Detrimental role of homocysteine in renal ischemia-reperfusion injury

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Prathapasinghe GA, Siow YL, O K. Detrimental role of homocysteine in renal ischemia-reperfusion injury. Am J Physiol Renal Physiol 292: F1354–F1363, 2007. First published January 30, 2007; doi:10.1152/ajprenal.00301.2006.—Ischemia followed by reperfusion is a major cause for renal injury in both native kidney and renal allografts. Hyperhomocysteinemia, a condition of elevated plasma homocysteine (Hcy) level, is associated with cardiovascular diseases. Recent evidence suggests that Hcy, at higher levels, may be harmful to other organs such as the kidney. In this study, we investigated the role of Hcy in ischemia-reperfusion-induced renal injury. The left kidney of a Sprague-Dawley rat was subjected to either 30-min or 1-h ischemia followed by 1- or 24-h reperfusion. Ischemia-reperfusion caused a significant increase in peroxynitrite formation and lipid peroxidation in kidneys, which reflected oxidative stress. The number of apoptotic cells in those kidneys was also markedly increased. Hcy levels were elevated 2.9- and 1.5-fold in kidneys subjected to ischemia alone or ischemia-reperfusion, respectively. Further investigation revealed that elevation of Hcy level in the kidney upon ischemia-reperfusion was due to reduced activity of cystathionine-β-synthase, a key enzyme in Hcy metabolism. Administration of anti-Hcy antibodies into the kidney not only abolished ischemia-reperfusion-induced oxidative stress and cell death in the kidneys but also restored renal function after 1 h of reperfusion. However, such a protective effect was not sustained after 24 h of reperfusion. In conclusion, ischemia-reperfusion impairs Hcy metabolism in the kidney. Hcy, at elevated levels, is capable of inducing oxidative stress and renal injury. Neutralization of Hcy with antibodies offers transient functional benefit against ischemia-reperfusion-induced oxidative stress and renal injury. These results suggest that Hcy may play a detrimental role in the kidney during ischemia-reperfusion.

oxidative stress; cystathionine-β-synthase; kidney function

ISCHEMIA-REPERFUSION INJURY is one of the most common causes for delayed function of renal allografts and is associated with poor long-term renal function (3). Complete and prolonged interruption of renal arterial blood flow occurs during renal transplantation or surgical procedures such as nephrolithotomy, parenchymal sparing surgery for renal tumors, and renal arterial surgeries. Prolonged renal ischemia can lead to acute renal failure (2). It has been estimated that ischemic insult, especially during renal transplantation, is responsible for 20–30% primary graft dysfunction (23). Our previous study demonstrated that ischemia-reperfusion caused oxidative stress leading to NF-κB activation and increased monocyte/macrophage infiltration into the kidney (28). Oxidative stress represents one of the important mechanisms underlying renal ischemia-reperfusion injury (21, 28).

Hyperhomocysteinemia, a condition of elevated plasma homocysteine (Hcy) level, is associated with cardiovascular and cerebral vascular disorders (6, 10). A recent epidemiological study revealed a positive association between the plasma Hcy level and development of chronic kidney disease in the general population (20). Hyperhomocysteinemia is a common clinical finding in patients with chronic kidney diseases and occurs almost uniformly in patients with end-stage renal disease (5, 26). A recent study indicated that elevated plasma total Hcy was independently associated with increased kidney allograft loss in humans (36). Hcy, at elevated levels, is associated with oxidative stress in extrarenal tissue (1, 34), as well as in the kidney (7, 38). The free or reduced sulfhydryl group of Hcy is highly reactive at the physiological pH, and in the presence of molecular oxygen, Hcy undergoes thiol oxidation reactions (12). Reactive oxygen species such as superoxide and hydrogen peroxide are produced during autooxidation of Hcy (18). It has been suggested that autooxidation of Hcy represents one of the mechanisms contributing to Hcy-induced cell injury (27, 33, 35). Our recent study demonstrated that levels of peroxynitrite and oxygen-derived free radicals are significantly elevated in kidneys isolated from hyperhomocysteinemic rats (38). Hcy is an intermediate amino acid formed during the metabolism of methionine. Hcy can be metabolized via two major pathways, namely, the remethylation pathway and transsulfuration pathway (8). In the remethylation pathway, Hcy is converted to methionine by using folate or betaine as methyl group donors. In the transsulfuration pathway, Hcy is irreversibly metabolized to cysteine. Although the kidney contains enzymes that are responsible for Hcy metabolism via remethylation and transsulfuration pathways (8), it is estimated that in the kidney, the majority of Hcy is metabolized through the transsulfuration pathway (13). In mammals, cystathionine-β-synthase (CBS) catalyzes the rate-limiting step in the transsulfuration pathway (15, 29).

