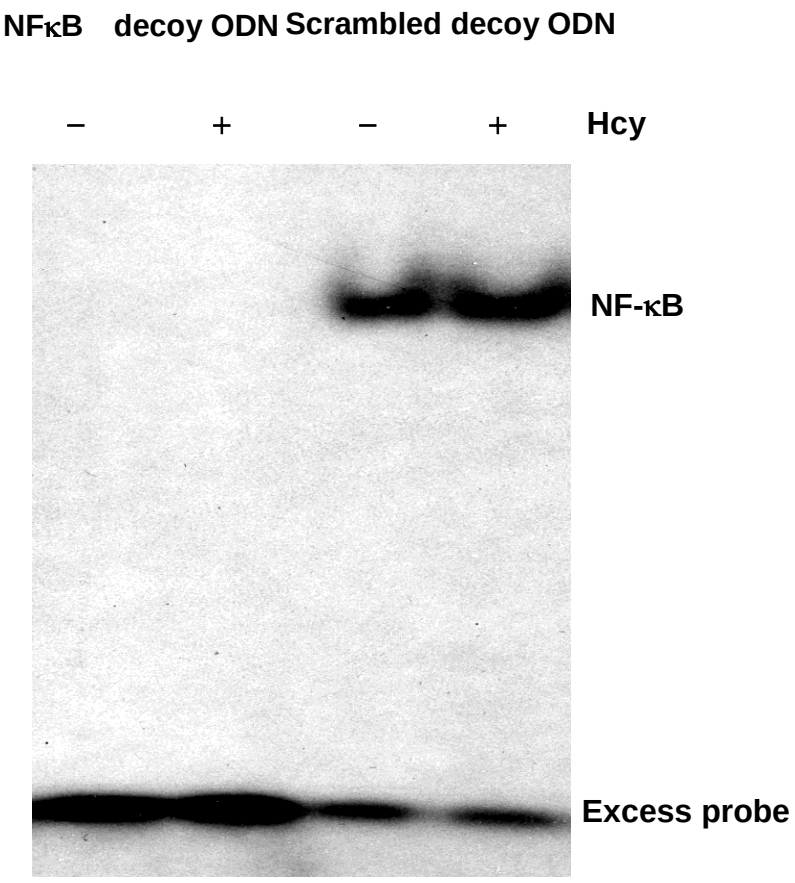

Figure 4.15 Effect of Hcy on NF-κB activation in tubular cells

Human kidney proximal tubular cells were transfected with decoy NF-κB oligodeoxynucleotides (decoy NF-κB ODN) or scrambled decoy ODN. Transfected cells were incubated in the absence or presence of Hcy (100 μM). After 15 min incubation, NF-κB/DNA binding activity was determined by EMSA.

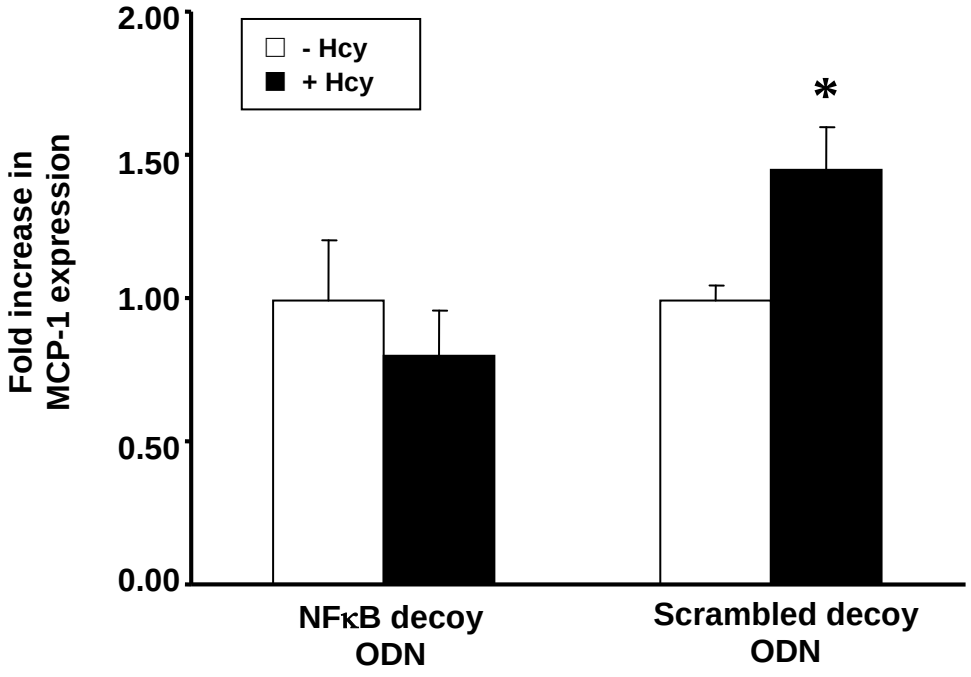

Figure 4.16 Effect of Hcy on MCP-1 expression in tubular cells

Human kidney proximal tubular cells were transfected with decoy NF-κB oligodeoxynucleotides (decoy NF-κB ODN) or scrambled decoy ODN. Transfected cells were incubated in the absence or presence of Hcy (100 μM). After 4h incubation, MCP-1 mRNA expression was determined by a real-time PCR detection system. Results are expressed as mean ± SEM (n = 5). *P<0.05 when compared with the value obtained from the control group.

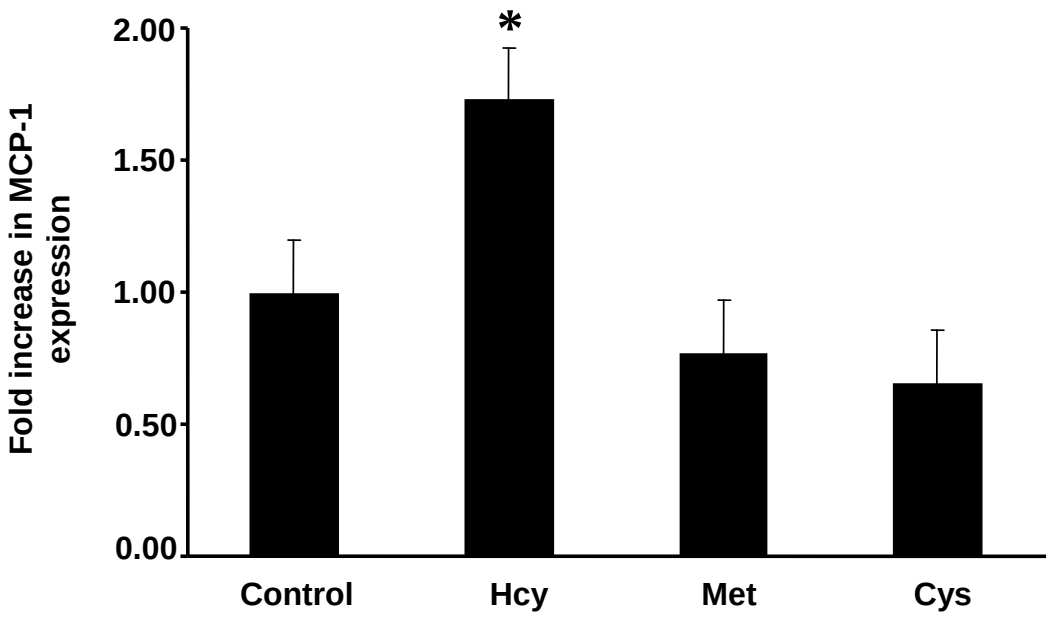

Figure 4.17 Effect of sulfur-containing amino acids on MCP-1 expression in tubular cells

Human kidney proximal tubular cells were incubated in the absence or presence of Hcy (100 μM), cysteine (Cys) (100 μM) or methionine (Met) (100 μM) for 4h. Results are expressed as mean ± SEM (n = 5). **P*<0.05 when compared with the value obtained from the control group.

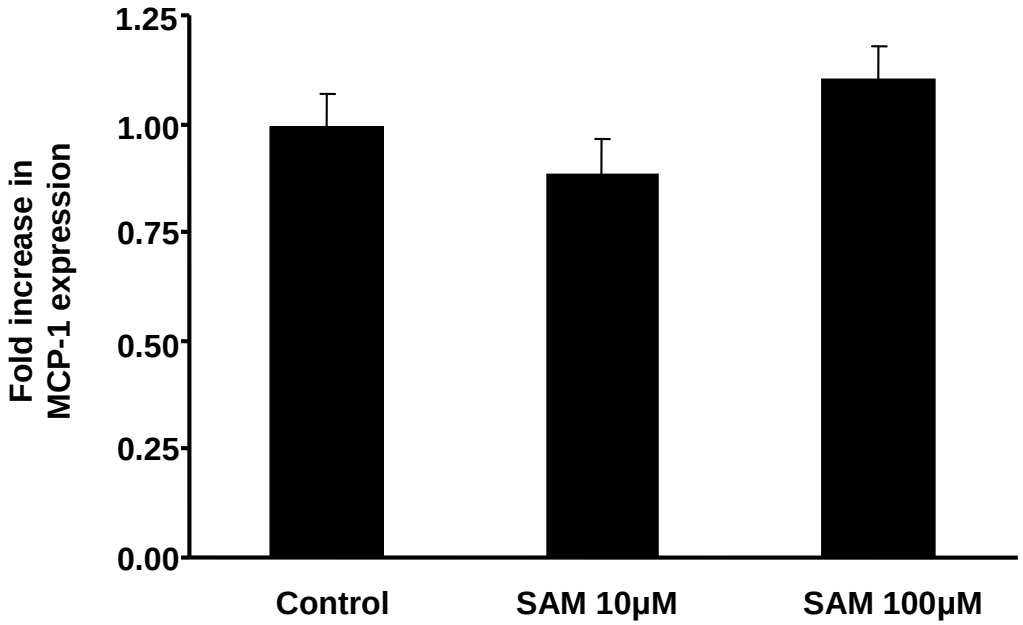

Figure 4.18 Effect of S-adenosylmethionine (SAM) on MCP-1 expression in tubular cells

Human kidney proximal tubular cells were incubated in the absence or presence of S-adenosyl-methionine (SAM, 10 or 100 μM) for 4h. MCP-1 mRNA expression was determined by a real-time PCR detection system. Results are expressed as mean ± SEM (n = 3).

4.5 Discussion

Results from the present study have clearly demonstrated that in the absence of other known risk factors, hyperhomocysteinemia can induce MCP-1 expression in rat kidneys. Activation of NF-κB due to increased phosphorylation of IκBα plays an important role in hyperhomocysteinemia-induced MCP-1 expression in the kidney tissue. Hcy-induced MCP-1 production in tubular cells leads to increased monocyte chemotaxis.

Increased chemokine expression in the tissue is one of the key steps in the inflammatory response. Chemokines including MCP-1 play an important role in leukocyte infiltration and activation during the inflammatory process. Similar to its role in the pathogenesis of atherosclerosis, elevated MCP-1 production contributes significantly to the recruitment of leukocytes into the kidney during the development of glomerulosclerosis (Anders *et al.*, 2001; Kuusniemi *et al.*, 2005; Lynn *et al.*, 2001; Lynn *et al.*, 2000; Rovin *et al.*, 1996; Sekiguchi *et al.*, 1997; Tang *et al.*, 1997; Wenzel *et al.*, 1997). An increase in chemokine expression is also responsible for leukocyte recruitment into allografts, which contributes to allograft rejection in kidney transplantation (Grandaliano *et al.*, 1997; Prodjosudjadi *et al.*, 1996; Robertson *et al.*, 2000). We previously reported that Hcy stimulated MCP-1 production in vascular smooth muscle cells (Wang *et al.*, 2000), endothelial cells (Sung *et al.*, 2001) and macrophages (Wang *et al.*, 2001). We have postulated that Hcy-induced MCP-1 expression in vascular cells may play an important role in the development of atherosclerosis in patients with hyperhomocysteinemia (Choy *et al.*, 2004; O & Siow, 2003). However, little information is available regarding the effect of hyperhomocysteinemia on chemokine expression in the kidney. The present study clearly demonstrated that Hcy, at elevated concentrations, also stimulated the

expression of MCP-1 mRNA and protein in the kidney. The MPO activity, used as an index of leukocyte infiltration, was increased in kidneys of hyperhomocysteinemic rats. Majority of patients with chronic renal disease displayed hyperhomocysteinemia (Ninomiya *et al.*, 2004; Wheeler, 1996). Epidemiological studies have revealed an inverse correlation between serum levels of Hcy and renal function (Ninomiya *et al.*, 2004). However, the impact of Hcy, at pathologically high concentrations, on the kidney itself is unknown. To the best of our knowledge, this is the first study demonstrating that hyperhomocysteinemia could induce MCP-1 expression in the kidney. It is plausible that such a stimulatory effect may play an important role in inflammatory response, which in turn, aggravates renal dysfunction in patients with hyperhomocysteinemia.

