Inhibition of cystathionine-β-synthase activity during renal ischemia-reperfusion: role of pH and nitric oxide

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Prathapasinghe GA, Siow YL, Xu Z, O K. Inhibition of cystathionine-β-synthase activity during renal ischemia-reperfusion; role of pH and nitric oxide. Am J Physiol Renal Physiol 295: F912–F922, 2008. First published August 13, 2008; doi:10.1152/ajprenal.00040.2008.—Our recent study (Prathapasinghe GA, Siow YL, O K. Am J Physiol Renal Physiol 292: F1354–F1363, 2007) indicates that homocysteine (Hcy) plays a detrimental role in ischemia-reperfusion-induced renal injury. Elevation of renal Hcy concentration during ischemia-reperfusion is attributed to reduced activity of cystathionine-β-synthase (CBS) that catalyzes the rate-limiting step in the transsulfuration pathway for the metabolism of the majority of Hcy in the kidney. However, the mechanisms of impaired CBS activity in the kidney are unknown. The aim of this study was to investigate the effects of pH and nitric oxide (NO) on the CBS activity in the kidney during ischemia-reperfusion. The left kidney of a Sprague-Dawley rat was subjected to ischemia-reperfusion. The CBS activity was significantly reduced in kidneys subjected to ischemia alone (15–60 min) or subjected to ischemia followed by reperfusion for 1–24 h. The pH was markedly reduced in kidneys upon ischemia. Injection of alkaline solution into the kidney partially restored the CBS activity in the kidneys subjected to ischemia-reperfusion. Further analysis revealed that reduction of CBS activity during reperfusion was accompanied by an elevation of NO metabolites (nitrate and nitrite) in the kidney tissue. Injection of a NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), restored the CBS activity in the kidneys subjected to ischemia-reperfusion. Treatment with PTIO could abolish ischemia-reperfusion-induced lipid peroxidation and prevent cell death in the kidney. These results suggested that metabolic acidosis during ischemia and accumulation of NO metabolites during reperfusion contributed, in part, to reduced CBS activity leading to an elevation of renal Hcy levels, which in turn, played a detrimental role in the kidney.

Complete and prolonged interruption of renal arterial blood flow (ischemia) occurs during renal transplantation and surgical procedures such as nephrolithotomy, parenchymal sparing surgery for renal tumors, and renal arterial surgeries. Prolonged renal ischemia results in acute renal failure (5). Warm ischemia especially during the cadaveric renal transplantation surgery is responsible for 20–30% of primary graft dysfunction (30). The kidney follows the restoration of blood supply (reperfusion) is associated with renal vascular and tubular damage. We (31) recently observed that the Hcy level in the rat kidney tissue was increased by 2.9-fold during ischemia. Hcy accumulation in the renal tissue was responsible for ischemia-reperfusion injury due to oxidative stress and cell death (31).

Homocysteine is an intermediate amino acid formed during the metabolism of methionine. The kidney contains enzymes that are responsible for Hcy metabolism via the remethylation pathway and the transsulfuration pathway (13). However, it has been estimated that in the kidney the majority of Hcy (78%) is metabolized through the transsulfuration pathway (18). The cystathionine-β-synthase (CBS) catalyzes the rate-limiting step in the transsulfuration pathway in which Hcy is irreversibly catabolized to cysteine (13). Results from our previous study (31) demonstrated that renal Hcy accumulation during ischemia-reperfusion was a result of impaired CBS activity in the kidney. However, the mechanism of CBS activity reduction in the kidney is not clear.

Mammalian CBS is a pyridoxal-5′-phosphate-dependent enzyme in which heme is a cofactor associated with the N-terminal domain (21). From acidic to physiological pH, heme in the CBS is in the ferric form Fe(III), while at alkaline pH the ferrous form Fe(II) predominates (28). The optimal activity of CBS in vitro is recorded at pH 8.5. There is a profound connection between the heme group of recombinant human CBS and pH in the redox-sensitive regulation of enzyme activity. Thus it has been suggested that heme regulates CBS activity through changes in the iron redox state (28). The kidney is a major organ with regard to the regulation of the acid-base balance (6). It maintains the acid-base balance by bicarbonate reabsorption (6, 45). In addition, systemic acidosis stimulates phosphate-dependent glutaminase and glutamate dehydrogenase, which are involved in the generation of bicarbonate and ammonia (NH3) from glutamine in the proximal tubules (10). Increased urinary excretion of ammonium ion is a major component by which the kidney responds to metabolic acidosis (11, 20).