Although hyperhomocysteinemia is often seen in patients with kidney disease or postrenal transplantation, it is not known whether Hcy contributes to renal injury during ischemia-reperfusion. The aim of the present study was to investigate the role of Hcy in ischemia-reperfusion-induced injury in rat kidney.

MATERIALS AND METHODS

Renal ischemia-reperfusion. Male Sprague-Dawley rats weighing 250–300 g were anesthetized with an injection of pentobarbital sodium (50 mg/kg) intraperitoneally. 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 30 min or 1 h with nontraumatic vascular clamp (Fine Science Tools, Vancouver, BC) as previously described (28). After

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the clamp was removed, reperfusion was confirmed visually and allowed for another 1 h. Both kidneys were harvested and collected in ice-cold potassium phosphate buffer. In some experiments, rats were subjected to nephrectomy of right kidney and 30-min ischemia of left kidney followed by 1- or 24-h reperfusion. A blood sample was drawn from the abdominal aorta before rats were killed. Plasma was separated by centrifugation of blood at 3,000 g for 20 min at 4°C. In one set of experiments, rats were injected with rabbit anti-Hcy polyclonal antibodies (250 μg, 1:20 dilution; Chemicon International, Temecula, CA) or rabbit IgG (250 μg, 1:20 dilution; Santa Cruz Biotechnology) through the left renal artery immediately after the induction of ischemia. Injection of anti-Hcy antibodies to the kidney via left renal artery immediately after the induction of ischemia would allow immediate access of antibodies to the kidney to neutralize Hcy generated inside the kidney during ischemia-reperfusion, to minimize antibody dilution in the circulation and to minimize systemic immune response since anti-Hcy antibodies were raised in rabbit. In another set of experiments, rats received l-Hcy (2.5 mg/kg) or vehicle (buffer used to prepare l-Hcy) via the left femoral vein when reperfusion started. l-Hcy was prepared from l-Hcy thiolactone which was hydrolyzed with 5 M NaOH at 37°C for 5 min and neutralized with 1 M KH₂PO₄ (31). A sham-operated group of rats was subjected to the same surgical procedure without inducing ischemia-reperfusion and were killed at corresponding time points. Results obtained from this group were used as controls. The Hcy concentrations in the plasma and kidney tissue homogenate were measured with the IMx Hcy assay which was based on fluorescence polarization immunoassay technology (Abbott Diagnostics, Abbott Park, IL) (1, 37). Plasma creatinine level was determined by using WAKO creatinine kit (Wako Chemical Industries) (39). Kidneys in ice-cold potassium phosphate buffer were bisected. One-half were preserved in 10% formalin for paraffin embedding. All procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and approved by the University of Manitoba Protocol Management and Review Committee.

**Determination of lipid peroxidation.** The degree of lipid peroxidation in the kidney tissue was determined by measuring malondialdehyde (MDA) levels with thiobarbituric acid reactive substances (TBARS) assay (22, 28). 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, 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 are expressed as a percentage of sham-operated group. The amount of MDA correlates to the degree of lipid peroxidation produced in the tissue.

