Effect of Hyperhomocysteinemia on Plasma or Tissue Adenosine Levels and Renal Function

Ya-Fei Chen, MD; Pin-Lan Li, MD, PhD; Ai-Ping Zou, MD, PhD

Background—Hyperhomocysteinemia (hHcys) is considered an independent risk factor for cardiovascular diseases. Recent studies in our laboratory have shown that hHcys produced glomerular dysfunction and sclerosis independently of hypertension. However, the mechanism mediating these pathogenic effects of homocysteine (Hcys) is poorly understood. Because Hcys and adenosine (Ado) are simultaneously produced via hydrolysis of S-adenosylhomocysteine (SAH), we hypothesized that hHcys may produce its pathogenic effects by decrease in plasma or tissue Ado concentrations.

Methods and Results—L-Hcys (1.5 μmol/min per kilogram) was infused intravenously for 60 minutes to produce acute hHcys in Sprague-Dawley rats. Plasma Hcys levels increased from 6.7±0.4 to 14.7±0.5 μmol/L, but Ado decreased from 141.7±15.1 to 52.4±6.8 nmol/L in these rats with acute hHcys. This hHcys-induced reduction of Ado was also observed in the kidney dialysate. In rats with chronic hHcys, plasma Ado levels were also significantly decreased. By kinetic analysis of the enzyme activities, decrease in renal Ado levels in hHcys was shown to be associated with inhibition of SAH hydrolase but not 5′-nucleotidase. Functionally, intravenous infusion of Hcys was found to decrease renal blood flow, glomerular filtration rate, and sodium and water excretion, which could be blocked by the Ado receptor antagonist 8-SPT.

Conclusions—These results strongly suggest that hHcys decreases plasma and tissue Ado concentrations associated with inhibition of SAH hydrolase. Decrease in plasma and tissue Ado may be an important mechanism mediating the pathogenic effects of Hcys. (Circulation. 2002;106:1275-1281.)

Key Words: risk factors ■ hemodynamics ■ adenosine

There is substantial evidence indicating that Hyperhomocysteinemia (hHcys) is an independent risk factor for cardiovascular diseases. Many recent epidemiological and clinical case-control studies have observed a positive association of hHcys with high blood pressure, arteriosclerosis, thrombosis, heart attack, and stroke. Animal experiments also demonstrated that hHcys could be a pathogenic factor responsible for arterial damages such as endothelial injury, cell proliferation, increased matrix formation, and arteriosclerosis. Recent studies in our laboratory have demonstrated that hHcys resulted in glomerular dysfunction and glomerular sclerosis independently of the elevations of arterial pressure, indicating that hHcys may be one of the important independent pathogenic factors resulting in glomerular injury in hypertension. However, the mechanism mediating the pathogenic effects of hHcys remains poorly understood.

Homocysteine (Hcys) is a sulfur-containing amino acid generated during the metabolism of methionine. During the process of methylation, S-adenosylmethionine (SAM) derived from methionine is converted to S-adenosylhomocysteine (SAH), which is additionally hydrolyzed to simultaneously produce Hcys and adenosine by SAH hydrolase in a variety of mammalian cells. However, it has been demonstrated that SAH hydrolase is bidirectional and favors the condensation of Hcys and adenosine. SAH hydrolase activity may, therefore, play a critical role in the regulation of tissue adenosine and Hcys concentrations. Indeed, previous studies have reported that under physiological conditions, myocardial adenosine levels are primarily determined by SAH hydrolase activity. When adenosine, Hcys, or both increased, SAH synthesis was markedly enhanced, resulting in reduction of adenosine levels in different tissues.

Given a wide variety of protective effects of adenosine in the cardiovascular homeostasis, regulation of various organ function, and cell growth or proliferation, it seems that adenosine has the opposite effects in different organ systems compared with Hcys. We hypothesized that elevations of plasma Hcys may reduce adenosine levels in plasma or tissues and consequently results in dysfunction of various organs such as the heart, vessels, and kidneys, thereby increasing the risk of cardiovascular diseases and other diseases. To test this hypothesis, the present study examined whether acute hHcys produces reduction of adenosine levels in plasma and renal tissues by simultaneously measuring

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plasma Hcys and adenosine levels using HPLC analysis. We also determined the possible mechanism by which hHcys decreases adenosine levels in renal tissue.