Nitric oxide (NO) readily reacts with iron that is either incorporated into the heme complex or in nonheme iron protein. It has been reported that recombinant human CBS binds NO in the ferrous Fe(II) state of heme, resulting in the inactivation of the enzyme (37). An increase in the tissue NO level has been reported in the rat kidney subjected to ischemia-reperfusion (31–33). The kidney contains all three isofoms of nitric oxide synthase (NOS). Neuronal (nNOS) and endothelial (eNOS) isofoms are mainly found in the macula densa and renal vasculature, respectively (8). The inducible form (iNOS) is expressed in the glomerulus, renal tubule, and arcuate arteries of the rat kidney (19). It was reported (22) that iNOS activity was increased dramatically during the first 24 h of
reperfusion in both the cortex and medulla of the rat kidney. Conversely, the eNOS activity decreased during reperfusion (22). After 6 h of reperfusion of the kidney, the plasma NO level was markedly increased due to the up-regulation of iNOS activity (8). Excessive production of NO, particularly from iNOS, or exogenous supply of NO before ischemia was found to play a detrimental role in ischemia-reperfusion-induced renal injury (4, 8, 27).

Since the CBS activity was significantly reduced in the kidney during ischemia-reperfusion, we hypothesized that the metabolic acidosis resulted from ischemia and that an elevation of tissue NO levels during reperfusion might play roles in regulating the CBS activity leading to Hcy accumulation in the kidney. Therefore, the aim of this study was to investigate the effect of an alkalinizing treatment during ischemia and NO scavenger during reperfusion on the CBS activity as well as on ischemia-reperfusion-mediated renal injury.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 250–300 g were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Rats were kept on a heat pad, and the rectal temperature was maintained at 37°C throughout the experimental procedure. Renal ischemia was induced by clamping the left renal artery for 15–60 min with nontraumatic vascular clamp (Fine Science Tools, Vancouver, Canada) as described previously (31, 36). Rats were subjected either to ischemia alone or ischemia followed by 1-, 6-, or 24-h reperfusion. In an ischemia alone group, rats were subjected to 45-min ischemia or ischemia with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Both kidneys in ice-cold potassium phosphate buffer were bisected. One-half was preserved in 10% formalin for paraffin embedding. The pH of tissue NO levels during reperfusion might play roles in regulating the CBS activity leading to Hcy accumulation in the kidney.

Measurement of CBS activity. The assay was based on a method developed by Mudd et al. (25) and modified by Taoka et al. (38). A 10% (wt/vol) tissue homogenate was prepared in 0.05 M potassium phosphate buffer (pH 6.9) followed by centrifugation at 18,000 g for 30 min at 4°C. The reaction was carried out in a reaction mixture containing 125 mM Tris-HCl (pH 8.5), 2.1 mM EDTA, 0.146 mM L-cystathionine, 41.7 mM Na-Hcy, 0.316 mM S-adenosylmethionine, 2.1 mM propargylglycine, and 0.42 mM pyridoxal-5’-phosphate. The reaction was initiated by adding 30 mM [14C]serine (PerkinElmer, Boston, MA) to the reaction mixture. After 1-h incubation at 37°C, ice-cold 15% trichloroacetic acid was added to stop the reaction. An aliquot of the reaction mixture was applied to a Bio-Rad AG 50W-X4 anion exchange column containing hydrogen-form resin. The column was washed with a series of water and 1 N HCl. Finally, cystathionine was eluted with 3 N NH4OH. The radioactivity associated with cystathionine was determined by using a Beckman liquid scintillation counter.

Measurement of NO activity. Total NO activity in the kidney was measured by an arginine to citrulline conversion assay (17). In brief, 10% (wt/vol) kidney tissue homogenate was prepared in 50 mM TEA-HCl (pH 7.4) containing 0.1 mM EDTA, 0.5 mM zinc ascorbate, 1 mM homocysteine, and 2 mM leupeptin. Hcy and the nitrocellulose membrane was probed with rabbit polyclonal antibody against L-arginine (Cayman Chemical). The reaction was carried out for 20 min at 4°C. The plasma creatinine level was determined by CA (to confirm the equal loading of proteins prepared from kidneys. The degree of lipid peroxidation in the kidney during ischemia-reperfusion, we hypothesized that the metabolic acidosis resulted from ischemia and that an elevation of tissue NO levels during reperfusion might play roles in regulating the CBS activity leading to Hcy accumulation in the kidney. Therefore, the aim of this study was to investigate the effect of an alkalinizing treatment during ischemia and NO scavenger during reperfusion on the CBS activity as well as on ischemia-reperfusion-mediated renal injury.