**Measurement of CBS activity.** The assay was based on a method developed by Mudd et al. (19) and modified by Taoka et al. (29). 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

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![Fig. 1](http://ajprenal.physiology.org/content/292/5/F1355/F1355)  
**Fig. 1.** Measurement of homocysteine (Hcy) concentrations and renal cystathionine-β-synthase (CBS) activity. **A:** left kidney was subjected to 1-h ischemia or 1-h ischemia followed by 1-h reperfusion (I/R). Hcy level in the kidney tissue homogenate was measured. In the control group (Sham), only the laparotomy was performed. **B:** plasma Hcy levels in different groups of rats were measured. **C:** kidney 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.

![Fig. 2](http://ajprenal.physiology.org/content/292/5/F1355/F1355)  
**Fig. 2.** Measurement of superoxide and nitric oxide (NO) metabolites in the kidney. Left kidney was subjected to 1-h ischemia or 1-h ischemia followed by 1-h reperfusion (I/R). **A:** kidney superoxide anion levels were measured. **B:** kidney NO metabolites (nitrite and nitrate) were determined. Results are expressed as means ± SE (n = 6). *P < 0.05 when compared with the value obtained from sham-operated group.
30 min at 4°C. The reaction was carried out in a reaction mixture containing 125 mM Tris·HCl (pH 8.3), 2.1 mM EDTA, 0.146 mM L-cystathionine, 41.7 mM DL-Hcy, 0.316 mM S-adenosylmethionine, 2.1 mM propargylglycine, and 0.42 mM pyridoxal phosphate. The reaction was initiated by adding 30 mM [1-14C] serine to the reaction mixture. After 1-h incubation at 37°C, ice-cold 15% perchloric acid was added to stop the reaction. An aliquot of the reaction mixture (0.5 ml) was applied to a Bio-Rad AG 50W-X4 anion exchange column.

Fig. 3. Immunohistochemical staining of nitrotyrosine in the kidney. Left kidney was subjected to sham operation (control), 1-h ischemia followed by 1-h reperfusion (I/R), ischemia-reperfusion with administration of anti-Hcy antibodies (I/R+HcyAb), or 1-h ischemia/1-h reperfusion with injection of nonspecific IgG (I/R+IgG). A: immunohistochemical staining for nitrotyrosine protein adducts was performed with anti-nitrotyrosine antibodies. After counterstaining with Mayer's hematoxylin, nitrotyrosine protein adducts were identified under light microscope with a magnification of ×400. Arrows point to the areas positively stained with nitrotyrosine protein adducts. As a negative control, immunohistochmical staining was performed by using nonspecific rabbit IgG as primary antibodies. B: intensity of nitrotyrosine staining was quantified. Results are depicted as means ± SE (n = 6). *P < 0.05 when compared with the value obtained from sham-operated group. #P < 0.05 when compared with the value obtained from I/R group.
portion of kidney was homogenized in 1:4 volumes of buffer containing 20 mM HEPES, 1 mM EDTA, and 0.1 mM PMSF followed by centrifugation at 3,000 g for 10 min at 4°C. The supernatant of kidney homogenate was incubated in a reaction mixture containing 10 μM dihydroethidium for 30 min at 37°C. The superoxide anion in the kidney homogenate caused oxidation of dihydroethidium leading to the formation of ethidium which was detected at an excitation of 475 nm and an emission of 610 nm using a fluorometer (Spectra Max Gemini, Molecular Devices, Sunnyvale, CA). The fluorescent signal produced by ethidium was proportional to the level of superoxide anion present in the kidney.

Determination of superoxide anion. The measurement of nitrite and nitrate was used to assess the nitric oxide (NO) levels in kidney tissue (30). 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 10 min of centrifugation at 400 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 Griess reaction method based on the azo coupling reaction (30). In brief, the supernatant was incubated with nitrate reductase to reduce nitrate to nitrite. Then, 12.5 mM sulfanilamide 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 diazoo-amino benzene in the reaction mixture was measured by a spectrophotometer at an absorbance of 520 nm. NaNO2 served as an internal standard.