Methods

HPLC Analysis of Homocysteine

Plasma total homocysteine (tHcys) was measured by fluorescence HPLC analysis as described previously. Briefly, rat (Sprague-Dawley) blood samples of 1 mL were collected into Vacutainer tubes containing sodium heparin (Becton Dickinson) and immediately centrifuged at 1000g for 10 minutes at 4°C. A 100-μL resulting plasma or standard solution mixed with 10 μL internal standard, 2-mercaptoethanol (ME) (2.0 mmol/L) solution, was treated with 10 μL of 10% tri-n-butylphosphine (TBP) solution in dimethylformamide at 4°C for 30 minutes. Then 100 μL of the supernatant was transferred into the mixture of 20 μL of 1.55 mol/L sodium hydroxide, 250 μL of 0.125 mol/L borate buffer (pH 9.5), and 100 μL of 1.0 mg/mL ABD-F solution. The resulting mixture was incubated at 60°C for 30 minutes to accomplish derivatization of plasma thiols. HPLC was performed with a Hewlett-Packard Model 1090 Series II system with an autosampler. Separation was carried out at ambient temperature on an analytical column, Supelco LC-18-DB (150×4.6 mm ID, 5 μm) with a Supelcosil LC-18 guard column (20×4.6 mm ID, 5 μm). Fluorescence intensities were measured with an excitation wavelength of 385 nm and emission wavelength of 412 nm by a Hewlett-Packard Model 1046A fluorosence spectrophotometer. The peak area of the chromatograms was quantified with a Hewlett-Packard 3392 integrator. The analytical column was eluted with 0.1 mol/L potassium dihydrogenphosphate buffer (pH 2.1) containing 6% acetonitrile (vol/vol) as mobile phase with a flow rate of 2.0 mL/min.

HPLC Analysis of Adenosine

Assay of adenosine in plasma and renal interstitial fluid by RP-HPLC was performed as described previously. In brief, a 500-μL blood sample was drawn into Vacutainer tubes (Becton Dickinson) and quickly mixed with 750 μL stop solution consisting of 0.6 μmol/L erthro-9-(2-Hydroxy-3-nonyl)adenine (EHNA), an adenosine deaminase (ADA) inhibitor, and 11 μmol/L dipyridamol, an inhibitor of adenosine uptake in cold sterile saline, before centrifugation at 30 000g at 4°C for 2 minutes. The medullary dialysates were lyophilized and resuspended in 30 μL and then directly injected onto HPLC.

Experimental Protocols

Protocol 1: Effects of Intravenous Infusion of L-Hcys on Plasma and Renal Intersitial Adenosine Concentrations

As described above, plasma adenosine concentrations were measured by HPLC before and after intravenous infusion of L-Hcys in Sprague-Dawley rats. To measure renal interstitial adenosine, in vivo microdialysis study was performed as described in our previous study and by others. Briefly, a microdialysis probe (Bioanalytical Systems) with 0.5-mm tip diameter, 1-mm dialysis length, and 20-kDa transmembrane diffusion cutoff was gently implanted into the renal outer medulla (5 mm in depth) from dorsal surface. The probe was connected to a microinfusion pump and perfused with PBS containing (in mmol/L) NaCl 205, Na2HPO4 40.5, and NaH2PO4 9.5 (pH 7.4; osmolarity, 550 mosM) at a rate of 2.0 μL/min throughout the experiment. Then L-Hcys solution (1.5 μmol/min per kilogram) was infused intravenously for 60 minutes and medullary dialysates and blood samples were collected for Hcys and adenosine analysis.

