Hyperhomocysteinemia Induces Renal Hemodynamic Dysfunction: Is Nitric Oxide Involved?

PATRICIA A. FISCHER,* GRACIELA N. DOMINGUEZ,* LUIS A. CUNIBERTI,† VERÓNICA MARTINEZ,‡ JOSÉ P. WERBA,† AGUSTÍN J. RAMIREZ,* and LUCAS D. MASNATTA*  

*Section of Pharmacology, Department of Physiology, and †Lipid and Atherosclerosis Research Laboratory, Department of Pathology, Favaloro University, Buenos Aires, Argentina.  

Abstract. Hyperhomocysteinemia is associated with endothelial dysfunction, although the underlying mechanism is unknown. Previous studies have shown that nitric oxide (NO) plays an important role in the regulation of systemic and renal hemodynamics. This study investigated whether hyperhomocysteinemia induces renal oxidative stress and promotes renal dysfunction involving disturbances of the NO-pathway in Wistar rats. During 8 wk, control (C) and hyperhomocysteinemic (HYC) groups had free access to tap water and homocysteine-thiolactone (HTL, 50 mg/kg per d), respectively. At 8 wk, plasma homocysteine concentration, renal superoxide anion (O_2^-), nitrotyrosine, and nitrite+nitrate levels, and renal function were measured. To assess NO involvement, the responses to L-Arginine (L-Arg, 300 mg/kg) and N^G^-nitro-L-arginine-methyl-ester (L-NAME, 20 \mu g/kg per min for 60 min) were analyzed. The HYC group showed higher homocysteine concentration (7.6 ± 1.7 versus 4.9 ± 1.0 \mu mol/L; P < 0.001), (O_2^-; production (157.92 ± 74.46 versus 91.17 ± 29.03 cpm · 10^4/mg protein), and nitrite+nitrate levels (33.4 ± 5.1 versus 11.7 ± 4.3 \mu mol/mg protein; P < 0.001) than the control group. Western blot analyses showed a nitrotyrosine mass 46% higher in the HYC group than in the controls. Furthermore, the HYC group showed lower GFR, renal plasma flow (RPF), and higher renal vascular resistance (RVR) than the controls. After L-Arg administration, the responses of GFR, RPF, and RVR were attenuated by 36%, 40%, and 50%, respectively; after L-NAME, the responses of RPF and RVR were exaggerated by 79% and 112%, respectively. This suggests a reduced NO bioavailability to produce vasodilation and an enhanced sensitivity to NO inhibition. In conclusion, hyperhomocysteinemia induces oxidative stress, NO inactivation, and renal dysfunction involving disturbances on the NO-pathway.

The endothelium plays an important role in the regulation of vascular tone involving vasorelaxing and vasoconstrictor factors (1,2). Nitric oxide (NO), one of the most important vasorelaxing factors, is produced from the amino acid L-Arginine by nitric oxide synthase (eNOS) in the endothelium (3,4). It stimulates cytosolic guanylate cyclase and increases guanosine 3', 5' -cyclic monophosphate (cGMP) content in vascular smooth muscle cells, thus inducing vascular relaxation (5,6).  

In the normal kidney, NO plays a key role in the homeostatic regulation of vascular, glomerular, and tubular function maintaining normal renal perfusion, GFR and renal vascular resistance, RVR (7,8).  

Increased superoxide anion radical (O_2^-) production inactivates the released NO, reducing NO bioavailability by peroxynitrite (ONOO^-) formation. Thus, a greater NO inactivation may reduce medullary blood flow, contributing to the development of renal failure (9).

Homocysteine is a sulfur-containing amino acid that is an intermediary product in methionine metabolism. Hyperhomocysteinemia is an independent risk factor of arteriosclerosis, involving coronary, cerebral, and peripheral arteries, and it is associated with an increased relative risk of cardiovascular events, comparable to hypercholesterolemia or smoking (10). Depending on the definition of hyperhomocysteinemia, 10 to 20% of the general population, more than 85% of patients with end-stage kidney disease (ESRD) (11–13), and up to 32% of the individuals having premature peripheral arterial disease (14) have increased homocysteine levels. On the other hand, hyperhomocysteinemia is a frequent finding in heart, liver, and renal transplant recipients and is associated with renal dysfunction, even though the etiologic factors have not been clearly identified (15–17). In patients with ESRD, plasma homocysteine levels may become 3 to 5 times higher than normal; even dialysis therapy or kidney transplantation fail to restore plasma homocysteine levels to a normal level (13).  