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Determination of NO metabolites in the kidney tissue homogenate. The measurement of nitrite and nitrate was used to assess the NO levels in kidney tissue (40). In brief, the kidney tissue was homogenized in a buffer containing 20 mM Tris (pH 7.4) and 2 mM EDTA. The supernatant was collected after a 10-min centrifugation at 1,500 g at 4°C. The supernatant was deproteinized with 0.3 N NaOH and 5% ZnSO4. After deproteinization, the amount of nitrite and nitrate was determined with a Griess reaction method based on the azo coupling reaction (40). In brief, the supernatant was incubated with nitrate reductase to reduce nitrate to nitrite. Then, 12.5 mM sulfuramidose in 6 N HCl and 12.5 mM N-(1-naphthyl)ethylenediamine were added to the nitrite solution to complete the azo coupling reaction. The diazino amino benzene in the reaction mixture was measured by spectrophotometer at an absorbance of 543 nm. NaNO2 was served as the standard.

Determination of iNOS protein. A portion of kidney was homogenized in lysis buffer containing 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2.1 μM leupeptin, 1 mM PMSF, and 1% (v/v) Triton X-100. The supernatant was collected after centrifugation of tissue homogenate at 6,000 g for 10 min. An equal amount of cellular proteins from each sample was separated by SDS/7.5% (wt/vol)-PAGE. The proteins in the gel were then transferred to a nitrocellulose membrane. The membrane was probed with rabbit polyclonal anti-iNOS antibodies (1:1,000; Calbiochem, Canada). Bands corresponding to iNOS protein were visualized with enhanced chemiluminescence reagents and exposed to Kodak BioMax Light film (43). Film was analyzed with Bio-Rad Quantity-One image analysis software (version 4.2.1). As an internal control, the nitrocellulose membrane was probed with rabbit polyclonal anti-β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) to confirm the equal loading of proteins prepared from kidneys.

Determination of lipid peroxidation. The degree of lipid peroxidation in the kidney tissue was determined by measuring the malondialdehyde (MDA) levels with thiobarbituric acid reactive substances.
assay (26, 36). Briefly, a 10% (wt/vol) kidney homogenate was prepared in 0.1 M KCl solution containing 3 mM 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, and 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 a spectrophotometer at an absorbance of 532 nm. MDA was used as the standard, and results were expressed as a percentage of sham-operated group. The amount of MDA correlates with the degree of lipid peroxidation in the tissue.

Histological and immunohistochemical staining. A portion of the kidney was immersion fixed in 10% neutral-buffered formalin overnight followed by embedding in paraffin. Sequential 5-μm paraffin-embedded cross sections were prepared. Hematoxylin and eosin staining was performed to examine histological changes in the kidney (36). Next, immunohistochemical staining was performed to detect nitrotyrosine protein adducts in the kidney (46). In brief, after deparaffinization, sections were incubated with primary antibodies, mouse anti-nitrotyrosine antibodies (1:100; Zymed Laboratories, San Francisco, CA). After overnight incubation with primary antibodies, sections were treated with 0.3% hydrogen peroxide (H2O2) for 20 min at room temperature to inhibit the endogenous peroxidase. Sections were incubated with biotin-conjugated anti-mouse immunoglobulins (1:200; DakoCytomation, Carpinteria, CA) as secondary antibodies followed by streptavidin-horseradish peroxidase conjugate (Zymed Laboratories). Sections were then treated with 3,3-diaminobenzidine-H2O2 colorimetric substrate solution. The attached peroxidase catalyzed the H2O2-mediated oxidation of 3,3-diaminobenzidine to yield a brown color. The area displayed brownish color indicating the nitrotyrosine protein adducts (46). The images were captured using an

![Fig. 1. Kidney tissue pH and cystathionine-β-synthase (CBS) activity during ischemia or ischemia-reperfusion. Left kidney was subjected to sham operation (Sham), ischemia (15, 45, or 60 min), or ischemia (45 min) followed by reperfusion for various time periods (1, 6, or 24 h). A: pH was determined in kidney tissue homogenate prepared in deionized water. B: CBS activity was determined. Results are expressed as means ± SE (n = 6). *P ≤ 0.05, when compared with the value obtained from sham-operated group.](http://ajprenal.physiology.org/)

![Fig. 2. Effect of pH on kidney CBS activity. Left kidney was subjected to sham operation (Sham), 45-min ischemia (I-45min), or 45-min ischemia followed by 6-h reperfusion (I-45min/R-6h). In 1 group of rats, an injection of (NH4)2HPO4 was given through the left renal artery immediately after induction of ischemia [I-45min/(NH4)2HPO4]. Another group of rats that received (NH4)2HPO4 during ischemia were allowed 6-h reperfusion [I-45min/R-6h/(NH4)2HPO4]. A: CBS activity was determined in the kidney tissue homogenate (n = 6). B: 10% (wt/vol) kidney tissue homogenate was prepared in 0.05 M potassium phosphate buffer at pH 6.5. After preincubation for 45 min, pH was adjusted at 8.5 in 1 group. CBS activity was determined with a reaction mixture at the corresponding pH (n = 3). *P ≤ 0.05, when compared with sham-operated group. †P ≤ 0.05, when compared with the value obtained from I-45min group.](http://ajprenal.physiology.org/)
Axioskop2 MOT microscope (Carl Zeiss Microimaging, Thornwood, NY) with an AxioCam camera and analyzed using Photoshop 6.0 (Adobe, San Jose, CA). For a negative control, nonspecific IgG was used as the primary antibody.

