Folic acid supplementation inhibits NADPH oxidase-mediated superoxide anion production in the kidney

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1Department of Animal Science, 2Department of Physiology, University of Manitoba, Manitoba; and 3Canadian Centre for Agri-food Research in Health and Medicine, St. Boniface Hospital Research Centre, 4Agriculture and Agri-Food Canada, Winnipeg, Canada

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Hwang S-Y, Siow YL, Au-Yeung KK, House J, O K. Folic acid supplementation inhibits NADPH oxidase-mediated superoxide anion production in the kidney. Am J Physiol Renal Physiol 300: F189–F198, 2011. First published October 27, 2010; doi:10.1152/ajprenal.00272.2010.—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. 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 nicotinamide adenine dinucleotide phosphate (NAPDH) 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 NAPDH 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 level 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 renal protective effect against oxidative stress.

Homocysteine; oxidative stress

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 identified a positive association between an elevation of Hcy level in the blood and the development of chronic kidney disease in the general population (21). 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 factor such as nuclear factor-κB (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 (13, 16, 17, 21, 38). 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 (4, 10, 11, 25). NADPH oxidase comprises membrane-bound components, termed cytochrome b55 (gp91phox and p22phox subunits), and cytosolic components (p47phox, p67phox, p40phox, and Rac 1/2 subunits). Under normal conditions, a small amount of superoxide anions generated inside cells can be scavenged by cellular antioxidant defense mechanisms. However, when there is an overproduction and insufficient removal of superoxide anions, oxidative stress occurs. The gp91phox is a catalytic subunit of the enzyme. Several homologs of gp91phox 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 (analog of gp91phox). 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 (9, 25). Our recent study showed that hyperhomocysteinemia can increase inducible nitric oxide synthase (iNOS) expression in the kidney leading to an increased NO production in rat kidneys.
was given to rats fed a regular diet (control) once a day for 7 days before euthanasia, in keratinocyte serum-free medium (GIBCO-BRL 17005–042) with 5 (ATCC). According to the instruction by ATCC, cells were cultured.

Animal Care and Use of Experimental Animals

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 (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), 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, 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 before euthanasia. After being on the experimental diets for 12 wk, rats were euthanized by injection of a high dose of pentobarbital sodium 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 (13, 31, 32). 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.

Cell culture. Human kidney cortex proximal tubular cells (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.

Determination of lipid peroxidation. The degree of lipid peroxidation in the kidney tissue including both cortex and medullar was determined by measuring malondialdehyde (MDA) levels with thio-barbituric acid-reactive substances (3, 27). Briefly, a portion of the kidney was homogenized in 0.1 M KCl solution containing 3 mM ethylenediaminetetraacetic acid (EDTA) followed by centrifugation at 600 g for 15 min at 4°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°C for 1 h, the amount of MDA formed in the reaction mixture was measured by spectrophotometer at absorbance of 532 nm. MDA was used as the standard, and results were expressed as a percentage of control group. The amount of MDA correlates to the degree of lipid peroxidation produced in the tissues.

Determination of NADPH oxidase activity, xanthine oxidase activity, and superoxide dismutase activity. The NADPH oxidase activity was measured by lucigenin chemiluminescence’s assay (15). A portion of the kidney (cortex and medullar) or tubular cells were homogenized in a 50-mM phosphate buffer (pH 7.0, 1:10, wt/vol) containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 3,000 rpm 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 (15). In one set of experiments, cellular fraction free of mitochondria was prepared (8). In brief, a portion of the kidney tissue including both cortex and medullar was homogenized in a 50-mM phosphate buffer (pH 7.0, 1:10, wt/vol) containing 1 mM EDTA and 1 mM PMSF. After centrifugation of the homogenate at 600 g for 10 min at 4°C, the supernatant was collected and then centrifuged at 7,000 rpm for 10 min at 4°C. After centrifugation, the supernatant was collected and used as mitochondria-free fraction (8) for measuring NADPH oxidase activity. Chemiluminescent signal (photon emission) was measured every 15 s for 3 min using a luminometer (Lumer LB9507, Berthold Technologies GmbH and 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 (18). 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 (18). A standard curve was prepared with xanthine (100 μM) and known serial concentrations of xanthine oxidase (Sigma, St. Louis, MO) 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. Superoxide dismutase (SOD) activity was determined as previously described (6). In brief, a portion of the kidney was homogenized in a 50-mM phosphate buffer (pH 7.8, 1:8, wt/vol) 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).