Several experimental and clinical studies have reported that hyperhomocysteinemia induces vascular oxidative stress and impaired vascular response to NO, denoting endothelial dysfunction (18). Moreover, Fu et al. (19) showed that homocysteine reacts with NO, inhibiting not only the endothelial-derived NO but also the bioactivity of exogenously supplied NO. The precise mechanism underlying the link between moderate hyperhomocysteinemia and endothelial dysfunction re-
mains to be determined. Various reports suggest that oxidative stress plays a major role (20,21). This is supported by recent studies showing that oral administration of antioxidants (e.g., vitamin C and vitamin E) prevents oral methionine load-induced impairment of dilatation of conduit arteries in response to acetylcholine and after release of forearm occlusion (18,22). On the other hand, there is evidence which suggests that hyperhomocysteinemia induces endothelial dysfunction by a mechanism involving an increased generation of reactive oxygen species and a decreased NO bioavailability (3,20,21).

Although the increased homocysteine level is able to impair endothelial-dependent vascular relaxation in coronary, brachial, or femoral arteries (23,24,19) and the renovascular bed is more sensitive to changes in endothelial function than other vascular beds (25), there are, to our knowledge, no reports showing the impact of hyperhomocysteinemia on renal hemodynamic function. Thus, the aim of the present work was to study whether hyperhomocysteinemia can induce renal oxidative stress and renal hemodynamic dysfunction involving disturbances on the NO pathway.

Materials and Methods

Animals

Three-month-old male Wistar rats ($n = 62; 350 to 400$ g) were used. The animals were maintained on a standard rat chow (Argentine Cooperative Association, Animal Nutritional Division) and tap water. They were housed under a 12/12 h day/night cycle at a steady temperature of 25°C. After 1 wk of acclimatization, they were randomly allocated to either a normal control (C, $n = 31$) or hyperhomocysteinemia (HYC, $n = 31$) group. Control and HYC groups had free access during 8 wk to either tap water or homocysteine thiolactone (HTL, 50 mg/kg per d), respectively (26).

Plasma Homocysteine Levels

At baseline conditions and at the end of the study, femoral arterial blood samples (200 $\mu$L) were withdrawn and immediately cooled in Eppendorf tubes containing 0.1% EDTA. Plasma was separated by centrifugation at 4°C and immediately frozen at −60°C until the day of analysis. Total plasma homocysteine levels were determined using the Bio-Rad HPLC kit (27).

Renal Oxidative Stress and NO Bioavailability

Superoxide anion radical production, thiobarbituric acid-reactive species (TBARS), and conjugated dienes (CD) were determined in fresh renal tissue. To estimate NO bioavailability, renal nitrotyrosine mass, total nitrate and nitrite (NOx), and cyclic GMP (cGMP) levels were also measured. For this purpose, 10 rats from each group were killed at 8 wk. After that, kidneys were isolated, cleaned in saline solution, and immediately frozen in liquid nitrogen, pulverized, and homogenized (25% wt/vol) in suitable solutions (see below).

Measurement of Superoxide Anion Radical Production

Renal homogenates were prepared in 50 mM phosphate-0.01 mM EDTA buffer (pH 7.4) using a glass-to-glass homogenizer followed by centrifugation (1000 × g) at 4°C for 10 min (28). The supernatants were maintained in ice until used. Protein content was measured according to the Lowry method (29).

In renal supernatants, superoxide anion radical production was determined by the increase of chemiluminescence produced in response to the addition of nicotinamide adenine dinucleotide (NADH) (100 $\mu$M), 5 $\mu$M of dark-adapted lucigenin (30), and in presence or absence of the nonenzymatic superoxide scavenger Tiron (10 $\mu$M), according to Trolliet et al. (31). The supernatant was incubated at room temperature for 5 min. Chemiluminescence was measured in a liquid scintillation counter (Packard-Tricarb 2200). A blank probe was run for each sample and subtracted before data transformation. Net superoxide elicited chemiluminescence was calculated from the Tiron inhibitable fraction and expressed as counts per min per mg protein (cpm/mg protein).

TBARS Measurement

Renal tissue was homogenized in 0.3 M Tris-HCl buffer, pH 7.4. TBARS were measured according to Ohkawa et al. (32). Protein content was determined by the Lowry method (29). Results were expressed as nmol of malondialdehyde per mg protein (nmol MDA/mg protein).

CD Measurement

Kidneys were homogenized in a saccharose (0.32 mM) and EDTA (3 mM) solution. Lipids were extracted with chloroform:methanol (3:1). The lipid extract was used to determine CD according to Davis et al. (33). Results were expressed as Abs/mg protein.