**Terminal deoxyribonucleotide transferase-mediated dUTP nick-end labeling.** Renal ischemia-reperfusion in animal models leads to both necrotic and apoptotic forms of cell death (31). DNA fragmentation and terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick-end labeling (TUNEL staining) was performed in renal sections as an index of cell injury (15, 47). Briefly, a portion of the kidney was fixed in 10% neutral-buffered formaldehyde overnight and then embedded in paraffin. DNA fragments in the sliced sections were labeled with 3 \( \mu \text{M} \) biotin-conjugated dUTP and 0.3 U/\( \mu \text{l} \) TdT (Roche Molecular Biochemical, Quebec, Canada) followed by counterstaining with Mayer’s hematoxylin. TUNEL-positive cells were recognized by focal nuclear staining (15, 47). As a positive control, a section of the sham-operated kidney was pretreated with DNase to mimic the appearance of apoptotic cells. The number of TUNEL-positive cells in the renal cortex and medulla was counted in 10 microscopic fields under a light microscope at a magnification of \( \times 400 \).

**Statistical analysis.** Results were analyzed using Student’s \( t \)-test and ANOVA followed by Newman-Keuls multiple comparison test as appropriate. Data are presented as means \( \pm \) SE. A \( P \) value \( \leq 0.05 \) was considered significant.

**RESULTS**

**Effect of pH fluctuation on kidney CBS activity.** To determine whether changes in tissue pH had any effect on CBS activity, rat kidneys were subjected to ischemia (15 to 60 min). The kidney tissue pH was decreased after ischemia for 15, 45, or 60 min (Fig. 1A). In correspondence, the CBS activity was measured (Fig. 1B). A: kidney tissue homogenate 10% (wt/vol) was prepared in 0.05 M potassium phosphate buffer (pH 6.9). Different concentrations of SNP were used as the source of NO. CBS activity was measured at pH 8.5 (\( n = 4 \)). B: left kidney was subjected to sham operation; 45-min ischemia followed by 6-h reperfusion (I-45min/R-6h); a 45-min ischemia followed by 6-h reperfusion with an intravenous injection of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO; I-45min/R-6h + PTIO); 45-min ischemia with an intrarenal arterial injection of \( (\text{NH}_4)\text{HPO}_4 \) during ischemia followed by 6-h reperfusion with an intravenous injection of PTIO [I-45min/R-6h + (\text{NH}_4)\text{HPO}_4 + PTIO]; or 45-min ischemia followed by 6-h reperfusion with an intravenous injection of 1.5 mg/kg of \( N^\text{G} \)-nitro-L-arginine methyl ester (\( L \)-NAME; \( n = 6 \)). C: kidney tissue homogenate 10% (wt/vol) was prepared in 0.05 M potassium phosphate buffer (pH 8.5). An aliquot of kidney tissue homogenate (150 \( \mu \text{l} \)) was preincubated for 15 min with SNP (200 \( \mu \text{M} \)) followed by addition of equal molar concentration of PTIO (200 \( \mu \text{M} \) SNP + 200 \( \mu \text{M} \) PTIO). In another set of treatments, an aliquot of kidney tissue homogenate (150 \( \mu \text{l} \)) was preincubated for 15 min with PTIO (200 \( \mu \text{M} \)) followed by addition of equal molar concentration of SNP (200 \( \mu \text{M} \) PTIO + 200 \( \mu \text{M} \) SNP). CBS activity was measured (\( n = 3 \)). D: left kidney was subjected to sham operation or 45-min ischemia followed by 6-h reperfusion (I-45min/R-6h). Inducible nitric oxide synthase (iNOS) protein level was determined by Western blotting analysis (\( n = 6 \)). Results are expressed as means \( \pm \) SE. \( \dagger P \leq 0.05 \), when compared with control group. \( * P \leq 0.05 \), when compared with the value obtained from sham-operated group. \# \( P \leq 0.05 \), when compared with the ischemia-reperfusion group. \( \ddagger P \leq 0.05 \), when compared with the group, which was preincubated with SNP first.
decreased in those kidney tissue samples (Fig. 1B). Although the kidney tissue pH was elevated during reperfusion (Fig. 1A), the CBS activity remained at the reduced levels (Fig. 1B). These results suggested that factors other than acidosis also affected the CBS activity during the reperfusion period. Phosphate and NH₃ are the two major buffering agents present in urine. Phosphate is freely filtered by glomeruli and travels down the tubules where it combines with H⁺ to form H₂PO₄⁻ (20, 45). Therefore, the (NH₄)₂HPO₄ solution was used as an alkalinizing agent to counteract the metabolic acidosis that was developed during ischemia. According to our preliminary studies, an intrarenal arterial injection of 250 μl of 10 mM (NH₄)₂HPO₄ was required to retain the kidney tissue pH at a value similar to sham-operated kidneys after 45 min of ischemia (data not shown). In the subsequent experiments, one dose (250 μl) of 10 mM (NH₄)₂HPO₄ was injected into rats via the left renal artery immediately after the onset of ischemia. Such a treatment significantly increased the CBS activity in kidneys subjected to ischemia alone or ischemia-reperfusion when compared with the untreated ischemia group (Fig. 2A). However, (NH₄)₂HPO₄ treatment in the ischemia-reperfusion group did not show any statistically significant increase in the CBS activity when compared with the untreated ischemia-reperfusion group (Fig. 2A). In vitro assays displayed that the pH-mediated inhibition of the CBS activity was completely reversible once the pH in the tissue homogenate was restored to 8.5 (Fig. 2B). The effect of pH on the CBS activity was further examined in the in vitro assay. The tissue homogenate was prepared from rat kidneys without surgery and used for CBS activity measurement. The CBS activity was determined in the reaction mixtures with pH ranging from 6.5 to 10. The optimum enzyme activity of CBS was recorded at pH 8.5 (Fig. 2C). These results suggested that metabolic acidosis during ischemia might contribute to the reduction of CBS activity in the kidney.