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 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’-GGGCCCTAGGTATGGTTGTA-3’ and (reverse) 5’-CTGGAAGAAGTTCGAGGCCTC-3’; rat p22phox (forward) 5’-TGTTGCAGAGTGCTCATC-3’ and (reverse) 5’-CTGCAGAGCTGAACATC3A-3’; which were used for rat GAPDH were (forward) 5’-TCAAGAAAGTGTGGAAGCAC-3’ and (reverse) 5’-AGTTGGA AGATTGGATTTG-3’. The primers for human NOX4 were (forward) 5’-CTTCGCTGTGGTTGACGT-3’ and (reverse) 5’-TGGTCCACACACAGAGAC-3’, human p22phox (forward) 5’-GCTCCCTAGTGGTGTA-3’ and (reverse) 5’-GGGCCCTAGGTATGGTTGTA-3’; and (reverse) 5’-CTGGAAGAAGTTCGAGGCCTC-3’. These primers are designed to amplify the coding sequence of each gene.
ward) 5′-GTCCCTGATTCTGTGCTTT-3′ (forward) and 5′-GAAA-
CACCTTCGGACCCCTGAT-3′ (reverse). Those 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 −ΔΔCt
, where Ct = (Ct target gene − CtgAPDH) treatment − (Ct target gene − CtgAPDH) control (19). 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).

Determination of intracellular superoxide anion levels. The intracellular superoxide anion level was measured by the nitroblue tetrazolium (NBT) reduction assay (1, 29). 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 prewarmed (37°C) Hank’s balanced salt solution. Cells were lysed with a phosphate buffer (80 mmol/l, pH 7.8) containing 5% sodium deoxycholate 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.

Transfection of cells with NOX4 and p22phox siRNAs. Cells were transfected with NOX4 and p22phox siRNA duplex oligoribonucleotides (Stealth RNAi, Invitrogen). Tubular cells were seeded in six-well plates and transfected with NOX4 and p22phox siRNAs according to the manufacturer’s instruction. For a negative control, cells were transfected with Stealth RNAi 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 p22phox mRNA, and the intracellular superoxide anion level was measured.

Histological staining. A portion of the kidney (cortex and medullar) was immersion-fixed in 10% neutral-buffered formalin over-night followed by embedding in paraffin. Sequential 5-μm 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 (32). Images were acquired using a Zeiss Axioskop2 microscope equipped with an Axiosvision digital camera. Using a camera mounted on microscope, slides were analyzed at ×200 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 formula followed by: mean glomerular volume = β/K(πr²)⁴/⁸. 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) (12).

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

RESULTS

Effect of folic acid on lipid peroxidation in the kidney during hyperhomocysteinemia. Rats that were fed a high-Met diet for 12 wk developed hyperhomocysteinemia. Those rats had a much higher level of serum tHcy than the control group (Fig. 1A). Supplementation of folic acid to rats fed a high-Met diet significantly lowered the serum tHcy levels (Fig. 1A). These results suggested that feeding a high-Met 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 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. 1B). Folic acid supplementation effectively reduced the MDA level in the kidney (Fig. 1B). Administration of apocynin, an inhibitor for NADPH oxidase, to hyperhomocysteinemic rats also reduced the MDA level in kidneys of hyperhomocysteinemic rats (Fig. 1B). Folic acid supplementation or apocynin injection to rats fed a regular diet did not affect the serum Hcy level (Fig. 1A) and the kidney MDA level (Fig. 1B). These results suggested that NADPH oxidase might be involved in increased free radical generation in the kidney leading to lipid peroxidation in hyperhomocysteinemic rats.

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. 2A). Folic acid supplementation reduced NADPH oxidase activity in kidneys of hyperhomocysteinemic rats (Fig. 2A). Treatment of hyperhomocysteinemic rats with...
apocynin, NADPH oxidase inhibitor, abolished NADPH oxidase activation in the kidney (Fig. 2A). Further analysis revealed that the mRNA levels of NADPH oxidase subunits, NOX4 and p22phox, were significantly elevated in the same kidney tissue (Fig. 2, B and C). These results suggested that the increased expression of NOX4 and p22phox 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 p22phox in the kidney of hyperhomocysteinemic rats (Fig. 2, B and C). Such an inhibitory effect of folic acid might contribute to a reduction of NADPH oxidase activity in those rats (Fig. 2A). Folic acid supplementation or apocynin injection to rats fed a regular diet did not affect the basal levels of NADPH oxidase activity, NOX4, and p22phox mRNA expression (Fig. 2). Another superoxide-generating enzyme xanthine oxidase was also significantly increased in kidneys of hyperhomocysteinemic rats while folic acid supplementation had an inhibitory effect (Fig. 3A). 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. 3B). 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. 3C). Folic acid supplementation reduced NADPH oxidase activity in this fraction (Fig. 3C).