Measurement of Nitrotyrosine

Renal tissue was homogenized in a solution containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, aprotinin, leupeptin, pepstatin (1 $\mu$g/mL each), 1 mM Na$_3$VO$_4$, and 1 mM NaF at 0 to 4°C using a sonic disruptor (Tekmar). Nitrotyrosine mass was measured by Western blot technique using an anti-nitrotyrosine monoclonal antibody (Upstate Biotechnology Inc., Lake Placid) according to Vaziri et al. (34) with some modifications. Renal homogenates (100 $\mu$g of protein) were size-fractioned on 4 to 20% Tris-Glycine gel (Novex Inc) at 120 V for 2 h. In preliminary experiments, we found that the protein concentrations to be measured were within the linear range of detection for our Western blot technique. After electrophoresis, proteins were transferred onto a polyvinylidine difluoride (PVDF) membrane (Amer sham Life Science Inc.) at 200 mA for 120 min using the Novex transfer system. The membrane was prehybridized in buffer A (10 mM Tris hydrochloride, pH 7.5, 100 mM NaCl, 0.1% Tween 20, and 5% nonfat milk powder) for 1 h and then hybridized overnight at 4°C in the same buffer containing 0.5 $\mu$g/mL anti-nitrotyrosine monoclonal antibody (clone 1A6, Upstate Biotechnology) or anti-actin monoclonal antibody at the final titer of 1:500 (SC-8432, Santa Cruz Biotechnology Inc.). The membrane was then washed in buffer A without nonfat milk for 30 min. This wash buffer was changed every 5 min. The membranes were subsequently incubated for 1 h in buffer A plus horseradish peroxidase-conjugated sheep anti-mouse IgG antibody at the final titer of 1:2000 (NA 931, Amersham Biosciences Inc.). Experiments were carried out at room temperature. The washing procedure was repeated before the membrane was developed with a light-emitting nonradioactive method using chemiluminescence (ECL) reagent (Amersham Biosciences Inc.). Once the membranes were incubated in the ECL reagent, they were exposed to a film (Hyperfilm ECL, Amersham Biosciences Inc.) from 20 s to 20 min to provide a hard-copy result. The autoradiographs were digitized using a scanner (HP ScanJet) and Adobe Photoshop software (Adobe Systems Inc.). The digitized image was then analyzed for total nitro-
tyrosinated proteins in each lane using QuantiScan (Microbial Systems Ltd, Biosoft Software). The area under the curve of densitometric analysis was quantified by Image J (Wayne Rasband, National Institutes of Health, Bethesda, MD). To evaluate equal protein loading, the membranes were stripped and reprobed with anti-actin monoclonal antibody. In addition, the transferred gels were stained with Coomassie Blue (Bio-Rad, Richmond, CA). To confirm the specificity of the anti-nitrotyrosine monoclonal antibody, positive and negative immunoblotting controls of nitrotyrosine (Upstate Biotechnology, NY, USA) were performed. Protein concentration was determined by the bicinchoninic acid protein assay kit (Pierce Biotechnology) (35).

Renal NOx Determination

NO is unstable and quickly oxidized; therefore, NOx determination is considered as a marker of NO synthesis (36). Renal tissue was homogenized in 0.3 M Tris-HCl buffer, pH 7.4. Supernatants were ultrafiltered using micropore filters (Ultrafree MC microcentrifuge device, Millipore), and NOx assay was performed using a commercial nitric oxide colorimetric assay kit (Roche Diagnostics GmbH). Protein concentration was determined by Lowry method (29). Results were expressed as μmol/mg protein.

Renal cGMP Determination

Kidneys were homogenized in ice-cold 50 mM Na3PO4 and 10% TCA. Levels of cGMP were measured according to Pradelles et al. (37) using a cGMP enzyme immunoassay kit (Cayman Chemical).

Renal Hemodynamic Function Determination

Hemodynamic studies were performed in conscious unrestrained rats as described previously (38). Briefly, these animals were anesthetized to cannulate both the femoral artery and the femoral vein. After recovery from surgery and in euolemic conditions, a priming dose of inulin (16 mg/kg) and sodium para-aminohippurate (PAH, 8 mg/kg) was administered. Immediately thereafter, a continuous intravenous infusion of 0.9% NaCl containing inulin (36 mg/ml) and PAH (11.6 mg/ml) was given at a rate of 0.0267 ml/min. After a 105-min equilibration period, baseline arterial blood samples were taken. Subsequently, still under intravenous infusion of inulin and PAH to maintain steady-state conditions throughout the study, the involvement of the NO pathway was investigated. For this purpose, vehicle (saline solution), L-Arg, and L-NAME treatments were performed. To evaluate the effect of NO synthesis induction on renal hemodynamics, a bolus of L-Arg (300 mg/kg, intravenously) was administered to both HYC (n = 7) and C groups (n = 7). Blood sampling was performed 30 min after L-Arg administration (39).