**Effect of NO on kidney CBS activity.** NO is a known inhibitor of CBS activity (37). The effect of NO on the kidney CBS activity was measured in the presence of various amounts of sodium nitroprusside (SNP), a known NO donor, in the reaction mixture. Addition of SNP to the assay reaction mixture displayed a dose-dependent inhibition of the kidney CBS activity (Fig. 3A). To further confirm the inhibitory effect of

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**Fig. 4.** The nature of NO-mediated inhibition of CBS activity. A: kidney tissue homogenate (10% wt/vol) was prepared in 0.05 M potassium phosphate buffer (pH 8.5). CBS activity was measured in a reaction mixture containing 200 μM SNP and PTIO at different concentrations (n = 3). B: kidney homogenate (10% wt/vol) was prepared in 0.05 M potassium phosphate buffer at different pH (6.5–10). CBS activity was measured at corresponding pH in the absence or presence of 200 μM SNP. C: left kidney was subjected to sham operation, 45-min ischemia, or 45-min ischemia followed by 1-, 6-, or 24-h reperfusion, or 45-min ischemia followed by 6-h reperfusion with (NH₄)₂HPO₄ injection through the left renal artery immediately after the induction of ischemia or intravenous injection of PTIO via femoral vein at the onset of reperfusion. Kidney tissue nitrite and nitrate levels were measured. D: kidney tissue NOS activity was determined (n = 6). Results are expressed as means ± SE. †P ≤ 0.05, when compared with the value obtained from control group. ‡P ≤ 0.05, when compared with the group treated with SNP alone. *P ≤ 0.05, when compared with the sham-operated group.
NO on the CBS activity, an NO scavenger (PTIO) was administered to rats. Injection of PTIO via the right femoral vein at the onset of reperfusion partially restored the CBS activity (Fig. 3B). Combined injection of (NH₄)₂HPO₄ during ischemia and injection of PTIO during reperfusion also significantly restored the CBS activity in the kidney (Fig. 3B). L-NAME was injected at three different doses: 10, 3, and 1.5 mg/kg (data not shown). Only at the lowest concentration (1.5 mg/kg) was L-NAME effective in regaining the CBS activity (Fig. 3B). In contrast to the inhibitory effect of acidic pH on the CBS activity, NO caused an irreversible inhibition (Fig. 3C). When the tissue homogenate was preincubated with SNP, the subsequent addition of PTIO as an NO scavenger was unable to restore the CBS activity (Fig. 3C). On the other hand, the preincubation with PTIO provided significant protection against the NO-mediated inhibition upon the subsequent addition of SNP (Fig. 3C). Western immunoblotting analysis revealed that there was a significant elevation in iNOS protein level in kidneys subjected to ischemia-reperfusion, while PTIO treatment did not affect the iNOS protein level in the tissue (Fig. 3D).