Protocol 2: Effects of L-Hcys on SAH Hydrolase Activity in the Homogenates From Renal Tissue

Renal tissue homogenates from rats were prepared as described previously. Different concentrations of L-Hcys (10 to 100 μmol/L) were incubated with renal tissue homogenate (100 μg) mixed with EHNA (0.03 mg/mL) and SAH (1.0 mmol/L) at 37°C for 60 minutes. The formation of adenosine was determined by HPLC analysis. In another group of experiments, different concentrations of SAH from 0.01 to 1.0 mmol/L were incubated with renal tissue homogenate in the presence of EHNA and S'-nucleotidase inhibitor, α, β-methylene-adenosine diphosphate (AOPCP) (100 μmol/L), adenosine production was determined, and both Km and Vmax of SAH hydrolase in renal homogenate were calculated. The Lineweaver-Burk plot was used to calculate Km and Vmax. To draw Lineweaver-Burk plot, the conversion rate of SAH product, Ado, was first obtained and then the reciprocal of these Ado conversion rates was taken to plot against the reciprocal of corresponding substrate concentrations, which produced a straight line with an intercept of 1/Vmax and a slope of 1/Km/Vmax. Through the regression equation of this Lineweaver-Burk plot, the Km and Vmax were calculated.

To determine the effect of L-Hcys on SAH hydrolase activity, L-Hcys (50 μmol/L) was added in the reaction mixture. In addition, the effects of L-Hcys on S'-nucleotidase activity were examined by determining its Km and Vmax as described above, but S'-AMP (0.01 to 1.0 mmol/L) was substituted for SAH.

Protocol 3: Effects of SAH Hydrolase Inhibitor on the Production of Adenosine and Hcys in Renal Tissue Homogenate

3-dezaadenosine (3-DZA) is a well-known SAH hydrolase inhibitor and widely used to study the biological activity of SAH hydrolase. This protocol was designed to examine whether hHcys-induced decrease in tissue adenosine production is a SAH hydrolase-mediated reaction. First, 3-DZA at different concentrations was added into the reaction mixture for measurement of SAH hydrolase activity, which confirms SAH hydrolase–mediated production of adenosine and Hcys. Then the effects of Hcys on adenosine production were examined in the presence of 3-DZA (50 μmol/L). These experiments aimed to demonstrate whether Hcys decreases adenosine production through SAH hydrolase.

Protocol 4: Effects of hHcys on MAP and Renal Function

Sprague-Dawley rats were anesthetized, and the surgery for renal function study was performed as we described previously. After surgery and equilibration period, continuous measurements of mean arterial pressure (MAP) and renal blood flow (RBF) were obtained throughout the experiment. To measure glomerular filtration rate (GFR), a 0.5-mL bolus of FITC-inulin (8.0 mg/mL) was given, then a steady intravenous infusion of FITC-inulin (4.0 mg/mL) at 3.0 mL/h was continued throughout the experiment. After a 1.5-hour equilibration period, two 20-minute timed collections of urine were made. Blood samples (100 μL) were taken in heparinized hematocrit tubes after each urine collection period. Then L-Hcys (0.375 to 1.5 μmol/min per kilogram) was infused intravenously for 60 minutes, and late urine and blood collections were repeated. Plasma and urine FITC-inulin, Na+, and K+ were analyzed according to previous methods. In another group of experiments, those rats were pretreated with adenosine receptor blocker, 8-sulphophenylthiophylline (8-SPT, 25 μg/min per kilogram IV), and then the effects of L-Hcys (0.375 to 1.5 μmol/min/kg) on renal hemodynamics and renal function were examined.

Protocol 5: Effect of Chronic hHcys on Plasma Adenosine Concentrations

In addition, we determined whether chronic hHcys results in decreased adenosine levels, chronic hHcys in Sprague-Dawley rats was produced, as recently described. A group of rats (n = 9) was given bottle water containing methionine (1g/kg per day) for 6 weeks. The dose of methionine was chosen based on previous studies showing that it can effectively produce hHcys. Methionine can be metabolized to produce Hcys via S-adenosylmethionine and S-adenosylhomocysteine. In addition, methionine and an intermediate product, S-adenosylmethionine, can inhibit Hcys methyltransferase and thereby block the metabolism of Hcys, increasing plasma Hcys levels. Another group of rats was given bottle water containing
without methionine. After 6-week methionine treatment, blood samples (0.5 mL) from these rats were collected for measurements of both plasma Hcys and adenosine concentrations, as we described previously.4,14

Statistics
Data are presented as mean±SEM. The significance of differences within and between groups in multiple groups of experiments was evaluated using ANOVA for repeated measures, followed by Duncan’s multiple range tests. The significance of differences between 2 groups was evaluated by Student’s t test. P<0.05 was considered statistically significant.