Terminal deoxynucleobucleotide transferase-mediated dUTP nick-end labeling assay. Renal ischemia-reperfusion in animal models can lead to both necrotic and apoptotic forms of cell death. Terminal deoxynucleobucleotide transferase-mediated dUTP nick-end labeling 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.

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. Immunohistochemical staining was performed to detect nitrotyrosine protein adducts in the kidney (38). 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 antibodies, sections were treated with 0.3% hydrogen peroxide. The level of superoxide anion in the kidney was determined as previously described (40). In brief, a portion of kidney was homogenized in 1:4 volumes of buffer containing 20 mM HEPES, 1 mM EDTA, and 0.1 mM PMSF followed by centrifugation at 3,000 g for 10 min at 4°C. The supernatant of kidney homogenate was incubated in a reaction mixture containing 10 μM dihydroethidium for 30 min at 37°C. The superoxide anion in the kidney homogenate caused oxidation of dihydroethidium leading to the formation of ethidium which was detected at an excitation of 475 nm and an emission of 610 nm using a fluorometer (Spectra Max Gemini, Molecular Devices, Sunnyvale, CA). The fluorescent signal produced by ethidium was proportional to the level of superoxide anion present in the kidney.

Determination of nitric oxide metabolites. The measurement of nitrite and nitrate was used to assess the nitric oxide (NO) levels in kidney tissue (30). 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 10 min of centrifugation at 400 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 Griess reaction method based on the azo coupling reaction (30). In brief, the supernatant was incubated with nitrate reductase to reduce nitrate to nitrite. Then, 12.5 mM sulfanilamide 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 diazoo-amino benzene in the reaction mixture was measured by a spectrophotometer at an absorbance of 520 nm. NaNO2 served as an internal standard.

Fig. 4. Determination of lipid peroxidation in the kidney and plasma creatinine levels. Left kidney was subjected to sham operation (control), 1-h ischemia/1-h reperfusion with or without injection of anti-Hcy antibodies (I/R+HcyAb), or injection of nonspecific IgG (I/R). Lipid peroxides in kidneys were determined by measuring the amount of MDA. Values are expressed as percentage increase of control (Sham: 1.36 ± 0.09 nmol MDA/mg protein). B: plasma creatinine levels were measured. Results are depicted as means ± SE (n = 6). *P < 0.05 when compared with the value obtained from sham-operated group. #P < 0.05 when compared with the value obtained from I/R group.

Fig. 5. Effect of Hcy injection on superoxide levels and lipid peroxidation in the kidney. Left kidney was subjected to sham operation (control), 1-h ischemia/1-h reperfusion (I/R), injection of 0.25 ml L-Hcy (2.5 mg/kg) without ischemia-reperfusion (Hcy), or injection of 0.25 ml vehicle (buffer used for L-Hcy preparation) via left femoral vein. Levels of superoxide (A) and lipid peroxide (B) in kidneys were determined. Results are expressed as means ± SE (n = 6). *P < 0.05 when compared with the value obtained from sham-operated group.
mainly through the transsulfuration pathway (13) in which CBS catalyzes the rate-limiting reaction. To investigate whether an increase in the Hcy level in kidneys during ischemia-reperfusion was a result of impaired transsulfuration pathway, the CBS enzyme activity was measured. The CBS activity in kidneys subjected to ischemia alone or ischemia followed by reperfusion was significantly lower than that in the sham-operated group (Fig. 1C). Reduction of Hcy metabolism due to impaired CBS activity during ischemia-reperfusion could lead to an elevation of Hcy level in the kidney and subsequently in the circulation.