The activation of NF-κB is intimately involved in inflammatory reaction. NF-κB activation has been implicated to play an important role in chemokine expression (Sung *et al.*, 2001; Wang *et al.*, 2001; Wang *et al.*, 2000). This transcription factor can be activated by diverse pathogenic signals. In general, a rapid phosphorylation and degradation of its inhibitor protein (IκBα) lead to nuclear translocation of NF-κB. Once inside the nucleus, the NF-κB binds to the promoter region of the target genes including MCP-1 and regulates gene expression. Several lines of evidence obtained from the present study suggested that the activation of NF-κB plays an important role in MCP-1 expression in the kidney during hyperhomocysteinemia. First, the levels of total IκBα was markedly reduced in the kidneys of hyperhomocysteinemic rats as a result of increased phosphorylation of this inhibitor protein. Second, results from the EMSA revealed that the binding activity of NF-κB/DNA was significantly increased, suggesting an activation of this transcription factor during hyperhomocysteinemia.

Pretreatment of hyperhomocysteinemic rats with a known NF-κB inhibitor reduced MCP-1 expression in the kidneys to the level similar to those found in the control group. Third, the involvement of NF-κB was further examined in proximal tubular cells transfected with NF-κB decoy oligodeoxynucleotide to inhibit the activation of this transcription factor. Indeed, inhibition of NF-κB activation completely abolished Hcy-induced MCP-1 expression in tubular cells. Taken together, these results indicated that NF-κB activation was necessary for Hcy-induced chemokine expression in the kidney. We previously demonstrated that Hcy could induce MCP-1 expression via NF-κB activation in vascular cells (Sung *et al.*, 2001; Wang *et al.*, 2000; Wang *et al.*, 2001; Wang *et al.*, 2002). Our results together with others suggest that Hcy-induced MCP-1 expression in vascular cells serve as one of the important mechanisms contributing to Hcy-induced atherosclerosis. The present study provides evidence that hyperhomocysteinemia activates NF-κB leading to increased MCP-1 expression in the kidney. Increased chemokine expression, in turn, facilitates leukocyte recruitment into the kidney. Those infiltrated cells are capable of producing inflammatory factors that exacerbate kidney injury. Taken together, these results suggest that Hcy-induced MCP-1 expression via NF-κB activation may serve as one of the mechanisms contributing to kidney injury.

In summary, the present study clearly demonstrates a direct link between hyperhomocysteinemia and the inflammatory response in the kidney. Our results suggest that activation of the NF-κB pathway is essential for Hcy-induced MCP-1 expression in the kidney. Increased chemokine expression may represent one of the important mechanisms that contribute to renal injury in patients with hyperhomocysteinemia.

V. Study 3: Role of Folic Acid Supplementation on Oxidative Stress-mediated Chemokine Expression in the Kidney

(Manuscript 3: Folic acid supplementation abolishes monocyte chemoattractant protein-1 expression in the kidney via nuclear factor-kappa B activation)

(In preparation for submission)

5.1 Abstract

Hyperhomocysteinemia, a condition of elevated blood homocysteine (Hcy) levels, is a metabolic disease. It is a common clinical finding in patients with chronic kidney disease and occurs almost uniformly in patients with end-stage renal disease. Inflammatory response has been implicated as one of the important mechanisms causing renal disease. Monocyte chemoattractant protein-1 (MCP-1) is a potent chemokine that is involved in the inflammatory response in renal disease. Folic acid supplementation is regarded as a promising approach for prevention and treatment of cardiovascular diseases associated with hyperhomocysteinemia due to its Hcy-lowering effect. However, its therapeutic effect on the kidney during hyperhomocysteinemia is not clear. The aim of this study was to examine the effect of folic acid supplementation on Hcy-induced MCP-1 expression and the molecular mechanism underlying such an effect in the kidney of hyperhomocysteinemic rats. Hyperhomocysteinemia was induced in male Sprague-Dawley rats fed a high-methionine diet for 12 wk with or without folic acid supplementation. A group of rats fed a regular diet was used as control. There was a significant increase in levels of MCP-1 mRNA in kidneys isolated from hyperhomocysteinemic rats. The NF-κB activity was significantly increased in those kidneys. Pretreatment of hyperhomocysteinemic rats with a NADPH oxidase inhibitor, apocynin, abolished hyperhomocysteinemia-induced MCP-1 expression in the kidney, indicating that hyperhomocysteinemia-induced superoxide anion generated by NADPH oxidase played a critical role in stimulating MCP-1 expression in the kidney of hyperhomocysteinemic rats. Folic acid supplementation could effectively abolish MCP-1 expression in those kidneys. Our results suggested that folic acid supplementation could abolish MCP-1 expression via inhibition of NADPH oxidase-

mediated oxidative stress in the kidney of hyperhomocysteinemic rats. Folic acid supplementation may offer renal protective effect not only against oxidative stress but also against an inflammatory response.

5.2 Introduction

Hyperhomocysteinemia is a metabolic disorder that is characterized by an elevation of homocysteine (Hcy) level in the circulation. It is very well known that hyperhomocysteinemia is an independent risk factor for cardiovascular diseases (Nygard *et al.*, 1997a; Clarke *et al.*, 1991; McCully, 1996). Hyperhomocysteinemia is also a common clinical finding in patients with chronic kidney disease and occurs almost uniformly in patients with end-stage renal disease. Although impaired kidney function is one of the common factors causing hyperhomocysteinemia, the adverse effect of Hcy, at pathological concentrations, on the kidney is not well documented. Recent epidemiological investigation has identified a positive association between an elevation of Hcy level in the blood and the development of chronic kidney disease in the general population (Ninomiya *et al.*, 2004). However, in contrast to Hcy-induced atherosclerosis, little information is available regarding the mechanism by which Hcy may exert adverse effects on the kidney.

Hcy is a sulfhydryl-containing amino acid formed during the metabolism of methionine to cysteine. Hcy, at abnormally high concentrations, elicits inflammatory response and impairs endothelial function via activation of transcription factors such as nuclear factor-kappaB (NF-κB), inducing oxidative stress and chemokine expression causing monocyte accumulation in the vascular endothelium and the kidney (Hwang *et al.*, 2008; Kumagai *et al.*, 2002; Li *et al.*, 2002; Zhang *et al.*, 2004). Transcriptional regulation of inflammatory response plays an important role in the development of kidney disease. Our recent studies clearly demonstrated that hyperhomocysteinemia caused an increased nitric oxide production leading to the formation of peroxynitrite, a potent oxygen free radical, in rat kidneys (Zhang *et al.*, 2004). Further analysis revealed that activation of NF-κB was involved in Hcy-

induced inducible nitric oxide synthase (iNOS) expression and monocyte chemoattractant protein-1 (MCP-1) expression in the kidney (Zhang *et al.*, 2004; Hwang *et al.*, 2008).

The NF-κB is a transcriptional factor that plays an important role in regulating gene expression of many inflammatory factors. NF-κB can be rapidly activated in response to stimuli leading to the upregulation of its target genes (Karin, 1999; Mercurio *et al.*, 1997; Simeonidis *et al.*, 1999). In the resting cell, NF-κB resides in the cytoplasm in an inactive form that is associated with an inhibitory protein (IκB). Among the several inhibitor proteins identified, IκBα is the best characterized form of IκB. Upon stimulation, there is a rapid phosphorylation of IκBα and subsequent degradation of IκBα by the proteasome, leading to the release of NF-κB. After dissociation from IκB, the free NF-κB can enter the nucleus. Once inside the nucleus, NF-κB binds to the κB binding motifs in the promoters or enhancers of its target genes (Karin, 1999; Mercurio *et al.*, 1997; Simeonidis *et al.*, 1999). Once activated, NF-κB is able to upregulate gene expression of many inflammatory molecules such as MCP-1. MCP-1 is a potent chemokine that stimulates the migration of leukocytes including monocytes into the intima of the arterial wall and other tissues such as kidney. In normal kidneys, little MCP-1 is detectable. However, MCP-1 gene expression is significantly increased in kidneys of patients and experimental animal models with kidney disease (Kuusniemi *et al.*, 2005; Rovin *et al.*, 1996; Sekiguchi *et al.*, 1997; Tang *et al.*, 1997; Wenzel *et al.*, 1997).

NF-κB is a redox-sensitive transcription factor. Therefore, reactive oxygen species (ROS) can induce phosphorylation and degradation of IκBα leading to NF-κB activation (Au-Yeung *et al.*, 2004). Nicotinamide adenine dinucleotide phosphate

(NADPH)-dependent oxidase is primarily responsible for intracellular superoxide anion generation. It is a major source of superoxide anion generation in the kidney (Geiszt *et al.*, 2000). Our previous study showed that activation of NADPH oxidase was responsible for hyperhomocysteinemia-induced oxidative stress in the kidney (Hwang *et al.*, 2011). However, it is not clear whether NADPH oxidase-mediated oxidative stress can play a role in stimulating MCP-1 expression via NF-κB activation in the kidney during hyperhomocysteinemia.

Folic acid is a synthetic form of the naturally occurring folate that is a water soluble B vitamin. Folate plays an important role in regulating Hcy metabolism. Hcy can be metabolized via two major pathways, namely, the remethylation pathway and the transsulfuration pathway. In the remethylation pathway, Hcy can be converted to methionine catalyzed by methionine synthase with folate as a co-substrate. In the transsulfuration pathway, Hcy is irreversibly converted to cystathionine by cystathionine-β- synthase. Factors that interrupt the steps in Hcy metabolic pathways can cause an increase in cellular Hcy levels and lead to its elevation in the blood (Kang *et al.*, 1992; Refsum *et al.*, 1998). Although folate reduces the concentration of plasma Hcy and has a protective potential against cardiovascular diseases, recent studies have suggested that folate may exert protective effects independent of its Hcy lowering effect. Our previous study demonstrated that Hcy-induced superoxide anion production via NADPH oxidase activation played a critical role in NF-κB activation in macrophages (Au-Yeung *et al.*, 2006). Pretreatment of cells with folic acid not only inhibited NADPH oxidase-mediated superoxide anion production but also attenuated Hcy-induced NF-κB activation and subsequent MCP-1 expression, indicating that the effect of folic acid was independent of its Hcy-lowering effect (Au-Yeung *et al.*, 2006). Furthermore, we also showed that folic acid supplementation effectively

abolished Hcy-induced superoxide anion generation via NADPH oxidase activation in kidneys isolated from hyperhomocysteinemic rats (Hwang *et al.*, 2011). However, the role of folic acid supplementation on Hcy-induced inflammatory response in the kidney remains to be investigated. Therefore, the aim of the present study was to investigate the effect of folic acid on Hcy-induced MCP-1 expression and underlying mechanism of such an effect in the kidney of hyperhomocysteinemic rats.