Fig. 2. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and mRNA levels of NADPH oxidase subunits in rat kidneys. Rats were fed with the same diets as in Fig. 1 for 12 wk. In one set of experiments, apocynin (4 mg/kg daily ip) was given to rats fed a high-Met diet (Met + apocynin) or fed a regular diet (control + apocynin) for 7 days before euthanasia. A: NADPH oxidase activity expressed as the amount of superoxide anion produced per mg of protein per min was measured by lucigenin chemiluminescence’s assay. NOX4 (B) mRNA and p22phox (C) mRNA levels were measured using a real-time PCR detection system. Results were expressed as means ± SE (n = 6). *P < 0.05 compared with control values. #P < 0.05 compared with values of high-Met-fed group.

Fig. 3. Xanthine oxidase activity, superoxide dismutase (SOD) activity, and NADPH oxidase activity in rat kidneys. Rats were fed with the following diets for 12 wk: a regular diet (control), a high-Met diet (1.7% Met), or a high-Met diet (1.7% Met) + folic acid (0.25%; Met + folic acid). Xanthine oxidase activity (A) and SOD activity (B) were measured in the kidney tissue. C: NADPH oxidase activity was measured in mitochondria-free fraction prepared from the kidney. Results were expressed as means ± SE (n = 6). *P < 0.05 compared with control values. #P < 0.05 compared with values of high-Met-fed group.
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 (25–250 μM) for 12 h. The intracellular level of superoxide anion was significantly increased in cells treated with Hcy at 50–250 μM (Fig. 4A). In the rest of cell culture experiments, Hcy at 100 μM 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. 4B). Apocynin treatment did not affect the basal level of intracellular superoxide anion (Fig. 4B). Next, to examine whether other superoxide-generating sources were also activated on 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. 4C), 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 P-450) did not affect intracellular superoxide anion levels on Hcy treatment (Fig. 4C). The NADPH oxidase activity was significantly increased in cells incubated with Hcy (Fig. 4D).

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 p22phox was significantly elevated in tubular cells incubated with Hcy (Fig. 5, A and B). To further examine the role of NOX4 and p22phox in Hcy-induced NADPH oxidase activation, tubular cells were transfected with NOX4 and p22phox siRNA, respectively. Transfection of cells with NOX4 siRNA or p22phox siRNA not only inhibited NOX4 (Fig. 6A) and p22phox (Fig. 6B) expression but also abolished Hcy-induced elevation of superoxide anion level in tubular cells (Fig. 6C). In accordance with the intracellular superoxide anion levels, transfection of cells with NOX4 siRNA or p22phox siRNA abolished the stimulatory effect of Hcy on NADPH oxidase activity while transfection of cells scramble siRNA did not affect the NADPH oxidase activity.

Fig. 4. Measurement of intracellular superoxide anion in tubular cells. Proximal tubular cells were incubated in the absence (control) or presence of Hcy (25–250 μM) for 12 h. A: intracellular superoxide anion levels were measured. B: cells were preincubated with 5-methyltetrahydrofolate (5-MTHF; 500 ng) or apocynin (300 μM) for 15 min followed by incubation with Hcy (100 μM) for 12 h. Intracellular superoxide anion levels were measured. C: cells were preincubated with rotenone (1 μM), cimetidine (100 μM), oxypurinol (10 μM), or apocynin (300 μM) for 15 min followed by incubation with Hcy (100 μM) for 12 h. Intracellular superoxide anion levels were measured. D: NADPH oxidase activity was measured by lucigenin chemiluminescence’s assay. Results were expressed as means ± SE (n = 6–8). *P < 0.05 compared with control values. #P < 0.05 compared with values of Hcy-treated group.
activity. These results suggested that Hcy-induced activation of NADPH oxidase was mainly mediated via increased NOX4 and p22phox expression in tubular cells.

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. 4D) and reduced the intracellular level of superoxide anion (Fig. 4C). Incubation of cells with 5-MTHF also reduced the mRNA levels of NOX4 and p22phox (Fig. 5, A and B). Hcy can be remethylated to Met via the enzymatic action of Met synthase which utilizes 5-MTHF as a methyl donor. Since folate in the form of 5-MTHF can methylate Hcy to Met, 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. 4B) or NOX4 and p22phox expression (Fig. 5). Next, to examine whether the reduction of superoxide anion by 5-MTHF was due to its Hcy-lowering effect, lipopolysaccharide (LPS) (23) 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. 7A) and intracellular superoxide anion level (Fig. 7B). Incubation of tubular cells with 5-MTHF effectively reduced the LPS-induced elevation of NADPH oxidase activity (Fig. 7A) and intracellular superoxide anion level (Fig. 7B). 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.