**Generation of superoxide and NO in the kidney.** Ischemia-reperfusion resulted in a significant elevation of superoxide and NO metabolite (nitrate and nitrite) levels in the kidney tissue homogenate (Fig. 2, A and B). Superoxide can interact rapidly with NO to form peroxynitrite, a potent oxidant. To determine whether there was an increase in peroxynitrite formation in the kidney subjected to ischemia-reperfusion, immunohistochemical staining was performed to detect nitrotyrosine, a biomarker for peroxynitrite. A significant increase in the intensity of nitrotyrosine protein adduct staining was found in kidneys subjected to ischemia-reperfusion (Fig. 3, A and B), indicating an increase in peroxynitrite formation. On the other hand, little nitrotyrosine protein adduct was detected in sham-operated kidneys (Fig. 3). Next, to determine whether Hcy played any role in ischemia-reperfusion-induced peroxynitrite formation in the kidney, anti-Hcy antibodies were injected into the left renal artery immediately after the induction of ischemia. Administration of anti-Hcy antibodies effectively abolished ischemia-reperfusion-induced peroxynitrite formation in the kidney (Fig. 3). As a control, one group of rats was given nonspecific antibodies (rabbit IgG). Administration of nonspecific antibodies into the kidney did not attenuate ischemia-reperfusion-induced peroxynitrite formation (Fig. 3A). These results indicated that elevation of tissue Hcy levels might be responsible for increased peroxynitrite formation in the kidney upon ischemia-reperfusion.

**Increased lipid peroxidation and plasma creatinine level in the kidney during ischemia-reperfusion.** Peroxynitrite can lead to tissue damage by oxidizing lipids to form lipid peroxides. Elevation of MDA level is an indicator for lipid peroxidation in the tissue. There was more than a twofold increase in the MDA level in kidneys subjected to ischemia-reperfusion, reflecting an increased renal lipid peroxidation (Fig. 4A). Administration of anti-Hcy antibodies into the kidney effectively reduced ischemia-reperfusion-induced lipid peroxidation to the basal level found in the sham-operated group (Fig. 4A). As a control, one group of rats received nonspecific antibodies (IgG) and such a treatment did not reduce ischemia-reperfusion-induced lipid peroxidation in the kidney (Fig. 4A). One-hour ischemia followed by 1-h reperfusion caused a significant increase in plasma creatinine level (0.43 ± 0.02 vs. 0.35 ± 0.01 mg/dl in the sham group). Administration of anti-Hcy antibodies reduced the plasma creatinine level to the basal level observed in the sham-operated group (Fig. 4B). Administration of nonspecific antibodies (IgG) did not reduce plasma creatinine level in rats subjected to ischemia-reperfusion (Fig. 4B). These results suggested that elevation of tissue Hcy level might contribute to ischemia-reperfusion-induced lipid peroxidation in the kidney and the renal dysfunction.

**RESULTS**

**Elevation of Hcy levels in the kidney and plasma.** Ischemia and ischemia-reperfusion were induced in the left kidney. The Hcy level in kidneys subjected to 1-h ischemia was significantly higher than that in the sham-operated group (24.68 ± 3.05 vs. 8.54 ± 0.52 nmol/g tissue in the sham group; Fig. 1A). The Hcy level remained elevated in kidneys during the 1-h reperfusion period (12.50 ± 0.69 nmol/g tissue; Fig. 1A). Ischemia alone did not elicit any significant change in the plasma Hcy level (Fig. 1B). However, the plasma Hcy level was significantly elevated in rats subjected to ischemia followed by reperfusion (5.18 ± 0.32 vs. 3.35 ± 0.29 μmol/l in the sham group; Fig. 1B). Hcy metabolism in the kidney is
Role of Hcy in oxidative stress in the kidney. To further examine the effect of Hcy on oxidative stress in the kidney, 1-Hcy (2.5 mg/kg) or vehicle was injected into rats via left femoral vein. In the absence of ischemia-reperfusion, injection of Hcy via intravenous route caused a significant increase in renal superoxide (Fig. 5A) and lipid peroxide levels (Fig. 5B).