5.3 Materials and Methods

5.3.1 Animal model

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) aged 8 wk were divided into 4 groups and maintained for 12 wk on the following diets: *1)* regular diet (Control), consisting of Lab Diet Rat Diet 5012 (PMI Nutrition International, St. Louis, MO), *2)* high-methionine (Met) diet, consisting of regular diet plus 1.7% (wt/wt) Met, *3)* high-Met plus folic acid diet, consisting of regular diet plus 1.7% (wt/wt) Met and 0.25% (wt/wt) folic acid (Met + folic acid), and *4)* in one set of experiments, high-Met-fed rats were injected with 4 mg/kg of apocynin, an inhibitor of NADPH oxidase (Met + apocynin; Calbiochem, San Diego, CA), intraperitoneally once a day for 7 days before euthanasia. After being on the experimental diets for 12 wk, rats were euthanized by injection of a high dose of sodium pentobarbital intraperitoneally. The portion of the rat kidney used for assays including PCR consisted of cortex and medullar. Results from our previous studies demonstrated that hyperhomocysteinemia could be induced in rats by feeding a high-Met diet (Woo *et al.*, 2008; Hwang *et al.*, 2008; Wu *et al.*, 2009). Total Hcy (tHcy) concentrations in the serum were measured with the IMx Hcy assay, which was based on fluorescence polarization immunoassay technology (Abbott Diagnostics, Abbott Park, IL). All procedures were performed in accordance with the *Guide for the Care and Use of Experimental Animals* published by Canadian Council on Animal Care and approved by University of Manitoba Protocol Management and Review Committee.

5.3.2 Measurement of MCP-1 mRNA level

Total RNAs were isolated from kidneys with TriZol reagent (Invitrogen Life Technologies, Carlsbad, CA). The MCP-1 mRNA was measured by a real-time polymerase chain reaction (PCR) analysis using an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). In brief, 2 μg of total RNA was converted to cDNA by reverse transcriptase. The reaction mixture of real-time PCR contained 0.4μM of 5'- and 3'- primer and 2μL cDNA product in iQ-SYBR Green supermix reagent (Bio-Rad). The primers used for rat MCP-1 were (forward) 5'-CAGAAACCAGCCAACTCTCA-3' and (reverse) 5'-AGACAGCACGTGGATGCTAC-3' (GenBank™/ Accession number: AF058786) and for rat GAPDH were (forward) 5'-TCAAGAAGGTGGTGAAGCAG -3' and (reverse) 5'- AGGTGGAAGAATGGGAGTTG-3' (GenBank™/ Accession number: NM_017008). All primers were synthesized by Invitrogen. The relative change in MCP-1 mRNA expression was determined by the fold change analysis in which fold change = $2^{-\Delta\Delta Ct}$, where Ct = $(Ct_{MCP\text{-}1} - Ct_{GAPDH})_{\text{ treatment}}$ - $(Ct_{MCP\text{-}1} - Ct_{GAPDH})_{\text{ Control}}$ (Livak & Schmittgen, 2001). Ct was the cycle number at which the fluorescence signal crossed the threshold, which was determined by iQ5 Optical System Software (version 2, Bio-Rad).

5.3.3 Immunohistochemical staining

For detection of MCP-1 protein, kidneys were excised from rats fed different types of diet. A portion of the kidney was immersion-fixed in 10% neutral-buffered formalin overnight and then embedded in paraffin. Sequential 5-μm paraffin-embedded sections were immunostained using rabbit polyclonal antibodies (1:100) as

primary antibodies against rat MCP-1 (PeproTech EC Ltd., Rocky Hill, NJ). Endogenous peroxidase was blocked with 0.3% H_2O_2 for 20 min. The secondary antibodies for immunostaining were biotin-conjugated anti- rabbit immunoglobulins (1:200) (Dako Canada, Inc., Mississauga, ON). Sections were then treated with 3,3-diaminobenzidine (DAB)-H_2O_2 colorimetric substrate solution. The attached peroxidase catalyzed the H_2O_2-mediated oxidation of DAB to yield brown color. The area displayed brownish color indicating the MCP-1 protein adducts. For a negative control, non-specific rabbit IgG was used as primary antibodies.

5.3.4 Electrophoretic mobility shift assay

Nuclear proteins were isolated and electrophoretic mobility shift assay (EMSA) was performed to determine the NF-κB/DNA binding activity (Au-Yeung *et al.*, 2004; Zhang *et al.*, 2004). In brief, nuclear proteins (10 μg) were incubated with the reaction buffer for 15 min at room temperature followed by incubation with ^{32}P-end-labelled oligonucleotide containing the consensus sequence for the κB binding site (5'-GGGGACTTTCC-3') (Promega, Madison, WI). The reaction mixture was separated in a non-denaturing polyacrylamide gel (6%) that was later exposed to X-ray film at –80^{o}C. The binding of labeled oligonucleotide to nuclear proteins was blocked by adding 100-fold excess unlabelled specific competitor oligonucleotide to the reaction mixture. This was to confirm that the binding of ^{32}P-end-labeled oligonucleotide to NF-κB was sequence-specific.

5.3.5 Myeloperoxidase activity assay

The myeloperoxidase (MPO) activity was measured in the kidney to assess the leukocyte infiltration into the tissue as described previously (Sung *et al.*, 2002;

Chatterjee *et al.*, 2000). A portion of the kidney was homogenized in 3mL of 50mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltri-methylammoniun bromide. The homogenate was centrifuged at 20,000 ×g for 20 minutes and the supernatant was collected. The reaction mixture containing 0.6 mM 3,3',5,5'-tetramethylbenzidine, 0.03 M hydrogen peroxide, 80 mM sodium phosphate buffer (pH 5.5) and an aliquot of supernatant was incubated at 25 °C for 15 minutes. The reaction was stopped by the addition of 1 mL of 0.5 M sulfuric acid. The absorbance was measured at 450 nm. Peroxidase (Sigma) was used as an external standard. MPO activity was expressed as mU MPO activity per mg protein.

5.3.6 Western immunoblot analysis

Kidney total IκBα and phosphorylated IκBα proteins levels were determined by a Western immunoblot analysis. For the measurement of total IκBα and phosphorylated IκBα proteins, kidney proteins (100 μg/mL) were separated by electrophoresis on a 12.5% SDS polyacrylamide gel. Proteins on the gel were then transferred to a nitrocellulose membrane. The membrane was probed with rabbit anti-IκBα polyclonal antibodies or anti-phospho-IκBα (Ser32) polyclonal antibodies (New England Biolabs Inc., Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies (Zymed, South San Francisco, CA) were used to develop the membranes. The IκBα and phospho-IκBα protein bands were visualized using enhanced chemiluminescence reagents and analyzed with a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst® version 1.1).

5.3.7 Statistical analysis

The results were analyzed using one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. Data were presented as the mean ± SEM. The level of statistical significance was set at $P < 0.05$.

5.4 Results

5.4.1 Effect of folic acid on MCP-1 expression in the kidney during hyperhomocysteinemia.

Rats that were fed a high-methionine diet for 12 wk developed hyperhomocysteinemia (Fig. 5.1). The high-methionine diet resulted in a significant increase in the serum tHcy levels compared with the control group. Supplementation of folic acid to rats fed a high-methionine diet significantly lowered the serum tHcy levels (Fig. 5.1). Next, the levels of MCP-1 expression in those kidneys were examined. There was a significant increase in MCP-1 mRNA levels in kidneys isolated from rats fed a high-methionine diet (Fig. 5.2). When pyrrolidine dithiocarbamate (PDTC), a known inhibitor for NF-κB activation (Sung *et al.*, 2002; Zhang *et al.*, 2004), was injected into the high-methionine fed rats, the renal levels of MCP-1 mRNA were significantly reduced to that of the control group (Fig. 5.2). In accordance with our previous studies, these results suggested that increased MCP-1 expression in the kidney during hyperhomocysteinemia might be mediated via NF-κB activation (Hwang *et al.*, 2008; Sung *et al.*, 2002). Folic acid supplementation effectively reduced the MCP-1 expression in kidneys of hyperhomocysteinemic rats (Fig. 5.1 and 5.2). Administration of apocynin, an inhibitor for NADPH oxidase, to hyperhomocysteinemic rats also reduced the MCP-1 expression in kidneys isolated from hyperhomocysteinemic rats (Fig. 5.1 and 5.2). These results suggested that NADPH oxidase might be involved in increased free radical generation in the kidney, leading to increased MCP-1 expression in hyperhomocysteinemic rats. We previously demonstrated that Hcy played a critical role in inflammatory response in macrophages by stimulating MCP-1 expression (Au-Yeung *et al.*, 2006). Furthermore, pretreatment of cells with folic acid significantly abolished Hcy-induced MCP-1 mRNA expression

(Au-Yeung *et al.*, 2006). In another study, we observed that folic acid supplementation effectively abolished Hcy-induced superoxide anion production via NADPH oxidase activation in kidneys of hyperhomocysteinemic rats (Hwang *et al.*, 2011). Therefore, these results suggested that supplementation of folic acid to rats fed a high-methionine diet not only might attenuate Hcy-induced NADPH oxidase activation but also reduce the MCP-1 expression in the kidney of hyperhomocysteinemic rats.

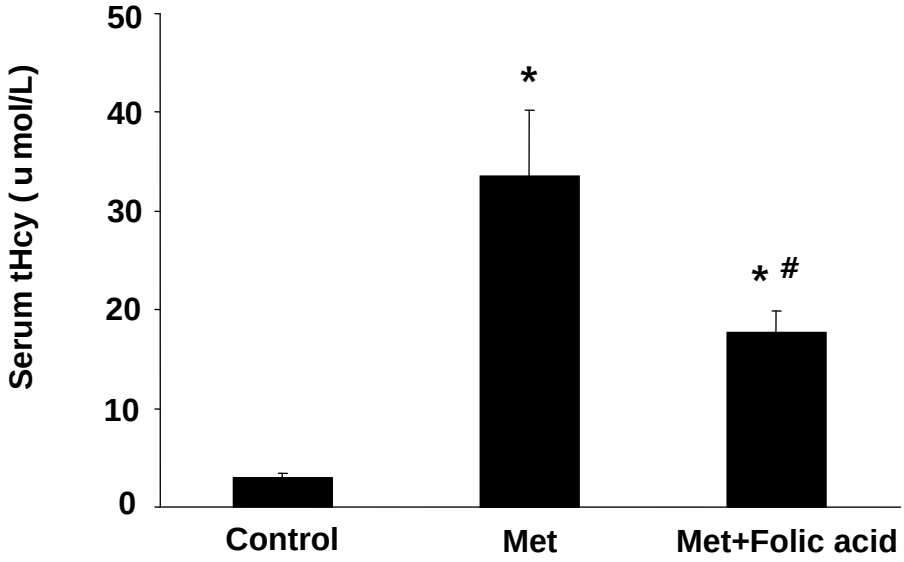

Figure 5.1 Measurement of serum total homocysteine (tHcy) levels

Rats were fed with the following diets for 12 wk: a regular diet (Control), a high-methionine diet (1.7% w/w Met), or a high-methionine diet plus folic acid (0.25% w/w; Met + Folic acid). Hcy levels in the serum were measured. Results were expressed as means ± SEM (n=4). *$P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the high-methionine fed group.