Results
Fluorescence HPLC Analysis of Standard and Plasma Hcys
HPLC chromatograms of ABD-F derivatized standards of several thiol compounds (panel A) and plasma thiols from Sprague-Dawley rat (panel B) with added internal standard are presented in Figure 1. Standard thiol solution contains 20 μmol/L cysteine (Cys), Hcys, and glutathione (GSH); 5 μmol/L cysteinylglycin (CysGly); and internal standard, 20 μmol/L ME. B, Typical HPLC chromatogram showing Hcys and other thiols in plasma from Sprague-Dawley rat.

Effects of hHcys on Plasma and Renal Interstitial Adenosine Concentrations
The results of these experiments are presented in Figure 2. When l-Hcys was infused intravenously for 60 minutes, plasma Hcys concentrations measured by HPLC were significantly increased from 6.7±0.4 of control to 14.7±0.5 μmol/L, a doubling of plasma Hcys levels (Figure 2A). In contrast, plasma adenosine concentrations were decreased from 141.5±15.1 of control to 52.4±6.8 nmol/L when l-Hcys was intravenously infused (Figure 2B). In parallel to the decrease in plasma adenosine levels, adenosine concentrations in the renal medullary interstitium were also decreased from 203±10.1 of control to 116.7±6.7 nmol/L (Figure 2C), as measured by microdialysis and HPLC analysis.

Effects of Hcys on SAH Hydrolase Activity in Renal Tissue Homogenate
To test whether hHcys-induced decreases in plasma or tissue adenosine are associated with SAH hydrolase activity, the effects of l-Hcys on the production of adenosine by this enzyme were examined. l-Hcys significantly inhibited adenosine production from SAH in renal tissue homogenates in a concentration-dependent manner. l-Hcys at 50 and 100 μmol/L decreased adenosine production by 52% and 91%, respectively. As shown in Figure 3A, l-Hcys at 50 μmol/L significantly reduced the activity of renal tissue SAH hydrolase.
lase. By Lineweaver-Burk plot analysis (inset in Figure 3A), the $K_m$ of SAH hydrolase decreased from 0.127±0.015 to 0.072±0.013 mmol/L, and $V_{max}$ decreased from 0.417±0.056 to 0.249±0.042 nmol/min per milligram protein when l-Hcys was added into the reaction mixtures. In another group of experiments, l-Hcys was found to have no effect on the activity of renal tissue 5'-nucleotidase (Figure 3B). Both $K_m$ (0.043±0.002 mmol/L) and $V_{max}$ (148.2±3.52 nmol/min per milligram protein) were not altered by addition of l-Hcys in the reaction mixtures.

Effect of SAH Hydrolase Inhibitor (3-DZA) on the Production of Adenosine and Hcys in Renal Tissue Homogenate

To additionally confirm that SAH hydrolase contributes to the production of both Hcys and adenosine and that the action of Hcys on plasma or tissue adenosine levels is related to the activity of this enzyme, we determined the effects of specific SAH hydrolase inhibitor, 3-DZA, on the production of adenosine using SAH as substrate and examined the effects of Hcys on adenosine production in the presence of 3-DZA. The results of these experiments are presented in Figure 4. SAH hydrolase inhibitor 3-DZA significantly decreased adenosine production in renal tissue homogenates incubated with SAH. In the presence of 3-DZA, l-Hcys had no additional effect on adenosine production in renal tissue homogenate.

Effects of hHcys on MAP, RBF, and GFR

The effects of hHcys on renal hemodynamics in rats are presented in Figure 5. Intravenous infusion of l-Hcys produced a slight but significant elevation of mean arterial pressure (Figure 5A) at a concentration of 1.5 μmol/min per kilogram. However, intravenous infusion of l-Hcys produced a dose-dependent decrease in RBF (Figure 5B) and GFR (Figure 5C). A significant decrease in both RBF and GFR was observed at an l-Hcys concentration of 0.75 μmol/min per kilogram. All of these l-Hcys–induced alterations of RBF
and GFR were blocked by pretreatment of rats with adenosine receptor antagonist 8-SPT (IV).