Next, the in vitro effect of Hcy was examined. The addition of 1-Hcy to the tissue homogenate prepared from normal kidneys caused a significant increase in superoxide (Fig. 6A) and lipid peroxide (Fig. 6B) levels. Such in vitro stimulatory effect by Hcy was in a concentration dependent manner (Fig. 6).

Role of Hcy in ischemia-reperfusion-induced apoptosis. The effect of ischemia-reperfusion on cell death was examined by TUNEL assay. Few TUNEL-positive cells were found in sham-operated kidneys (Fig. 7). However, there was a marked increase in the number of TUNEL-positive cells in the cortex and medulla of kidneys subjected to ischemia-reperfusion (Fig. 7). It is known that ischemia-reperfusion can cause kidney cell death by necrosis as well as apoptosis. Necrotic cell death is characterized by cell swelling and nuclear swelling, whereas apoptotic cell death is characterized by cell shrinkage and nuclear chromatin condensation (16, 17).

![Fig. 7. TUNEL staining of kidney tissue.](image-url)

Fig. 7. TUNEL staining of kidney tissue. Left kidney was subjected to sham operation (control), 1-h ischemia/1-h reperfusion (I/R), or 1-h ischemia/1-h reperfusion (I/R) with anti-Hcy antibodies (I/R+HcyAb). Ischemia-reperfusion induced cell death was assessed by TUNEL staining. As a positive control, a section of sham-operated kidney was pretreated with DNase to mimic the appearance of TUNEL-positive cells. A: after counterstaining with Mayer’s hematoxylin, TUNEL-positive cells were identified under light microscope with a magnification of ×400. Representative photos were depicted and each group contained 6 rats. Insert: image of normal cell, apoptotic cell, and necrotic cells were enlarged. Filled arrow points to the apoptotic cells with condensed nuclear materials while open arrow points to the necrotic cells and arrowhead points to normal cell. The numbers of necrotic cells and apoptotic cells in cortex (B) and in medulla (C) were counted. Data represent the average of 10 high-power fields (HPF) under light microscope. Results are depicted as means ± SE (n = 6). ∗P < 0.05 when compared with the value obtained from sham-operated group. #P < 0.05 when compared with the value obtained from I/R group.
Most of the TUNEL-positive cells found in the ischemia-reperfusion group were necrotic cells rather than apoptotic cells. Taken together, these results suggested that Hcy, at an elevated level, contributed to ischemia-reperfusion-induced cell death both by necrosis and apoptosis in the kidney. Neutralization of tissue Hcy with anti-Hcy antibodies could attenuate 1-h ischemia/1-h reperfusion-induced cell death in kidneys. We also examined whether ischemia for a shorter period (30 min vs. 60 min) had differential effect on renal CBS activity and lipid peroxidation in the kidney. The left kidney was subjected to ischemia for 30 min followed by reperfusion for 60 min. The CBS activity was significantly reduced in kidneys subjected to 30-min ischemia alone or 30-min ischemia/60-min reperfusion compared with the sham-operated group (Fig. 8A). Such a treatment also caused a significant increase in lipid peroxidation in the kidney (Fig. 8B). These results suggested that ischemia for 30 or 60 min followed by reperfusion for 60 min could inhibit CBS activity and induce lipid peroxidation in the kidney.

Effect of prolonged ischemia-reperfusion and nephrectomy on kidney injury. We then examined the effect of a shorter period of ischemia (30 min) in the left kidney followed by reperfusion for 1 or 24 h in combination with right nephrectomy on renal function and oxidative stress. In one set of experiments, ischemia in the left kidney was induced for 30 min followed by reperfusion for 1 h with right nephrectomy. In this group of rats, there was a significant increase in plasma creatinine level and kidney lipid peroxide content compared with the sham-operated group (Fig. 9A). Administration of anti-Hcy antibodies to the left kidney exerts a protective effect as both creatinine level and lipid peroxide content were reduced to the levels similar to the sham group (Fig. 9A). As a control, one group of rats received nonspecific antibodies (IgG) and such a treatment had no protective effect on renal function (Fig. 9A). In another set of experiments, ischemia in the left kidney was induced for 30 min followed by reperfusion for 24 h with right nephrectomy. There was a significant increase in plasma creatinine level and kidney lipid peroxide content compared with the sham-operated group (Fig. 9B). Administration of anti-Hcy antibodies to the left kidney did not significantly reduce plasma creatinine levels or kidney lipid peroxide content to that similar to the sham group (Fig. 9B). Injection of Hcy resulted in a further increase in plasma creatinine level and lipid peroxidation in kidneys subjected to 30-min ischemia followed by reperfusion for 1 h (Fig. 9A) or 24 h (Fig. 9B).