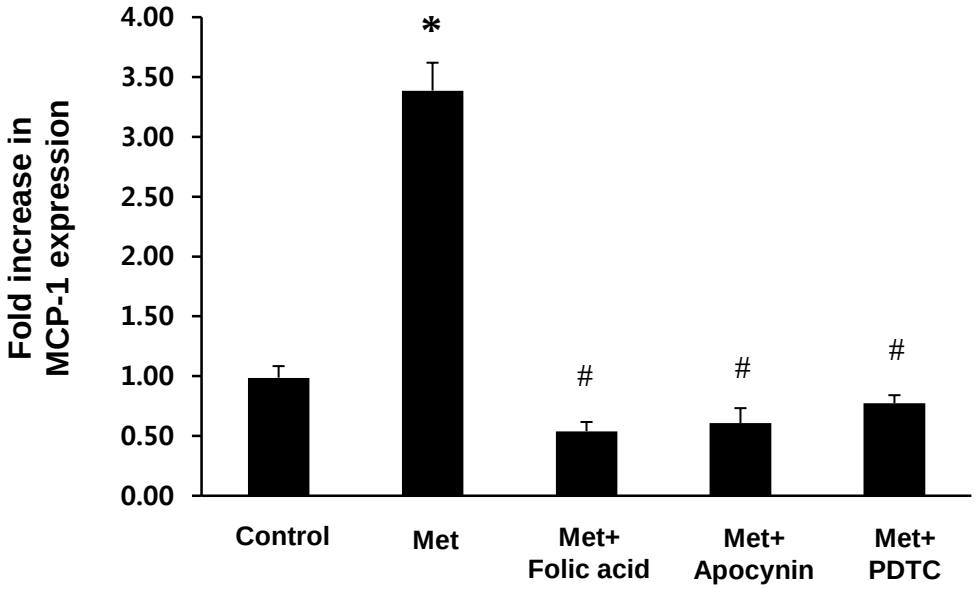

Figure 5.2 Expression of monocyte chemoattractant protein-1 (MCP-1) mRNA in rat kidneys

Rats were fed with the following diets for 12 wk: a regular diet (Control), a high-methionine diet (1.7% Met), or a high-methionine diet plus folic acid (0.25%; Met + Folic acid). In one set of experiments, apocynin (4 mg/kg, daily, ip), a known inhibitor of NADPH oxidase, was given to rats fed a high-methionine diet (Met+Apocynin) for 7 days prior to euthanasia. Another set of experiments, pyrrolidine dithiocarbamate (PDTC, 100 mg/kg, daily, ip) was given to rats fed a high-methionine diet (Met+PDTC) for 3 days before euthanasia. Total RNAs were prepared from rat kidneys and the MCP-1 mRNA levels were measured by a real-time PCR detection system. Results were expressed as mean ± SEM (n=4). $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the high-methionine fed group.

5.4.2 Effect of folic acid on the distribution of MCP-1 protein and leukocyte infiltration in the kidney during hyperhomocysteinemia.

The distribution of MCP-1 protein in the kidney was examined by immunohistochemical analysis. Compared with the control group, kidneys isolated from rats fed a high-methionine diet displayed an increased staining for MCP-1 protein in the cortex and medulla (Fig. 5.3). Folic acid supplementation effectively attenuated the staining for MCP-1 protein in kidneys of hyperhomocysteinemic rats (Fig. 5.3). As a negative control, the immunohistochemical staining was performed with nonspecific anitibodies (IgG) and no positive staining was observed (Fig. 5.3). Leukocyte infiltration was assessed by measuring the MPO activity in the kidney. The MPO activity was significantly increased in kidneys isolated from hyperhomocysteinemic rats, indicating an elevation of leukocyte infiltration into the kidney during hyperhomocysteinemia (Fig. 5.4). Folic acid supplementation effectively reduced the MPO activity in kidneys of hyperhomocysteinemic rats (Fig. 5.4). Administration of apocynin, a known inhibitor for NADPH oxidase, to hyperhomocysteinemic rats also reduced the MPO activity in kidneys isolated from hyperhomocysteinemic rats (Fig. 5.4). These results suggested that NADPH oxidase might be involved in increased free radical generation in the kidney, leading to increased leukocyte infiltration in hyperhomocysteinemic rats.

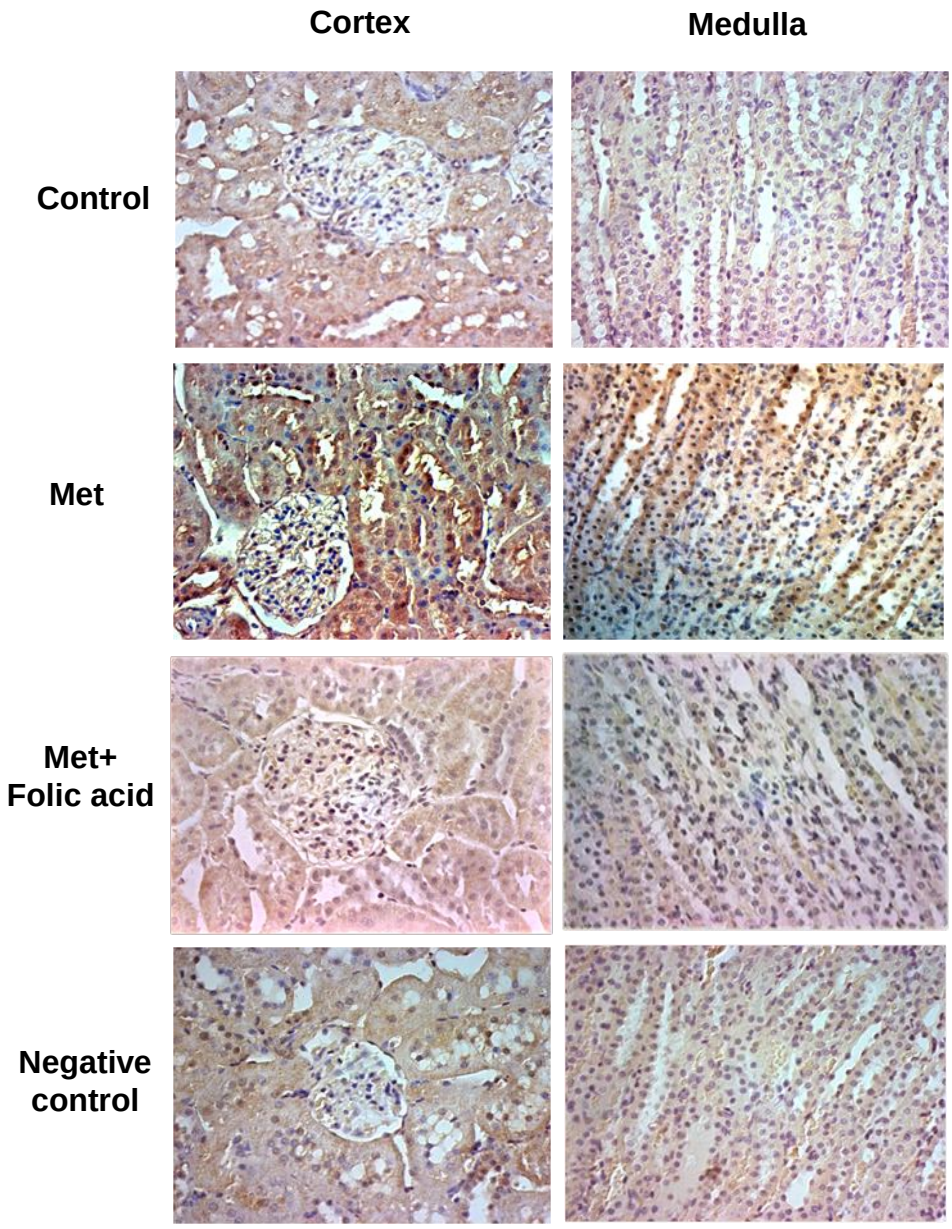

Figure 5.3 Immunohistochemical staining of MCP-1 protein in rat kidneys

Rats were fed with following diets for 12 wk: a regular diet (Control), a high-methionine diet (1.7% Met), or a high-methionine diet plus folic acid (0.25%; Met + Folic acid). Immunohistochemical staining for MCP-1 protein was performed with anti-MCP-1 antibodies. After counterstaining with Mayer's hematoxylin, MCP-1 protein was identified under light microscope at a magnification of ×400. As a negative control, immunohistochemical staining was performed by using nonspecific rabbit IgG as primary antibodies. All staining analyses were performed in kidneys isolated from 4 rats of each group. Representative photos are shown.

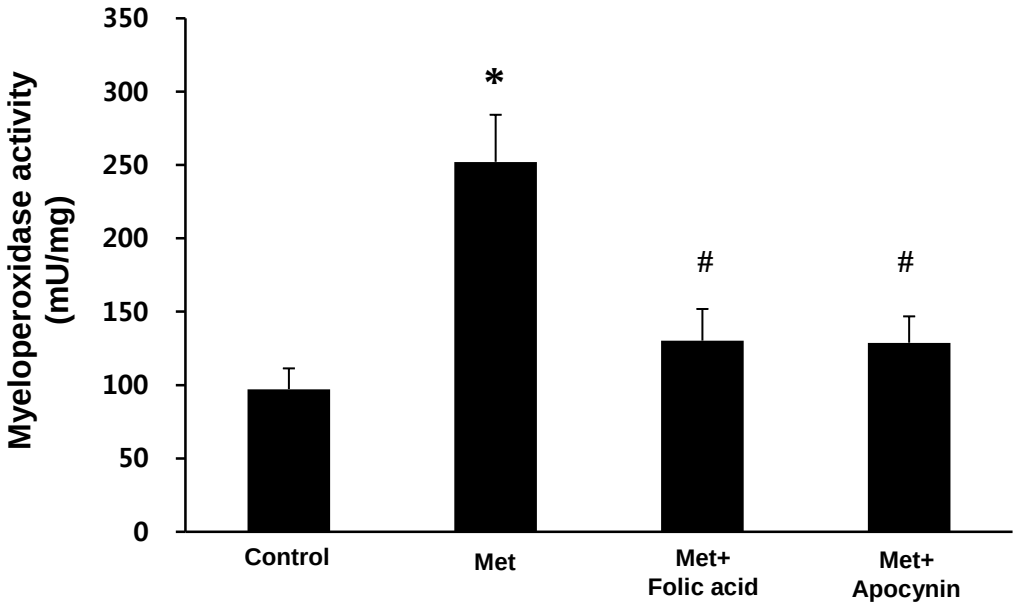

Figure 5.4. Determination of myeloperoxidase (MPO) activity in rat kidneys

Rats were fed with the following diets for 12 wk: a regular diet (Control), a high-methionine diet (1.7% Met), or a high-methionine diet plus folic acid (0.25%; Met + Folic acid). In one set of experiments, apocynin (4 mg/kg, daily, ip) was given to rats fed a high-Met diet (Met + Apocynin) for 7 days before euthanasia. MPO activity was determined to evaluate leukocyte infiltration. Results were expressed as means ± SEM (n=4). $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of high-Met-fed group.

5.4.3 Effect of folic acid on NF-κB activation in the kidney during hyperhomocysteinemia

The NF-κB activation in the kidney was examined by EMSA. The NF-κB/DNA binding activity was significantly elevated in kidneys isolated from rats fed a high-methionine diet compared with the control (Fig. 5.5). Western immunoblot analysis was performed to determine the levels of phosphorylated IκBα protein and total IκBα protein in the kidney. There was a significant increase in the level of phosphorylated IκBα protein (Fig. 5.6) and, as a consequence, a significant reduction in the level of total IκBα protein (Fig. 5.7) in kidneys isolated from rats fed a high-methionine diet. These results suggested that Hcy-induced NF-κB activation was mediated via increased phosphorylation and degradation of IκBα protein in the kidney. Supplementation of folic acid to the rats of hyperhomocysteinemia effectively abolished Hcy-induced phosphorylation of IκBα protein and hence prevented NF-κB activation (Fig. 5.5-5.7). To investigate further the link between hyperhomocysteinemia-induced oxidative stress and NF-κB activation and subsequent MCP-1 expression, we treated rats fed a high-methionine diet with apocynin, a known NADPH oxidase inhibitor. Such treatment also completely blocked Hcy-induced IκBα phosphorylation and NF-κB activation in kidneys of hyperhomocysteinemic rats (Fig. 5.5-5.7), indicating that the NADPH oxidase system might be activated upon Hcy treatment and such an activation might be necessary for Hcy-induced IκBα phosphorylation. Therefore, these results suggested that Hcy-induced superoxide anion production via NADPH oxidase might play a critical role in inflammatory response in the kidney. Folic acid supplementation effectively

antagonized hyperhomocysteinemia-induced oxidative stress and subsequent chemokine expression via both its Hcy-dependent and Hcy-independent effects.