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. 8). Folic acid supplementation reduced the glomerular size in rats fed a high-Met diet (Fig. 8). 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.

Fig. 5. Measurement of NOX4 and p22phox mRNA levels in tubular cells. 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 preincubated with 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. NOX4 (A) mRNA and p22phox (B) mRNA levels were measured. Results were expressed as means ± SE (n = 4–6). *P < 0.05 compared with control values. #P < 0.05 compared with values of Hcy-treated group.

Fig. 6. Determination of mRNA levels of NADPH oxidase subunits and superoxide anion production in siRNA-transfected tubular cells. Proximal tubular cells were transfected with NOX4 and p22phox siRNAs or with a negative control siRNA. Transfected cells were incubated for 12 h in the absence (control) or presence of Hcy (100 μM). NOX4 (A) and p22phox (B) mRNA levels were measured. C, 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 means ± SE (n = 4–6). *P < 0.05 compared with control values.
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. Third, the involvement of NADPH oxidase was further confirmed by using siRNA transfection technique. Transfection of tubular cells with NOX4 siRNA or p22phox 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 cytchrome P-450 did not significantly affect intracellular superoxide anion levels on 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 wk) 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 showed an increase in NOX4 and p22phox expression in the kidney of streptozotocin-induced diabetic rats (7). It has been suggested that increased NADPH oxidase-mediated superoxide generation contributes to the development of diabetic nephropathy (7). It has also been shown that Hcy, at elevated levels, can induce inflammatory response and apoptosis in kidney cells and animal models (24, 33–35). Hcy treatment was shown to increase collagen production and cell proliferation in rat mesangial cells (33). Such a stimulatory effect by Hcy could be blocked by inhibition of NADPH oxidase activation (33). Recently, the deletion of the gp91phox gene in mice was reported to protect hyperhomocysteinemia-induced kidney cell injury (36). In a rat model of hyperhomocysteinemia induced by folate-free diet, glomerulosclerosis was characterized by enhanced oxidative stress, mesangial

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 p22phox. 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 nonphagocytic cells. Excessive activation of NADPH oxidase can lead to overproduction 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 p22phox subunits in the kidney. Second, the level of lipid peroxides in the kidney of hyperhomocysteinemic rats was increased.

Fig. 7. Determination of NADPH oxidase activity and intracellular superoxide anions in lipopolysaccharide (LPS)-stimulated tubular cells. Proximal tubular cells were incubated in the absence (control) or presence of LPS (10 μg) for 12 h. In one set of experiments, cells were preincubated with 5-MTHF (500 ng) for 15 min followed by incubation with LPS (10 μg). A: NADPH oxidase activity was measured by lucigenin chemiluminescence assay. B: intracellular superoxide anion levels were determined by NBT reduction assay. Results were expressed as means ± SE (n = 6–8). *P < 0.05 compared with control values. #P < 0.05 compared with values of LPS group.
expansion, podocyte dysfunction, and fibrosis. When these rats with hyperhomocysteinemia were treated with apocynin, an inhibitor of NADPH oxidase, the glomerular injury was attenuated (34, 35). In a recent study, Zhang et al. (37) demonstrated that NMDA receptor mediates NADPH oxidase-dependent superoxide anion production induced by Hcy in mesangial cells, which may contribute to hyperhomocysteinemia-induced glomerulosclerosis. Our recent study demonstrated that hyperhomocysteinemia causes activation of NF-\(\kappa\)B, induction of iNOS, and chemokine expression in rat kidneys (13, 38). NF-\(\kappa\)B is a transcriptional factor that plays an important role in regulating gene expression of various inflammatory factors in the kidney and is a redox-sensitive transcription factor (13, 38). We also observed that ROS can induce phosphorylation and degradation of IkB, an inhibitory protein normally associated with NF-\(\kappa\)B, leading to NF-\(\kappa\)B activation in endothelial cells and in macrophages (1, 29). 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 cosubstrate in the remethylation pathway catalyzed by Met synthase to convert Hcy to Met. Therefore, folic acid supplementation is regarded as a promising approach in reducing the mortality of cardiovascular disease (5, 20). 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-Met diet supplemented with excess folic acid. However, the level of serum Hcy was still considerably higher (4-fold higher) in this group of rats compared with 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 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 (26). In our previous study, folic acid supplementation was shown to reduce oxidative stress and alleviate liver injury in hyperhomocysteinemic rats (30). Moreover, we 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 (2, 28). Superoxide anion can react with NO 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 p22phox expression and hence abolish renal oxidative stress in hyperhomocysteinemic rats.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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