DISCUSSION

Ischemia followed by reperfusion caused oxidative stress and cell death in the kidney. The novel findings of this study are 1) ischemia-reperfusion impairs Hcy metabolism leading to Hcy accumulation in the kidney and subsequently in the plasma; 2) Hcy, at elevated levels, contributes to ischemia-reperfusion-induced lipid peroxidation, apoptotic and necrotic cell death in the kidney; and 3) administration of anti-Hcy antibodies protects the kidney against short-term ischemia-reperfusion injury.

Several lines of evidence obtained from the present study suggested that Hcy played an important role in ischemia-reperfusion-induced oxidative stress in the kidney. First, there was a significant elevation of Hcy level in kidneys subjected to ischemia alone or ischemia followed by reperfusion. This was in accordance with the reduced activity of CBS, an enzyme responsible for regulating the rate-limiting step in Hcy metabolism via transulfuration pathway. The CBS activity remained at the diminished level after 24 h of reperfusion. Second, the level of two biochemical markers for oxidative stress (peroxynitrite, lipid peroxide) and a renal functional marker (plasma creatinine level) were increased in rats subjected to ischemia-reperfusion. Administration of anti-Hcy antibodies to kidneys effectively abolished ischemia-reperfusion-induced peroxynitrite formation and lipid peroxidation during the first hour of reperfusion. Third, the addition of Hcy to the kidney homogenate caused a significant increase in lipid peroxidation in a concentration dependent manner. It has been suggested that auto-oxidation of Hcy and formation of Hcy mixed disulfides may contribute to the reactive oxygen species pool. Hcy, when added to plasma, undergoes autooxidation, which was accompanied by the generation of H2O2 or superoxide anion (11). In the present study, elevation of renal superoxide was observed only during the reperfusion period, even though an increase in renal Hcy level was observed during the ischemia and ischemia-reperfusion. This is not surprising because the thiol oxidation reactions require the presence of molecular oxygen and Hcy remained in the reduced form until reperfusion started. During reperfusion, reoxygenation occurred in the
kidney. In the presence of oxygen, Hcy could undergo thiol oxidation reactions to generate reactive oxygen species, including superoxide, in the kidney. Superoxide avidly reacts with NO to form peroxynitrite. Increased peroxynitrite formation is believed to be responsible for ischemia-reperfusion-induced oxidative stress in the kidney (32).

It is reported that renal arterial ischemia causes an abrupt increase in tissue NO concentration (24). Such an increase in renal NO concentration during ischemia is primarily originated from thiol-dependant tissue stores (25). In the present study, besides an elevation of superoxide level, there was a significant increase in the level of NO metabolites in kidneys subjected either to ischemia or to ischemia-reperfusion. In accordance, there was a significant increase in peroxynitrite formation and lipid peroxidation in the same kidneys. An increase in the number of TUNEL-positive cells in the medulla and cortex after ischemia-reperfusion provided further evidence for renal injury. Administration of anti-Hcy antibodies to the kidney not only abolished lipid peroxidation but also effectively blocked 1-h ischemia/1-h reperfusion-induced necrotic and apoptotic cell death. Anti-Hcy antibody treatment prevented the renal injury, presumably, by forming immune complexes with Hcy, which, in turn, made Hcy unavailable for the thiol oxidation reactions. Thus generation of reactive oxygen species might have been attenuated. Taken together, these results suggested that elevation of tissue Hcy levels contributed to oxidative stress and apoptosis in the kidney during 1-h ischemia/1-h reperfusion. However, a single dose of anti-Hcy antibody injection had no effect on lipid peroxidation and plasma creatinine level in rats subjected to a longer duration of reperfusion. This might be due to diminished CBS activity during the 24-h reperfusion period throughout which Hcy level in the kidney remained elevated. A single administration of antibodies might not be sufficient to neutralize excess Hcy accumulated in the kidney during a prolonged period of reperfusion. On the other hand, results obtained from the present study did not rule out the possibility that mechanisms other than Hcy might be involved in oxidative stress and cell death in the kidney during 24-h reperfusion. The mechanisms by which prolonged ischemia-reperfusion causes renal injury remain to be further investigated in a future study.