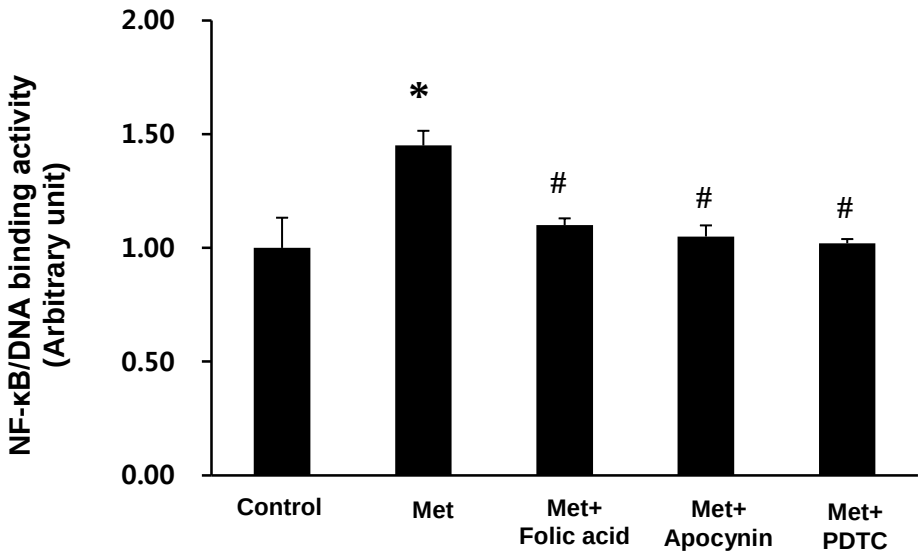

Figure 5.5 NF-κB activation in rat kidneys

Rats were fed with the following diets for 12 wk: a regular diet (Control), a high-methionine diet (1.7% Met), or a high-methionine diet plus folic acid (0.25%; Met + Folic acid). In one set of experiments, apocynin (4 mg/kg, daily, ip) was given to rats fed a high-Met diet (Met + Apocynin) for 7 days before euthanasia. Another set of experiments, pyrrolidine dithiocarbamate (PDTC; 100 mg/kg, daily, ip) was given to rats fed a high-Met diet (Met + PDTC) for 3 days before euthanasia. NF-κB/DNA binding activity was determined by EMSA. Results were expressed as means ± SEM (n=4). *$P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the high-Met-fed group.

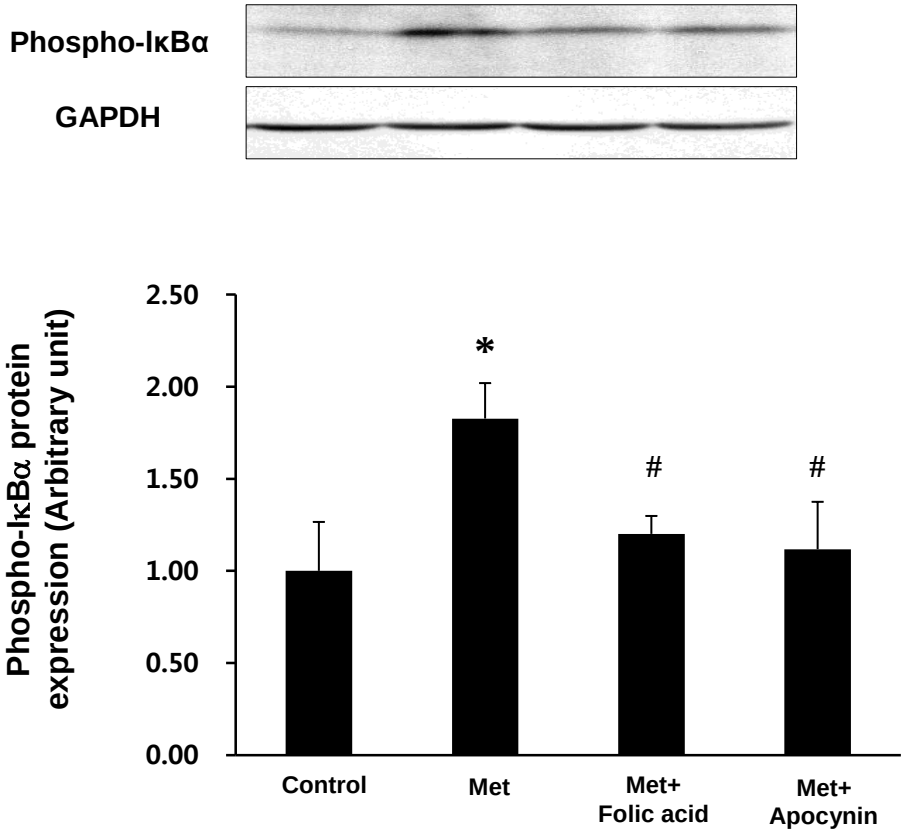

Figure 5.6 Phospho-IκBα protein levels in rat kidneys

Rats were fed with the following diets for 12 wk: a regular diet (Control), a high-methionine diet (1.7% Met), or a high-methionine diet plus folic acid (0.25%; Met + Folic acid). In one set of experiments, apocynin (4 mg/kg, daily, ip) was given to rats fed a high-Met diet (Met + Apocynin) for 7 days before euthanasia. Levels of phospho-IκBα protein were measured using Western immunoblot analysis. Results were expressed as means ± SEM (n=4). $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the high-Met-fed group.

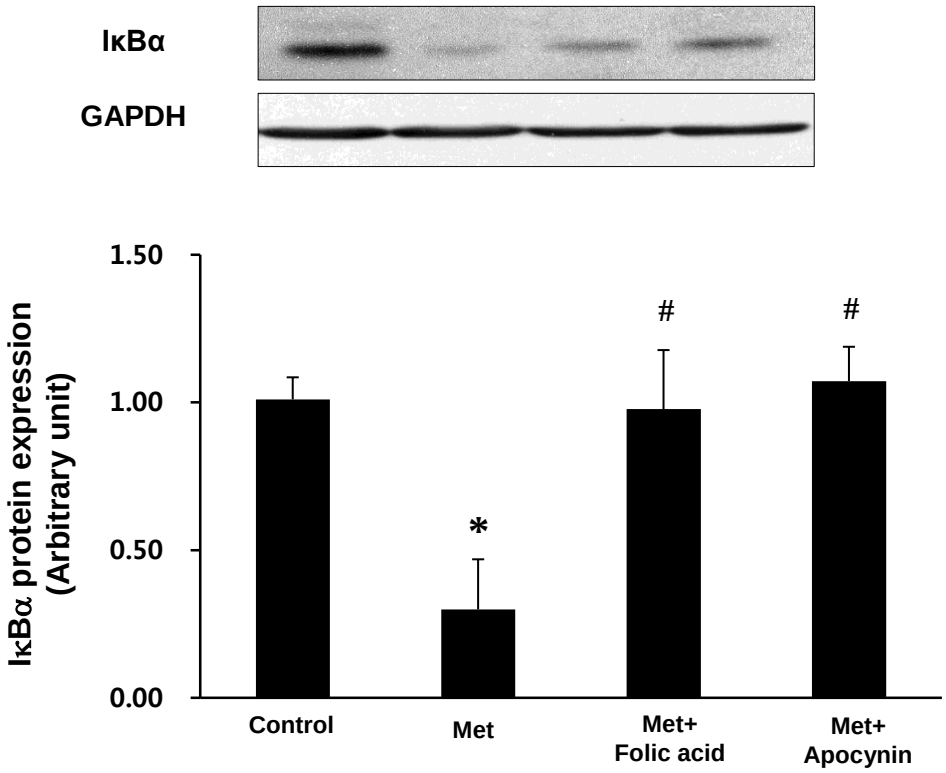

Figure 5.7 Total IκBα protein levels in rat kidneys

Rats were fed with the following diets for 12 wk: a regular diet (Control), a high-methionine diet (1.7% Met), or a high-methionine diet plus folic acid (0.25%; Met + Folic acid). In one set of experiments, apocynin (4 mg/kg, daily, ip) was given to rats fed a high-Met diet (Met + Apocynin) for 7 days before euthanasia. Levels of total IκBα protein were measured using Western immunoblot analysis. Results were expressed as means ± SEM (n=4). *$P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with values of the high-Met-fed group.

5.5 Discussion

The present study, for the first time, clearly demonstrated that Hcy-induced superoxide anion generation via NADPH oxidase activation might play an important role in MCP-1 expression via NF-κB activation in kidneys of hyperhomocysteinemic rats. Folic acid supplementation could effectively abolish Hcy-induced NF-κB activation and MCP-1 expression in those kidneys. Results from the present study showed that activation of NF-κB due to increased phosphorylation of IκBα might play an important role in Hcy-induced MCP-1 expression in the kidney of hyperhomocysteinemic rats. Moreover, our previous studies showed that Hcy-induced MCP-1 production in human proximal tubular cells and rat kidney mesangial cells led to increased monocyte chemotaxis (Cheung *et al.*, 2008; Hwang *et al.*, 2008).

Increased chemokine expression in the tissue is one of the key steps in the inflammatory responses. Chemokines including MCP-1 play an important role in leukocyte infiltration during the inflammatory process. As our laboratory and others demonstrated, similar to its role in the pathogenesis of atherosclerosis, elevated MCP-1 production might significantly contribute to the recruitment of leukocytes into the kidney during the development of glomerulosclerosis (Hwang *et al.*, 2008; Cheung *et al.*, 2008; Lynn *et al.*, 2001; Lynn *et al.*, 2000; Rovin *et al.*, 1996; Sekiguchi *et al.*, 1997; Tang *et al.*, 1997). The present study clearly showed that folic acid supplementation could significantly abolish Hcy-induced NF-κB activation and consequent MCP-1 expression in kidneys isolated from hyperhomocysteinemic rats. Furthermore, folic acid supplementation was able to reduce the MPO activity, used as an index of leukocyte infiltration, in those kidneys. To the best of our knowledge, this is the first study demonstrating that folic acid supplementation can attenuate MCP-1 expression in the kidney during hyperhomocysteinemia. It has been demonstrated that

a majority of patients with chronic kidney disease display hyperhomocysteinemia (Ninomiya *et al.*, 2004; Wheeler, 1996). Epidemiological studies have revealed an inverse correlation between serum levels of Hcy and kidney function (Ninomiya *et al.*, 2004). Therefore, it can be postulated that such an inhibitory effect of folic acid on MCP-1 expression may play a beneficial role in preventing and/or treating inflammatory response, which can aggravate kidney dysfunction in patients with hyperhomocysteinemia.

The activation of NF-κB is intimately involved in inflammatory reaction. NF-κB activation has been implicated to play an important role in chemokine expression (Wang *et al.*, 2000; Wang *et al.*, 2001; Sung *et al.*, 2001). This transcription factor can be activated by diverse pathogenic signals. In general, a rapid phosphorylation and degradation of its inhibitor protein (IκBα) lead to nuclear translocation of NF-κB. Once inside the nucleus, the NF-κB binds to the promoter region of the target genes including MCP-1 and regulates gene expression. Our previous studies showed that the activation of NF-κB might play an important role in MCP-1 expression in the kidney during hyperhomocysteinemia (Cheung *et al.*, 2008; Hwang *et al.*, 2008).

Hcy has been shown to activate NF-κB via its ability to alter redox thiol status of the cell generated by ROS (Outinen *et al.*, 1999; Au-Yeung *et al.*, 2006; Cheung *et al.*, 2008; Hwang *et al.*, 2008). ROS are often generated in cells as by-products or as second messengers in many metabolic and signaling transduction pathways. Intracellular ROS can be scavenged by the antioxidative defense system. However, when ROS are above a certain level, antioxidants and ROS scavengers would no longer be sufficient for their removal. Therefore, as a result, ROS can accumulate and as a consequence oxidative stress can occur. Hcy is readily oxidized

due to its thiol group. The oxidation of two molecules of Hcy can result in the formation of disulfide linked Hcy, two protons and two electrons. When an oxygen molecule is reduced by these electrons, ROS such as superoxide anion are produced. Eventually this auto-oxidation of Hcy molecules generating ROS is related to the generation of various harmful events such as lipid peroxidation and hydroxyl radical production (Hwang *et al.*, 2011; Tien *et al.*, 1982; Rowley & Halliwell, 1982). In patients with end-stage renal disease, oxidative stress often correlates with impaired kidney function. Our previous study clearly showed that a rapid increase of superoxide anion level induced by Hcy in rat mesangial cells, which preceded NF-κB activation and MCP-1 expression. Pretreatment of those cells with superoxide dismutase (SOD), a scavenger of ROS, effectively reduced Hcy-induced superoxide anion production and completely blocked Hcy-induced NF-κB activation (Cheung *et al.*, 2008). Furthermore, a reduction in intracellular superoxide anion levels by SOD significantly reversed Hcy-induced MCP-1 expression, suggesting that oxidative stress might play an important role in the upregulation of MCP-1 expression by Hcy in renal mesangial cells (Cheung *et al.*, 2008). Therefore, it can be postulated that Hcy, at pathophysiologic concentrations, causes an increase in intracellular levels of superoxide anion, promoting phosphorylation of the inhibitory protein IκBα. As a result, NF-κB is activated and translocated into the nucleus where it can stimulate MCP-1 gene expression and MCP-1 protein production, leading to enhanced monocyte chemotaxis.