The nature of the protective effect of PTIO on CBS activity was further examined in vitro. In the presence of the NO donor SNP (200 μM), PTIO had stoichiometrically a greater NO scavenging capacity at higher SNP:PTIO levels (Fig. 4A). PTIO, at the equal molar of SNP (200 μM), showed the

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**Fig. 5.** Effect of NO scavenger on kidney tissue Hcy concentration, lipid peroxidation, and plasma creatinine levels. Left kidney was subjected to sham operation, 45-min ischemia followed by 6-h reperfusion, or 45-min ischemia followed by a 6-h reperfusion with an intravenous injection of PTIO. A: kidney tissue Hcy concentration, plasma Hcy concentration (B), lipid peroxidation (C), and plasma creatinine levels were measured after 6-h (D) and 24-h (E) reperfusion. Results are expressed as means ± SE (n = 6). *P ≤ 0.05, when compared with the value obtained from sham-operated group. #P ≤ 0.05, when compared with value obtained from I-45min/R-6h group. †P ≤ 0.05, when compared with the value obtained from I-45min/R-24h group.
maximum restoration of the SNP-mediated inhibition of CBS activity (Fig. 4A). The NO-mediated inhibition of CBS activity was significantly greater at alkaline conditions (Fig. 4B), which, presumably, explained why CBS activity was not restored during the reperfusion period. Next, the levels of NO metabolites were determined in the kidney tissue. Since NO is unstable and quickly oxidized, the tissue level of NO is determined by measuring the level of its metabolites, namely, nitrate and nitrite (14). A significant increase in the NO metabolite levels was observed in the kidney tissue during the reperfusion periods (Fig. 4C). There appeared to be an inverse relationship between the CBS activity (Fig. 1B) and the NO level in the kidney during the reperfusion periods (Fig. 4C). The renal NOS activity was increased in the ischemia-reperfusion group (Fig. 4D). Ischemia alone did not seem to affect the NOS activity (Fig. 4D); however, the level of NO metabolites was increased (Fig. 4C). The elevation of the NO metabolite level during ischemia might be derived from tissue stores (33). Neither the alkaline solution nor PTIO treatment resulted in a decrease in the NO metabolite level nor in the NOS activity or protein level in the kidney tissue (Fig. 4, C and D). These results suggest that an elevation of tissue NO levels might contribute to the reduced CBS activity in the kidney subjected to ischemia followed by reperfusion.

Effect of NO scavenging on oxidative stress and apoptosis in the kidney. There was a significant elevation of Hcy levels in kidneys subjected to 45-min ischemia followed by 6-h reperfusion (Fig. 5A). Administration of PTIO reversed the tissue Hcy levels to that of the sham-operated group (Fig. 5A). The alkalinization treatment during ischemic period did not result in a decrease in tissue Hcy levels after 6-h reperfusion period (data not shown). The low level of Hcy accumulation in the kidney subjected to ischemia-reperfusion was disproportionate to the degree of CBS activity inhibition. This might be due to the efflux of Hcy from the kidney tissue to the circulation compartment during the reperfusion period. As shown in Fig. 5B, ischemia-reperfusion-induced elevation of plasma Hcy was much greater than that in the kidney tissue (Fig. 5B). Removal of the right kidney and impaired function of the left kidney during the reperfusion period might have contributed to such a discrepancy. The PTIO treatment also prevented lipid peroxidation, as determined by measuring the level of MDA in the kidney tissue (Fig. 5C). Kidney function was determined by plasma creatinine measurements. The PTIO treatment showed a significant reduction in the plasma creatinine level (Fig. 5D). Kidney function was further tested by measuring plasma creatinine after 24-h reperfusion. A second dose of PTIO (first dose was administered at the onset of reperfusion) was administered via intraperitoneal route at after a 12-h reperfusion to these rats. Plasma creatinine level remained significantly decreased in PTIO-treated group (0.729 ± 0.018 mg/dl) when compared with the 24-h untreated ischemia-reperfusion group (0.867 ± 0.026 mg/dl; Fig. 5E). NO can react with superoxide anion to form peroxynitrite. Ischemia-reperfusion caused a significant increase in the intensity of nitrotyrosine protein adduct staining, indicating an increased peroxynitrite formation. Rats treated with PTIO displayed much less nitrotyrosine protein adduct staining in the kidney tissue (Fig. 6A). TUNEL-positive cells were present in both the cortex and medulla upon ischemia-reperfusion, indicating cell death (due to apoptosis and necrosis) in the kidney (Fig. 6B). The PTIO treatment caused a significant reduction in the count of TUNEL-positive cells (Fig. 6B). The gross and microscopic appearance of kidney sections was also examined. Administration of PTIO retained the gross appearance of ischemia-reperfusion kidneys similar to the sham-operated group (Fig. 6C). Hematoxylin and eosin stained tissue sections showed massive extravasations of blood cells in the medulla region of the ischemia-reperfusion group (Fig. 6C). The PTIO treatment caused a marked reduction in blood cell infiltration (Fig. 6C). TUNEL staining was also performed in kidneys subjected to 45 min of ischemia followed by reperfusion for 24 h. The number of TUNEL-positive cells was significantly elevated in kidneys after reperfusion for 24 h (Fig. 7). The PTIO treatment significantly reduced the number of dead cells in kidney tissue (Fig. 7).