Effects of hHcys on Renal Function

hHcys-induced changes in renal water and sodium excretion are presented in Figure 6. hHcys (IV) dose-dependently decreased urine flow rate (UV) (Figure 6A) and sodium excretion (U\text{Na}\text{V}) (Figure 6B), but it had no effect on potassium excretion (U\text{K}\text{V}) (Figure 6C). Compared with the value before infusion of hHcys, UV decreased by 24%, and urine sodium excretion decreased by 45% during hHcys (1.5 μmol/min per kilogram). However, these hHcys-induced alterations of UV and U\text{Na}\text{V} were blocked by pretreatment of rats with adenosine receptor antagonist 8-SPT (IV).

Changes in Plasma Adenosine Levels in Rats With Chronic hHcys

In rats with a 6-week high-methionine diet, marked sclerotic changes in aorta and glomeruli were observed, as described in our recent studies.4 As shown in Figure 7, plasma Hcys levels were increased, but adenosine concentration was significantly reduced, suggesting that a low level of plasma adenosine exists in rats with hHcys.

Discussion

The important finding in the present study was that hHcys significantly decreased plasma adenosine concentrations. These results indicate that hHcys may produce detrimental actions or increase the risk of cardiovascular disease through decreased adenosine levels. It is well known that adenosine plays a critical role in the control of cardiovascular homeostasis and other organ functions.8 There is substantial evidence indicating that adenosine dilates coronary and cerebral arteries, increases blood flow in microcirculation, inhibits platelet aggregation, and decreases proliferation or growth of smooth muscle or mesangial cells.8,19 All of these effects of adenosine can protect cardiovascular and other organ systems from ischemic or atherosclerotic injuries. Decrease in adenosine levels may lead to vasoconstriction, arteriosclerosis, thrombosis, and other cardiovascular disorders. These pathological changes resulting from decreased adenosine are similar to those during hHcys. Therefore, the deficiency of adenosine may be one of the important mechanisms resulting in pathological changes in cardiovascular and other organ systems during hHcys.

Recent studies have indicated that the effects of adenosine on cardiovascular and organ functions mainly depend on its concentrations in the extracellular space.20 To determine whether hHcys also decreases tissue interstitial concentrations of adenosine, we additionally determined adenosine levels in the renal interstitium. Consistent with the changes in plasma adenosine levels, renal interstitial adenosine was significantly reduced by hHcys. This decrease in plasma and tissue adenosine in response to acute hHcys may be associated with increased adenosine consumption through SAH hydrodase–mediated condensation of adenosine with increased Hcys. Indeed, previous studies have demonstrated that SAH synthesis was markedly enhanced by intravenous infusion of Hcys in rats or addition of Hcys in renal tissue homogenates.21-24 Furthermore, SAH accumulation in the
myocardium and brain tissues was also found when adenosine was coadministered with Hcys.25 These results strongly suggest that elevations of plasma Hcys may enhance the consumption of adenosine to synthesize SAH. This SAH synthesis may result in decrease in adenosine levels in plasma and tissue interstitial fluid.

To test this hypothesis, the present study examined the effects of Hcys on adenosine production via SAH hydrolase in tissue homogenates by in vitro analysis of the enzyme activities. It was found that Hcys markedly inhibited adenosine production from SAH in renal tissue homogenates, but it had no effect on the production of adenosine from 5'-AMP. These results support the view that elevated Hcys reduces adenosine levels by inhibiting the conversion of SAH to adenosine through SAH hydrolase. This probably represents a product feedback inhibition on SAH hydrolase. By kinetic analysis, we found that Hcys decreased both $K_m$ and $V_{max}$ of SAH hydrolase. Although $K_m$ reduction by Hcys may indicate an increased binding of the substrate to SAH hydrolase, $V_{max}$ decrease represents a reduction of the number of SAH converted into adenosine per minute by this enzyme. This inhibitory effect of Hcys may be associated with its allosteric interactions on the enzyme with SAH, because Hcys may bind to different sites of SAH hydrolase from SAH.21,22 As an allosteric inhibitor, Hcys may reduce adenosine production but not alter or even increase the binding of SAH (decreased $K_m$) to the enzymes.21

To additionally determine whether SAH hydrolase is responsible for Hcys and adenosine production in the kidney, the effects of 3-DZA, a relatively specific SAH hydrolase inhibitor, on the production of adenosine from SAH were examined. The results showed that 3-DZA did concentration-dependently decrease adenosine production from SAH in renal tissue homogenates. In the presence of 3-DZA, Hcys had no additional effect to decrease adenosine production from SAH. These results provide additional important evidence that SAH hydrolase present in the kidney contributes to the production of adenosine and Hcys and that inhibition of this SAH hydrolase accounts for reduction of adenosine levels during hHcys.