In the present study, several lines of evidence clearly indicated that folic acid supplementation could exert its inhibitory effect on Hcy-induced MCP-1 expression via its inhibition of NADPH oxidase activation in kidneys isolated from hyperhomocysteinemic rats. Firstly, administration of apocynin to

hyperhomocysteinemic rats completely blocked Hcy-induced MCP-1 expression, indicating that NADPH oxidase was involved in Hcy-induced inflammatory responses. Folic acid supplementation could effectively abolish Hcy-induced NF-κB activation and MCP-1 expression in kidneys isolated from hyperhomocysteinemic rats. Secondly, folic acid supplementation resulted in a reduction of phosphoIκBα protein, followed by an increase in the levels of this inhibitor protein in kidneys isolated from hyperhomocysteinemic rats. Finally, folic acid supplementation could effectively reduce the MPO activity in those kidneys, indicating a reduction of leukocyte infiltration into the kidney during hyperhomocysteinemia. Our previous study demonstrated that lipopolysaccharide treatment caused a significant elevation of NADPH oxidase activity and intracellular superoxide anion level in human kidney proximal tubular cells (Hwang *et al.*, 2011). Incubation of tubular cells with 5-methytetrahydrofolate effectively abolished the lipopolysaccharide-induced elevation of NADPH oxidase activity and intracellular superoxide anion level, suggesting that the inhibitory effect of 5-methyltetrahydrofolate on Hcy-induced superoxide anion production in tubular cells was not merely dependent on Hcy reduction. We also demonstrated that folic acid supplementation could suppress NADPH oxidase activity by inhibiting the expression of NOX4 and $p22^{phox}$, alleviating oxidative stress in the kidney of hyperhomocysteinemic rats and tubular cells (Hwang *et al.*, 2011). Taken together, it can be postulated that folic acid supplementation may alleviate oxidative stress by inhibiting NADPH oxidase activation, which in turn may reduce phosphorylation of IκBα protein. Hence, an increase in the levels of this inhibitor protein may prevent NF-κB activation and subsequent MCP-1 expression in kidneys isolated from hyperhomocysteinemic rats.

Folic acid supplementation is regarded as a promising approach for patients with cardiovascular diseases associated with hyperhomocysteinemia (Kang *et al.*, 1992; Refsum *et al.*, 1998). Studies demonstrated that folic acid supplementation may have a therapeutic implication in patients with cardiovascular diseases associated with hyperhomocysteinemia. The 5-methyltetrahydrofolate, which is formed from folic acid via the action of 5, 10-methylenetetrahydrofolate reductase, is the active form of folic acid. Since 5-methyltetrahydrofolate serves as a co-substrate in the remethylation pathway catalyzed by methionine synthase to convert Hcy to methionine, folic acid supplementation is regarded as a promising approach in reducing blood Hcy levels. It has been obvious that there is an inverse correlation between folate and Hcy levels in the circulation. However, data from large prospective randomized clinical trials failed to demonstrate a beneficial effect of folic acid supplementation in reducing the mortality of cardiovascular diseases (Bonaa *et al.*, 2006; Lonn *et al.*, 2006). In the HOPE-2 and NORVIT secondary prevention studies, participants were more than 55 years of age with cardiovascular disease, hypertension, myocardial infarction, and diabetes, in which cardiovascular damaging effects might be irreversible. This might contribute to the observation that despite a reduction in plasma Hcy levels by folic acid supplementation in conjunction with vitamin B_6 and B_{12}, there was no significant cardiovascular benefit. However, the same studies revealed that Hcy lowering effect with folic acid in conjunction with vitamin B_6 and B_{12} decreased the risk of stroke (Bonaa *et al.*, 2006; Lonn *et al.*, 2006).

One recent clinical trial reported that in females MCP-1 concentrations were positively correlated with Hcy and negatively correlated with both serum and red blood cell folate (Hammons *et al.*, 2009). Another study also showed that in patients with hyperhomocysteinemia folic acid treatment for 6 months decreased the levels of

Hcy, interleukin-8, and MCP-1 (Wang *et al.*, 2005). In addition, in that study folic acid treatment significantly reduced the elevated levels of ROS, NADPH oxidase, and chemokines in response to Hcy in cultured human monocytes (Wang *et al.*, 2005). In experimental animal studies, folic acid supplementation not only reduced Hcy levels and MCP-1 concentrations released from both plasma and peripheral blood mononuclear cells of rats with hyperhomocysteinemia but also attenuated MCP-1 expression in the aorta of rats with hyperhomocysteinemia (Li *et al.*, 2007). Another study also showed that folic acid supplementation reduced Hcy levels in the blood and expression of vascular cell adhesion molecule-1 in aorta of hyperhomocysteinemic rats (Li *et al.*, 2006). Our previous studies showed that folic acid supplementation could prevent dietary-induced hyperhomocysteinemia in rats (Wang *et al.*, 2002; Hwang *et al.*, 2011; Woo *et al.*, 2006a). Consequently, the Hcy-stimulated MCP-1 expression in the aortic endothelium was abolished (Wang *et al.*, 2002). Folic acid treatment also abolished the NADPH oxidase-mediated superoxide anion generation in kidneys of hyperhomocysteinemic rats as well as human kidney proximal tubular cells (Hwang *et al.*, 2011). The inhibitory effect of folic acid on Hcy-induced NADPH oxidase activation led to a reduction of superoxide anion generation in macrophages (Au-Yeung *et al.*, 2006). Subsequently, the Hcy-induced NF-κB activation and MCP-1 expression were inhibited in those cells (Au-Yeung *et al.*, 2006). Such an inhibitory effect of folic acid on inflammatory responses appeared to be independent of its Hcy-lowering action. Antagonizing Hcy-induced inflammatory responses by folic acid may represent one of the important mechanisms that contribute to the beneficial effect of folic acid supplementation in kidney disease associated with hyperhomocysteinemia.

In conclusion, results obtained from the present study clearly indicate that Hcy-induced superoxide anion production via NADPH oxidase is responsible for NF-κB activation and subsequent MCP-1 expression in kidneys isolated from hyperhomocysteinemic rats. Oxidative stress-mediated NF-κB activation and chemokine expression represents one of the important mechanisms underlying Hcy-induced inflammatory responses in the kidney. Therefore, targeting the overproduction of superoxide anions may represent a promising strategy against kidney disease.

VI. Overall Discussion

The present study clearly demonstrated that (1) hyperhomocysteinemia could cause oxidative stress and renal injury via NADPH oxidase-mediated superoxide anion production; (2) hyperhomocysteinemia could stimulate a potent chemokine MCP-1 expression by activating the transcription factor NF-κB; (3) administration of apocynin, an inhibitor of NADPH oxidase, could reverse hyperhomocysteinemia-induced MCP-1 expression, indicating that Hcy-induced NADPH oxidase activation might play an important role in inflammatory response; and (4) folic acid supplementation could offer a renal protective effect by preventing oxidative stress and inflammatory response in the kidney during hyperhomocysteinemia.

Hyperhomocysteinemia was successfully induced in Sprague-Dawley rats by feeding with a high-methionine diet. The serum total Hcy level in rats fed a high-methionine diet as a model of hyperhomocysteinemia was significantly higher than that of the control. In the present study, there was an increase in lipid peroxidation indicating that oxidative stress occurred in the kidney of hyperhomocysteinemic rats. Our results clearly demonstrated that Hcy could cause oxidative stress and renal injury via increased superoxide anion production and decreased enzymatic antioxidant activities in enzymes such as SOD. Inhibition of Hcy-induced NADPH oxidase activation abolished renal injury. These results suggested that NADPH oxidase-mediated superoxide anion production could lead to renal injury during hyperhomocysteinemia.

Oxidative stress plays an important role in different types of renal disorders. Superoxide anion has a very short half-life and is rapidly converted to hydrogen peroxide (H_2O_2), which can lead to the formation of hydroperoxide radicals ($^{\bullet}OH$). Hydroperoxide radical can attack reactive lipids such as unsaturated fatty acids,

leading to the formation of lipid hydroperoxides, which can be further oxidized to form lipid peroxide radicals in the presence of oxygen. Oxidative stress can lead to direct tissue damage and stimulate inflammatory reactions, which, in turn, contribute to tissue injury. Therefore, oxidative stress is thought to be one of important mechanisms for Hcy-induced kidney disease.

Several lines of evidence from this thesis suggested that hyperhomocysteinemia-induced renal injury was mediated via oxidative stress. First, there was a significant increase in the levels of lipid peroxides in the kidney of hyperhomocysteinemic rats, indicating that oxidative stress occurred. Our previous study clearly demonstrated that there was a significant increase in peroxynitrite formation in the kidney of hyperhomocysteinemic rats (Zhang *et al.*, 2004). An increase in the level of peroxynitrite could be due to an elevation of superoxide anion and/or nitric oxide levels. Peroxynitrite is a potent oxidant that can damage cells by modifying lipids, proteins, and DNA. Therefore, there was a significant increase in the levels of lipid peroxides and nitrotyrosine protein adducts in the kidney of hyperhomocysteinemic rats (Zhang *et al.*, 2004). Second, our present study demonstrated that NADPH oxidase activation caused an increase in superoxide anion production in the kidney during hyperhomocysteinemia. The involvement of NADPH oxidase in renal oxidative stress was further confirmed by the experiments using an inhibitor of NADPH oxidase, apocynin. The activity of NADPH oxidase, superoxide anion levels, and lipid peroxidation were significantly reduced to the basal levels in the kidney of hyperhomocysteinemic rats administrated with apocynin, indicating that NADPH oxidase-mediated superoxide anion production played an important role in oxidative stress-induced renal injury during hyperhomocysteinemia. Our previous study demonstrated that the activities of hepatic antioxidant enzymes such as SOD

and catalase were decreased whereas glutathionine peroxidase remained unchanged in the liver of hyperhomocysteinemic rats (Woo *et al.,* 2006a). In the present study, the activity of renal antioxidant enzyme SOD was significantly decreased in the kidney of hyperhomocysteinemic rats, elucidating another mechanism for the increased superoxide anion production and lipid peroxidation.

According to epidemiological studies, hyperhomocysteinemia is involved in atherogenesis. The recruitment of monocytes into the arterial wall is an early event during the development of atherosclerosis. It has been demonstrated that MCP-1 plays an important role in monocyte chemotaxis. The mechanism by which an elevated homocysteine level stimulates monocyte infiltration and macrophage accumulation during atherogenesis is not fully known. However, our previous study clearly demonstrated that Hcy, at pathophysiological concentrations, induced the phosphorylation of IκBα protein and reduced the expression of IκBα mRNA and protein, which, in turn, activated NF-κB in macrophages (Wang *et al.*, 2001). The activation of NF-κB was necessary for Hcy-induced MCP-1 gene expression leading to enhanced MCP-1 protein production and subsequent monocyte chemotaxis, suggesting one of the important mechanisms by which Hcy caused atherosclerosis (Wang *et al.,* 2001). We also reported that Hcy stimulated MCP-1 expression in cultured endothelial cells leading to enhanced monocyte adhesion to endothelial cells (Sung *et al.*, 2001).

Apart from NF-κB, activation of AP-1, another transcription factor, could stimulate MCP-1 expression in mammalian cells. NF-κB has been shown to participate in Hcy-induced MCP-1 expression in vascular smooth muscle cells, macrophages, rat mesganial cells and human tubular cells (Au-Yeung *et al.,* 2004; Hwang *et al.,* 2008; Wu *et al.,* 2009; Zhang *et al.,* 2004). However, Hcy did not

stimulate NF-κB activation in hepatocytes or in the liver of hyperhomocysteinemic rats (Woo *et al.*, 2008). It has been shown that activation of either one of those transcription factors, AP-1 and NF-κB, is sufficient to enhance MCP-1 gene expression.