DISCUSSION

We previously demonstrated that ischemia-reperfusion-induced renal injury was, in part, mediated by Hcy (31). The reduced CBS activity, initially as a consequence of ischemia, remained in the diminished state throughout 24-h reperfusion (31). The present study identified two factors, namely, pH and NO, that affected the CBS activity during ischemia and reperfusion. Decreased pH in the kidney during ischemia caused the initial inhibition of CBS activity, while the elevation of NO levels prevented restoration of CBS activity during reperfusion. Furthermore, the administration of the NO scavenger could restore the CBS activity significantly in kidneys subjected to ischemia-reperfusion. Renal injury was substantially minimized in rats given NO scavenger as reflected by histo-
REGULATION OF CYSTATHIONINE-β-SYNTHASE ACTIVITY

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A

Cortex Medulla

Sham

I-45 min/R-6h

I-45 min/R-6h + PTIO

Negative control

B

Cortex Medulla

Sham

I-45 min/R-6h

I-45 min/R-6h + PTIO

Positive control

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**A**

**Nitrotyrosine staining Mean intensity (% of Sham)**

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<th>Sham</th>
<th>I-45 min/R-6h</th>
<th>I-45 min/R-6h + PTIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrotyrosine staining Mean intensity (% of Sham)</td>
<td>±</td>
<td>+</td>
<td>#</td>
</tr>
</tbody>
</table>

**B**

**TUNEL positive Cell count/100μm²**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>I-45 min/R-6h</th>
<th>I-45 min/R-6h + PTIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUNEL positive Cell count/100μm²</td>
<td>*</td>
<td>+</td>
<td>* #</td>
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</table>

**C**

Cortex Medulla

Sham

I-45 min/R-6h

I-45 min/R-6h + PTIO
Effect of an alkalizing treatment during ischemia on CBS activity. First, we examined the effect of pH on renal CBS activity during ischemia-reperfusion. The anaerobic metabolism during ischemia could result in metabolic acidosis in the kidney (29). Metabolic acidosis was severe when the kidney ischemia was prolonged. There was an inverse relationship between the degree of acidosis and the CBS activity (Fig. 1). Injection of an alkaline solution (NH₄)₂HPO₄ into the kidney at the onset of ischemia improved the CBS activity compared with the ischemia group (Fig. 2A). However, such a treatment was unable to restore the CBS activity to the level found in the control group, perhaps due to the limited availability of free NH₃ in the solution. NH₃ in a solution can cross cell membranes predominantly in their uncharged form and can consume cytosolic H⁺, resulting in an increase in intracellular pH (6). Some other factors, which include carbon monoxide (CO), might have also contributed to the inhibition of CBS activity during ischemia (37). It was reported that CO-mediated inhibition of CBS activity was reversible (39). Therefore, if CO played a role in the impairment of CBS activity during ischemia, it might not exert an inhibitory effect throughout the reperfusion period, since the aerobic metabolism was restored once reperfusion started. Similarly, the pH-mediated inhibition of CBS activity was also reversible (Fig. 2B). The kidney tissue pH was increased to the level higher than the normal physiological level during reperfusion. The response of kidney to an acute acidosis is as such that the withdrawal of the cause of acute acidosis follows a rise in intracellular pH above its initial level (6). This agrees with the observation in the present study (Fig. 1A). Kidney CBS activity exhibited a pH optimum of 8.5 (Fig. 2C). Interestingly, neither the elevation of tissue pH during reperfusion nor the alkaline treatment at the induction of ischemia was able to restore the CBS activity in the kidney after 6-h reperfusion period (Figs. 1 and 2A). These results suggest that factor(s) other than pH change might also be responsible for reduced CBS activity in the kidney particularly during reperfusion.