The kidney is one of the major sites for removal of Hcy from the circulation. It has been estimated that the kidney is responsible for removal of ~20% Hcy from the blood circulation mainly through the transsulfuration pathway while the excretion of Hcy in urine appears to be negligible in healthy rats (4). In the present study, kidney Hcy level was significantly increased during the ischemic phase while the plasma Hcy level remained unchanged. During the reperfusion phase, an elevation of plasma Hcy level was observed. The elevation of Hcy

Fig. 9. Effect of ischemia for 30 min followed by reperfusion for 1 or 24 h on renal function and lipid peroxidation. Right kidney was surgically removed (right nephrectomy). A: left kidney was subjected to sham operation, 30-min ischemia/1-h reperfusion (I/R), 30-min-ischemia/1-h reperfusion with anti-Hcy antibodies injected into the left renal artery immediately after the induction of ischemia (I/R+HcyAb), 30 min-ischemia/1-h reperfusion with nonspecific rabbit IgG injected into the left renal artery immediately after the induction of ischemia (I/R+IgG), or 30-min ischemia/1-h reperfusion with l-Hcy (2.5 mg/kg in 250 µl) injection into the left femoral vein when reperfusion started (I/R/HS). B: left kidney was subjected to sham operation, 30-min ischemia/24-h reperfusion (I/R), 30 min-ischemia/24-h reperfusion with anti-Hcy antibodies injected into the left renal artery immediately after the induction of ischemia (I/R+HcyAb), 30 min-ischemia/24-h reperfusion with nonspecific rabbit IgG injected into the left renal artery immediately after the induction of ischemia (I/R+IgG), or 30-min ischemia/24-h reperfusion with l-Hcy (2.5 mg/kg in 250 µl) injection into the left femoral vein when reperfusion started (I/R/HS). Plasma creatinine level was measured and lipid peroxides in kidneys were determined by measuring the amount of MDA. Results are expressed as means ± SE (n = 4). *P < 0.05 when compared with the value obtained from sham-operated group. #P < 0.05 when compared with the value obtained from I/R group.
level in the circulation was likely a result of excess Hcy exported from the kidney during the reperfusion phase. The elevation of Hcy levels in the kidney upon renal ischemia-reperfusion appeared to be due to impaired Hcy metabolism through the transsulfuration pathway. It has been reported that in rat kidney majority of Hcy (up to 78%) is metabolized via the transsulfuration pathway while small amount of Hcy is remethylated to methionine (13). This finding concurred with our current observation that the activity of CBS, the enzyme responsible for the rate-limiting reaction in the transsulfuration pathway, was markedly decreased during ischemia. CBS activity remained at the diminished level throughout the reperfusion phase. Such a reduction in CBS activity caused Hcy accumulation in the kidney. Taken together, these results suggested that accumulation of Hcy in the kidney during ischemia-reperfusion was likely caused by a reduction of CBS activity.

To the best of our knowledge, the present study demonstrated, for the first time, that Hcy is elevated in the kidney upon renal ischemia-reperfusion. The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. J Biol Chem 1997; 272: 212–219.