Chronic kidney disease is recognized as a common condition that elevates the risk of cardiovascular disease along with kidney failure and other complications. Patients with chronic kidney disease are considered to be in the highest risk group for subsequent cardiovascular disease events. Therefore, there has been growing interest in the association between kidney disease and cardiovascular disease. Accelerated atherosclerosis in kidney disease has consistently been implicated due to a higher prevalence of traditional risk factors such as diabetes, hypertension, and dyslipidemia. However, these traditional risk factors have not fully accounted for the high risk of cardiovascular disease in patients with kidney disease. Recent studies have supported the notion that nontraditional risk factors such as decreased nitric oxide, oxidative stress, and inflammation may play an important role in the development of cardiovascular disease in patients with kidney disease. In chronic kidney disease patients, progressive deterioration of renal function might lead to accumulation of Hcy that can induce oxidative stress and development of glomerulosclerosis.

Folic acid supplementation can lower the blood Hcy levels in hyperhomocysteinemia and is, therefore, thought to be beneficial for individuals with cardiovascular disease. In the present study, supplementation of folic acid in the diet lowered the serum total Hcy levels in hyperhomocysteinemic rats. Folic acid supplementation was also able to inhibit Hcy-induced oxidative stress and to prevent oxidative stress-mediated renal injury. The results of the present study showed a protective role of folic acid in restoring renal injury during hyperhomocysteinemia.

Oral folic acid supplementation was shown to improve endothelium-dependent vascular function in patients with mild hyperhomocysteinemia (Doshi *et al.*, 2001). Although the underlying mechanisms of such an effect of folic acid have not been fully investigated, it has been suggested that the beneficial effect of folic acid on cardiovascular diseases contributes to Hcy-lowering effect as well as its ability to antagonize oxidative stress (Doshi *et al.*, 2001). Although in the present study folic acid supplementation led to a significant reduction in the serum total Hcy level, the serum Hcy level in hyperhomocysteinemia plus folic acid group was still higher than that observed in the control group. However, supplementation of folic acid completely abolished hyperhomocysteinemia-induced superoxide anion production and lipid peroxidation in the kidney of hyperhomocysteinemia. Furthermore, the present study clearly demonstrated that folic acid supplementation not only reduced oxidative stress but also reduced the size of glomeruli in the kidney. Moreover, the protective effect of folic acid supplementation in the kidney might be mediated, in part, via its direct effect on oxidative stress. In the present study, LPS-induced NADPH oxidase activity and superoxide anion production were significantly reduced by treatment of 5-MTHF in human tubular cells, indicating the possibility of a direct effect of folic acid against oxidative stress. Our present study also showed that folic acid supplementation restored an antioxidant enzyme SOD in the kidney of hyperhomocysteinemic rats.

Studies have suggested that the beneficial effect of folic acid is mediated by its effect on endothelial function. Oral folic acid supplementation (5 mg) could restore the impaired endothelium-dependent vasodilation in familial hypercholesterolemia patients (Verhaar *et al.*, 1998). It has been suggested that folic acid supplementation can restore the enzymatic coupling of eNOS (Antoniades *et al.*, 2009). Such a restoration resulted in amelioration of nitric oxide-mediated endothelial function by

preventing superoxide anion production from the uncoupling eNOS (Antoniades *et al.*, 2009). These studies suggested that the beneficial effect of folic acid supplementation on endothelial function was independent of its Hcy-lowering effect (Antoniades *et al.*, 2009; Doshi *et al.*, 2001). However, a recent study showed that patients with folic acid treatment had an increased risk of in-stent restenosis and the need for target-vessel revascularization (Lange *et al.*, 2004). That study included 636 patients who had undergone successful coronary stenting and the patients were supplemented with either folic acid (1 to 1.2 mg) along with vitamin B_6 (48 mg) and vitamin B_{12} (60 μg) or a placebo daily for 6 months (Lange *et al.*, 2004). The study showed that folic acid treatment lowered the risk of restenosis in patients with significantly elevated Hcy levels in the circulation (Lange *et al.*, 2004).

Folic acid supplementation could exert its inhibitory effect on Hcy-induced MCP-1 expression via its inhibition on NADPH oxidase activation in kidneys isolated from hyperhomocysteinemic rats. First, administration of apocynin to hyperhomocysteinemic rats completely blocked Hcy-induced MCP-1 expression, indicating that NADPH oxidase was involved in Hcy-induced inflammatory responses. Folic acid supplementation could effectively abolish Hcy-induced NF-κB activation and MCP-1 expression in kidneys isolated from hyperhomocysteinemic rats. Second, folic acid supplementation resulted in a reduction of IκBα protein, followed by an increase in the levels of this inhibitor protein in kidneys isolated from hyperhomocysteinemic rats. Finally, folic acid supplementation could effectively reduce the MPO activity in those kidneys, indicating a reduction of leukocyte infiltration into the kidney during hyperhomocysteinemia.

Our previous study demonstrated that lipopolysaccharide treatment caused a significant elevation of NADPH oxidase activity and intracellular superoxide anion

level in human kidney proximal tubular cells. Incubation of tubular cells with 5-methytetrahydrofolate effectively abolished the lipopolysaccharide-induced elevation of NADPH oxidase activity and intracellular superoxide anion level, suggesting that the inhibitory effect of 5-methyltetrahydrofolate on Hcy-induced superoxide anion production in tubular cells was not merely dependent on Hcy reduction. We also demonstrated that folic acid supplementation could suppress NADPH oxidase activity by inhibiting the expression of NOX4 and $p22^{phox}$, alleviating oxidative stress in the kidney of hyperhomocysteinemic rats and tubular cells (Hwang *et al.*, 2011). Taken together, it can be postulated that folic acid supplementation may alleviate oxidative stress by inhibiting NADPH oxidase activation, which in turn may reduce phosphorylation of IκBα protein. Hence, an increase in the levels of this inhibitor protein may prevent NF-κB activation and subsequent MCP-1 expression in kidneys isolated from hyperhomocysteinemic rats.

Our previous study demonstrated the involvement of Hcy in ischemia-reperfusion injury (Prathapasinghe *et al.*, 2007). A significant portion of the rate-limiting enzyme in the transsulfuration pathway was associated with the endogenous hydrogen sulfide (H_2S) production in the kidney. H_2S production, which is metabolically related to Hcy, was significantly impaired during renal ischemia-reperfusion (Xu *et al.*, 2009). One recent study reported that administration of exogenous H_2S 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).

However, it can be argued that the Hcy-lowering effect by folate might lead to the decrease in plasma H_2S level which might mask the beneficial effect of Hcy reduction. Inadequate H_2S levels could induce hypertension and subsequent

cardiovascular events. Our previous study demonstrated a renoprotective effect when Hcy was lowered by restoring CBS activity (Prathapasinghe *et al.*, 2007).

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. This might be due to the fact that the addition of a cofactor in excess could not increase the rate of an enzyme reaction (Bonaa *et al.*, 2006). Nonetheless, a co-substrate could increase the rate of an enzymatic reaction as long as the other substrates and cofactors were adequate. This was observed in both the 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).

Histological staining was performed to examine the effect of hyperhomocysteinemia on morphological changes in the kidney. The glomerular size was significantly increased in kidneys of hyperhomocysteinemic rats as indicated by larger mean glomerular volumes. An increase in the glomerular size might be one of the histological changes associated with the glomerular hypertrophy. Therefore, our results demonstrated that hyperhomocysteinemia not only increased oxidative stress but also enlarged the size of glomeruli in the kidney of hyperhomocysteinemic rats. Folic acid supplementation reduced the glomerular size in rats fed a high-methionine diet. Moreover, folic acid supplementation was able to attenuate oxidative stress and chemokine expression. Therefore, it can prevent kidney injury. Based on our studies, targeting the overproduction of superoxide anions may represent a promising strategy against hyperhomocysteinemia-induced kidney disease. Folic acid supplementation

may offer a protective effect against oxidative stress and oxidative stress-mediated chemokine expression via a reduction of Hcy levels as well as regulation of superoxide anion production and metabolism.

VII. Conclusion and Future Perspectives

7.1. Study 1: Folic acid supplementation inhibits NADPH oxidase-mediated superoxide anion production in the kidney

Results from our research demonstrate that hyperhomocysteinemia induces NADPH oxidase-mediated superoxide anion generation in the kidney leading to oxidative stress. Folic acid supplementation can effectively inhibit NADPH oxidase activation through inhibition of NOX4 and $p22^{phox}$ expression and hence abolish renal oxidative stress in hyperhomocysteinemic rats.

7.2. Study 2: Hyperhomocysteine stimulates monocyte chemoattractant protein-1 (MCP-1) expression in the kidney via nuclear factor-kappa B activation (NF-κB)

This part of the study clearly demonstrates a direct link between hyperhomocysteinemia and inflammatory response in the kidney. Our results suggest that activation of the NF-κB pathway is essential for Hcy-induced MCP-1 expression in the kidney. Increased chemokine expression may represent one of the important mechanisms that contribute to renal injury in patients with hyperhomocysteinemia.

7.3. Study 3: Folic acid supplementation abolishes monocyte chemoattractant protein-1 expression in the kidney via nuclear factor-kappa B activation

Results from our research demonstrate that Hcy-induced superoxide anion production via NADPH oxidase is responsible for NF-κB activation and subsequent MCP-1 expression in kidneys of hyperhomocysteinemic rats and in human kidney proximal tubular cells. Oxidative stress-mediated NF-κB activation and chemokine expression represent one of the important mechanisms underlying Hcy-induced inflammatory responses in the kidney. Targeting the overproduction of superoxide anions may represent a promising strategy against kidney disease. Antagonizing Hcy-

induced inflammatory responses by folic acid may represent one of important mechanisms that contribute to the beneficial effect of folic acid supplementation in kidney disease associated with hyperhomocysteinemia and/or with other risk factors.

In conclusion, the present study has clearly demonstrated that Hcy induces oxidative stress and inflammatory responses in the kidney during hyperhomocysteinemia. Folic acid supplementation is able to protect the kidney from oxidative stress-mediated inflammation during hyperhomocysteinemia. Hcy-induced oxidative stress and inflammatory responses may represent an important mechanism for kidney disease. Investigating the molecular mechanisms of renal injury during hyperhomocysteinemia may suggest a new perspective for the role of hyperhomocysteinemia in renal pathophysiology and develop therapies.