Effect of an NO scavenger on CBS activity. Next, we investigated whether NO played a role in the reduction of CBS activity in the kidney upon ischemia-reperfusion. It was reported that NO was able to inhibit CBS activity (12, 37). In the present study, CBS activity was inhibited in vitro when a known NO donor (SNP) was added to the enzyme assay mixture (Fig. 3A). However, preincubation of kidney tissue homogenate with an NO scavenger (PTIO) prevented the subsequent inhibition of CBS activity by SNP (Fig. 3C), suggesting a low affinity interaction between NO and CBS (37). However, PTIO was unable to restore the CBS activity when kidney tissue homogenate was first incubated with SNP (Fig. 3C), indicating the irreversibility of NO-mediated inhibition on CBS. Levels of NO metabolites remained elevated in the kidney tissue during ischemia and reperfusion periods (Fig. 4C), while a significant increase in the NOS activity was detected in the kidney during reperfusion period (Fig. 4D). In humans, both urinary and serum nitrite and nitrate levels are significantly elevated in renal transplant recipients who subsequently develop acute renal failure. The elevation of NO metabolite levels occurs before clinical diagnosis of acute renal failure in these patients and subsides with successful therapeutic intervention (2). Administration of either PTIO or L-NAME to the kidney at the onset of reperfusion showed a significant recovery of CBS activity after 6-h reperfusion when compared with the untreated ischemia-reperfusion group (Fig. 3B). The NO-mediated inhibition of CBS activity was greater toward alkaline pH (Fig. 4B). Therefore, similar to the in vitro observation, it was plausible that an interaction of NO with CBS might be responsible for the reduction of CBS activity during reperfusion. The use of NOS inhibitors showed a promising renal protective effect against ischemia-reperfusion injury in the rat (7, 8). Moreover, iNOS knockout mice were protected...
against ischemia-reperfusion-mediated acute renal failure (23). In the present study, PTIO was used to counteract the excess NO generated in the kidney during ischemia-reperfusion. A recent study (24) showed that PTIO treatment prevented the acute renal failure associated with septic shock in the rat. At higher NO concentrations, the stoichiometric ratio of the interaction between NO and PTIO reaches two, while at steady state one NO molecule reacts with one PTIO molecule (16). In addition to PTIO, the effect of the NOS inhibitor L-NAME was examined at three different doses (10, 3, and 1.5 mg/kg). L-NAME at the lowest concentration (1.5 mg/kg) showed a protective effect (Fig. 3B). These results suggested that NO in excess amount could be hazardous and that maintaining the NO at physiological levels was important for renal function. Taken together, the protective effect of PTIO might be mediated via its ability to scavenge excess NO produced in the kidney tissue during ischemia-reperfusion (22).

Effect of the restoration of CBS activity on renal injury. Finally, we explored whether the correction of kidney tissue CBS activity could ameliorate the ischemia-reperfusion-mediated renal injury. The inhibition of CBS activity during ischemia-reperfusion caused Hcy accumulation in the kidney tissue during ischemia (31). Partial restoration of CBS activity by PTIO treatment resulted in a significant decrease in kidney tissue Hcy level (Fig. 5A). However, the level of NO metabolites as measured by tissue nitrite and nitrate level was not reduced in the PTIO-treated group (Fig. 4C). This discrepancy might be attributed to the nature of the reaction between PTIO and NO. Reaction of PTIO with NO results in the formation of NO2 (1). The method we used in the present study to determine kidney tissue NO was based on the measurement of NO metabolites (nitrate and nitrite). The restoration of CBS activity had a protective effect against kidney tissue injury, as assessed by the formation of nitrotyrosine protein adducts (Fig. 6A), lipid peroxidation (Fig. 5C), and apoptotic and necrotic cell count (Fig. 6B). In addition, the renal function, as determined by the plasma creatinine level, was improved when the CBS activity was partially restored in the PTIO-treated group (Fig. 5, D and E). These findings further support the notion that Hcy, at elevated levels, contributes to kidney ischemia-reperfusion injury (31). The reperfusion caused an exportation of intracellular Hcy into the plasma where it could be oxidized into homocysteine, Hcy mixed disulfides, and reactive oxygen species (31, 35). Thus the generation of reactive oxygen species should be an extracellular phenomenon during reperfusion. An increase in levels of reactive oxygen species was found in the renal venous blood during kidney ischemia-reperfusion (9). Reactive oxygen species can impair the integrity of the endothelium of blood vessels (41). During reperfusion, the integrity of the renal vasculature was compromised, which resulted in a massive extravasation of intravascular fluid and blood cells into a previously ischemic area (42). As shown in Fig. 6C, the renal medulla was extensively infiltrated with blood cells in ischemia-reperfused kidneys. Conversely, the treatment of PTIO during ischemia or reperfusion partially retained the CBS activity and prevented the extravasation of blood cells. The renal protective effect of PTIO treatment as measured by lipid peroxidation and histological examination was greater than its effect on the restoration of CBS activity. NO is a reactive nitrogen species and at higher levels can exert oxidative stress via its reaction with superoxide anion to form a potent radical, peroxynitrite. The scavenging of NO by PTIO might have ameliorated the direct cytotoxic effect by peroxynitrite.

In summary, our results suggest that the inhibition of CBS activity during ischemia-reperfusion is biphasic. Metabolic acidosis exerted an inhibition on CBS activity during ischemia, while NO binding to CBS prevented the restoration of the enzyme activity during reperfusion. A treatment with an NO scavenger partially restored the CBS activity, which, in turn, substantially ameliorated ischemia-reperfusion-induced renal injury. Regulation of CBS activity may have a therapeutic potential for renal ischemia-reperfusion injury.

GRANTS

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REFERENCES

Regulation of cystathionine-β-synthase activity