In in vivo animal experiments, we examined the effects of acute hHcys on renal hemodynamics and renal functions. It was demonstrated that RBF and GFR were significantly decreased by intravenous infusion of l-Hcys in a dose-dependent manner, suggesting that hHcys leads to the abnormality of renal hemodynamics. After pretreatment of rats with an antagonist of adenosine receptors (both A1 and A2 receptors), 8-SPT, l-Hcys–induced actions on renal hemodynamics were substantially blocked. Therefore, the effect of hHcys on renal glomerular perfusion and filtration may be associated with decreased adenosine in the kidney. Although previous studies reported that adenosine constricted preglomerular arterioles, the effects of adenosine on RBF and GFR were varied, depending on the doses, administration methods, or resources of adenosine used in different experiments.26–27 Recent studies have shown that renal microvascular responses to adenosine include vasoconstriction at low concentrations and vasodilation at higher concentrations.28 Long-term intrarenal infusion of adenosine induces an initial transient vasoconstriction followed by a sustained renal vasodilation,29 thereby predominantly increasing total RBF.30 These results have suggested that relatively long-term infusion of adenosine produces renal vasodilation and increases RBF. Based on these data, therefore, hHcys-induced decrease in endogenous adenosine may be one of the important mechanisms mediating Hcys-induced decreases in RBF and GFR. In addition, Hcys has been reported to decrease NO levels in the endothelium11 and increase oxidative stress in the artery wall.32 These Hcys-induced biochemical alterations may also contribute to its effects on renal hemodynamics.

The present study also demonstrated that elevations of plasma Hcys reduced urine flow and urinary sodium excretion, which was also blockable by adenosine receptor antagonist 8-SPT. This reduction of urinary sodium excretion may be simply attributable to the decrease in GFR. However, previous studies have indicated that adenosine is a potent diuretic or natriuretic paracrine or autocrine when given intra-arterially and that its effect may be associated with the direct inhibition of tubular ion transport activity.26–27 Recent studies in our laboratory demonstrated that adenosine dilated medullary vessels, increased renal medullary blood flow, and thereby produced natriuretic effects.11 It is possible that Hcys-induced decrease in plasma and renal interstitial adenosine may be one of the important mechanisms reducing renal water and sodium excretion during hHcys.

By determining plasma adenosine concentrations in rats with hHcys induced by 6-week high-methionine diet, we found that chronic elevations of plasma Hcys concentration resulted in a sustained low level of plasma adenosine, which is consistent with those obtained from rats with acute hHcys. We have recently reported that rats with hHcys induced by feeding a high-methionine diet, as done in the present study, exhibited arteriosclerotic changes and glomerular dysfunction and sclerosis.9 This sclerotic effect of hHcys may be associated with decreased adenosine. Indeed, recent studies in our laboratory and by others have demonstrated that decreased adenosine levels are associated with enhanced proliferation or growth of vascular smooth muscle cells and sclerotic changes in arteries or glomeruli.19,33,34 More recently, it has been reported that hHcys is one of the most important mechanisms resulting in cardiovascular complications in chronic renal diseases.35 In this regard, Hcys-induced renal dysfunction or glomerular injury observed in the present study may represent an early pathological abnormality resulting in arteriosclerosis and cardiovascular diseases.

In summary, the present study demonstrated the following: (1) acute and chronic hHcys significantly decreased plasma levels of adenosine in rats; (2) Hcys markedly decreased adenosine levels in renal tissue by inhibiting SAH hydrolase activity; and (3) elevations of plasma Hcys significantly increased arterial blood pressure but decreased RBF, GFR, and renal water/sodium excretion. In conclusion, elevations of plasma Hcys reduce adenosine levels in plasma and tissue interstitium, and decreased adenosine levels may contribute to hHcys-induced renal dysfunction. These results suggest that decreases in plasma and tissue adenosine levels may represent an important mechanism mediating the pathogenic ef-
ffects of hHcys on the artery wall or other organs such as the kidney.

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