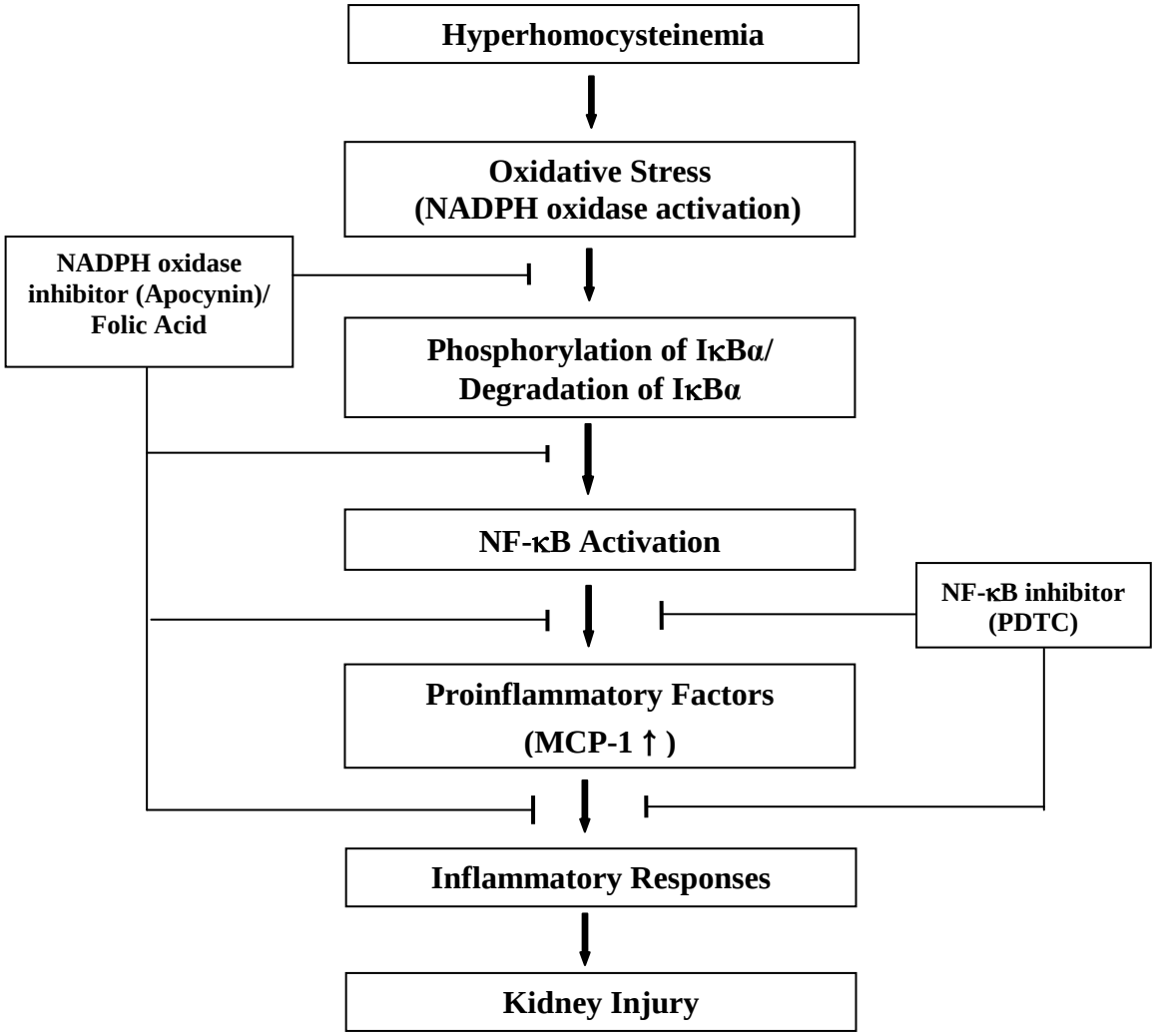

Figure 7.1 Proposed mechanism of hyperhomocysteinemia-induced kidney injury

Hyperhomocysteinemia causes kidney injury via oxidative stress-mediated inflammatory response characterized by the induction of a proinflammatory factor (MCP-1) expression. Oxidative stress caused by hyperhomocysteinemia results in the activation of NF-κB and subsequent elevation of MCP-1 expression. Inhibition of oxidative stress by apocynin, an inhibitor of NADPH oxidase, or prevention of NF-κB activation by PDTC, a known inhibitor of NF-κB activation, is able to abolish MCP-1 expression in the kidney of hyperhomocysteinemic rats. Folic acid supplementation can effectively inhibit NADPH oxidase activation and hence abolish MCP-1 expression in the kidney of hyperhomocysteinemic rats.

7.4. Future Perspectives

Studies have suggested that glutathione depletion is the underlying mechanism of increased oxidative stress during hyperhomocysteinemia (Robert *et al.*, 2005). This hypothesis was based on the CBS-deficient hyperhomocysteinemia model. A decrease or an absence of CBS activity in this model yielded a decrease in the level of cystathionine which could be converted to cysteine and glutathione. Therefore, it was suggested that glutathione depletion would be due to the decreased synthesis of cysteine from cystathionine (Robert *et al.*, 2005). Our previous study showed that in the diet-induced hyperhomocysteinemic rat model, hepatic CBS activity was the same as that of the control group (Woo *et al.*, 2006a). In that study, total glutathione level in the liver of hyperhomocysteinemic rats was also similar to that of the control group. However, only the levels of the oxidized form of glutathione (GSSG) were increased. Glutathione reductase is the enzyme reducing GSSG to GSH which is the reduced form of glutathione. However, the CBS activity and antioxidant enzyme activities such as catalase and glutathionine remain to be elucidated in the hyperhomocysteinemic kidney.

Oxidative stress is a result of a decrease in antioxidant enzyme activities. Our previous study showed that the activities of hepatic catalase and SOD were significantly decreased in hyperhomocysteinemic rats and folic acid supplementation was able to restore those antioxidant enzyme activities (Woo *et al.*, 2006a). However, the mechanism by which folic acid supplementation can restore antioxidant enzymes still remains to be elucidated. Redox systems including antioxidant enzymes provide protection against ROS-induced injury. Nuclear factor-erythroid-2-related factor 2 (Nrf2) plays an important role in the induction of genes encoding various antioxidant enzymes such as catalase, glutathione peroxidase, and SOD (Li *et al.*, 2008; Surh *et*

al., 2008). Nrf2 is present in the cytoplasm as an inactive complex bound to a repressor molecule known as Kelch-like ECH-associated protein 1 (Keap1). Keap1 contains reactive cysteine residues that serve as sensors of the intracellular redox state. Therefore, oxidative modification in these cysteine residues results in dissociation of Nrf2 from Keap1 and its translocation to the nucleus. In the nucleus, Nrf2 binds to regulatory sequences termed antioxidant response elements located in the promoter region of genes encoding the antioxidant enzymes. The Nrf2-mediated regulation of cellular antioxidant genes plays an important role in defense against oxidative stress (Li *et al.*, 2008; Surh *et al.*, 2008). Activation of Nrf2 and the consequent upregulation of its target genes not only counteract oxidative stress but also attenuate tissue inflammation. Genetic disruption of Nrf2 signaling augments the expression of proinflammatory mediators and activation of these proinflammatory mediators is under the control of NF-κB (Yoh *et al.*, 2008; Jin *et al.*, 2008a; Jin *et al.*, 2008b). Therefore, it may be possible that there is cross-talk between Nrf2 and NF-κB. Understanding the molecular mechanisms of folic acid supplementation involved in hyperhomocysteinemia-induced renal injury can lead to the development of possible therapeutic strategies for patients with kidney diseases.

Hyperhomocysteinemia-related kidney disease was associated with decreased endogenous hydrogen sulfide (H_2S) production. H_2S has been recognized as a toxic gas. However, research has been shown that H_2S has protective roles in cardiovascular disease similar to the actions of nitric oxide and carbon monoxide, which are considered two gaseous transmitters. Additionally, H_2S supplementation has been shown to protect against hyperhomocysteinemia-induced renal damage partially through its antioxidant and anti-inflammatory properties. Hcy and H_2S are two important molecules produced in the body during the metabolism of sulfur amino acids.

In mammalian systems, two enzymes, CBS and CGL, in the transulfuration pathway catalyze conversion of H_2S from Hcy. Since Hcy is one of the precursors of endogenous H_2S generation, increase of plasma Hcy level would elevate in the plasma H_2S level. However, due to a negative feedback mechanism, increased plasma Hcy may inhibit its metabolizing enzymes, CBS and CGL, resulting in low production of H_2S production. Recent study has shown that methionine-induced superoxide production is significantly reduced by NaHS, a donor of H_2S. Moreover, H_2S combined with either apocynin, an inhibitor of NADPH oxidase, or SOD may synergistically protect the cell damage by decreasing ROS production and by increasing antioxidant effects. However, a potential role of H_2S combined with folic acid supplementation in preventing hyperhomocysteinemia-induced oxidative stress and inflammation by scavenging ROS and/or enhancing antioxidants in the kidney is unknown. Therefore, it remains to be investigated whether folic acid combined with H_2S would have synergistic effects on scavenging ROS and enhancing antioxidants via activation of Nrf2 and consequent upregulation of the antioxidant enzymes such as catalase and SOD in rat kidneys of hyperhomocysteinemia.

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IX. Appendices

Appendix I

Reagents/Chemical	Vendor
Acrylamide/bis 30% solution (37.5:1)	Bio-Rad
Adenosine triphosphate-[^{32}P]	PerkinElmer
2-Thiobarbituric acid	Sigma-Aldrich
3,3',5,5'-tetramethylbenzidine	Sigma-Aldrich
Acetic Acid, Glacial	Fisher
Ammonium phosphate	Sigma-Aldrich
Bio-Rad Protein Assay	Bio-Rad
Bovine Serum Albumin (BSA)	EMD
Calcium Chloride	Fisher
DL-Homocysteine	Sigma-Aldrich
DNA, Salmon testes	Sigma-Aldrich
Dnase I	Amersham
Ethidium Bromide	Sigma-Aldrich
Ethylene glycol tetraacetic acid	Sigma-Aldrich
Ethylenediaminetetraacetic acid	Sigma-Aldrich
Flavin adenine dinucleotide Disodium salt Hydrate	Sigma-Aldrich
Flavin mononucleotide	Sigma-Aldrich
Folic acid	Sigma-Aldrich
Hematoxylin, Harris	Sigma-Aldrich
Hematoxylin, Mayers	Sigma-Aldrich
HEPES	Fisher
Hydrochloric acid	Fisher
Hydrogen peroxide 30%	Fisher
Hydroxylamine hydrochloride	Sigma-Aldrich
L-Cysteine	Sigma-Aldrich
Leupeptin	Sigma-Aldrich
L-Homocysteine thiolactone	Sigma-Aldrich
L-Lactate Dehydrogenase	Sigma-Aldrich
Manganese (II) Chloride tetrahydrate	Sigma-Aldrich
Methanol	VWR
N-(1-Napthyl)ethylenediamine dihydrochloride (NEDA)	Sigma-Aldrich
N-ethylmalemide (NEM)	Sigma-Aldrich
Nitrate Reductase	Sigma-Aldrich
Pentobarbital	Sigma-Aldrich
Pepstatin A	Sigma-Aldrich
Phenylmethanesulfonyl fluoride (PMSF)	Sigma-Aldrich
Potassium Chloride	Sigma-Aldrich
Potassium Phosphate dibasic (K2HPO4)	Sigma-Aldrich
Potassium Phosphate monobasic (KH2PO4)	Sigma-Aldrich
Proteinase K	Sigma-Aldrich
Pyridoxal-5'-phosphate hydrate	Sigma-Aldrich
Resin AG 50W-X8 Sodium form	Bio-rad
Resin AG 50W-X8 Hydrogen form	Bio-rad

S-adenosylmethionine (SAM)	Sigma-Aldrich
Sodium Azide	Fisher
Sodium Chloride	VWR
Sodium Citrate	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	Fisher
Sodium hydrosulfide hydrate	Sigma-Aldrich
Sodium Hydroxide	Fisher
Sodium L-ascorbate	Sigma-Aldrich
Sodium Nitrite	Fisher
Sodium Nitroprusside	Sigma-Aldrich
Sodium phosphate dibasic (Na2HPO4)	Sigma-Aldrich
Sodium phosphate monobaisc (NaH2PO4)	Fisher
Sodium Pyruvate	Fisher
Sodium thiosulfate	Sigma-Aldrich
Sulfanilamide	Sigma-Aldrich
Trichloracetic acid	EMD
Tris	Invitrogen
Triton X-100	Sigma-Aldrich
Xylene	EMD
Zinc Acetate dihydrate	Sigma-Aldrich
β-Nicotinamide adenine dinucleotide 2'-phosphate reduced (NADPH)	Sigma-Aldrich
β-Nicotinamide adenine dinucleotide, reduced form	Sigma-Aldrich

APPENDIX II

Equipment

Model	Manufacturer
Axioskope2 MOT microscope	Carl Zeiss Microimaging
Centrifuge 5804R	Eppendorf
Gel Doc	Bio Rad
IMx	Abbot
LS 6500 Multi-purpose Scintillation counter	Beckman Instruments
Lumet LB9507	Berthold Technologies
Microtome	Therma
Minispin	Eppendorf
MRX TC Revelation	Dynex
My Cycler	Bio Rad
Spectra Max Gemini	Molecular devices
Spetrophotometer DU 800	Beckman Coulter

APPENDIX III

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