On the other hand, NO production from HYC (n = 7) and C (n = 7) groups was inhibited by L-NAME administration. For this purpose, the infusion was changed to another one containing the same concentration of inulin and PAH plus L-NAME (0.075 mg/ml per 100 g body wt) at the same infusion rate (20 μg/kg per min for 60 min, intravenous infusion). Previous studies have shown that this dose induces a significant increase (more than 20%) in RVR in normal rats without changing mean arterial pressure, MAP (39). Blood samples were taken 60 min after the change. Sham rats from HYC (n = 7) and C (n = 7) groups were subjected to the same surgical instrumentation and received identical infusion of inulin and PAH at the same rate for 105 min. Immediately thereafter, a bolus or infusion of vehicle was given and blood samples were taken.

Blood samples were used for hematocrit, inulin, and PAH determinations. Clearances of inulin and PAH were calculated as described previously (40,41).

BP and heart rate were monitored throughout the study via an arterial catheter connected to a Statham pressure transducer (Gould Instrument) and attached to a polygraph recorder (model 2400S, Gould Instrument).

Chemicals and Reagents

Ether (Sigma Chemical Co) and Ketamine (G & M S.A.) were used as anesthetics. Lucigenin, EDTA, NADH, Tiron, L-Arg, L-NAME, and HTL were purchased from Sigma. Sterile and non-pyrogenic inulin (Cypres Pharmaceutical Co, West Carlsbad, CA) and PAH ampules for intravenous administration (Merck & Co. Inc) were used.

Statistical Analyses

All values are expressed as mean ± SD. Comparisons between HYC and C groups were done using the unpaired t test. To compare the responses to L-Arg, L-NAME, and vehicle within one group, percent changes from baseline were analyzed by one-way ANOVA followed by the Scheffe test. Differences between values were considered to be statistically significant when P < 0.05.

Results

Animals

No significant differences were found in body weight or fluid or food consumption between HYC and C groups at 8 wk.

Plasma Homocysteine Levels

Basal plasma homocysteine concentration did not differ between the two groups. After 8 wk of HTL administration, total plasma homocysteine levels were 55% higher in HYC group compared with the C group (7.6 ± 1.7 μmol/L versus 4.9 ± 1.0 μmol/L; P < 0.001).

Renal Oxidative Stress and NO Bioavailability

Renal parameters of oxidative stress are shown in Figure 1. In the HYC group, the production of O2· ((+74%), CD (+40%), and TBARS (+75%) were significantly higher when compared with the C group. Greater concentrations of NOx (33.4 ± 5.1 versus 11.7 ± 4.3 μmol/mg protein; P < 0.001) and cGMP (5.3 ± 1.5 versus 2.8 ± 1.5 pmol/mg protein; P < 0.05) were observed in HYC rats, showing that NO synthesis was 185% higher, whereas the cGMP production was only 88% higher compared with the C group.

A representative Western blot analysis using anti-nitrotyrosine monoclonal antibody is shown (Figure 2A). Higher levels of nitrotyrosinated proteins were observed in HYC rats. By quantitative densitometric analysis, HYC group showed a 46% higher mass of nitrotyrosinated proteins as compared with controls (Figure 2B). Positive and negative immunoblotting controls of nitrotyrosine were shown in Figure 3, denoting the high specificity of anti-nitrotyrosine monoclonal antibody.

Renal Hemodynamic Function Determination

Under baseline conditions, MAP and RVR were significantly higher in the HYC group than in controls (Figure 4, A
and D). Conversely, GFR (Figure 4B) and RPF (Figure 4C) were significantly lower in the HYC than in the C group.

In sham rats from both HYC and C groups, vehicle treatment failed to induce any significant alterations of systemic and renal hemodynamics (data not shown). To evaluate whether NO was involved in the renal hemodynamic effects induced by hyperhomocysteinemia, percent changes from baseline in GFR, RPF, and RVR after L-Arg and L-NAME treatments were analyzed. L-Arg induced a percent increase in GFR in both groups, but this was 36% lower in the HYC group (Figure 5A). On the other hand, the GFR reductions induced by L-NAME were not different between the HYC and C groups. In addition, after L-Arg treatment, the RPF increase (Figure 5B) was 40% smaller. The RPF decrease in response to L-NAME was 79% higher in the HYC compared with the C group. Finally, L-Arg decreased RVR in both groups, although the reduction was 50% smaller in the HYC group, and the RVR increase induced by L-NAME was 112% higher in HYC than in C rats (Figure 5C).

**Discussion**

The balance between vasodilating and vasoconstricting agents produced by the endothelium plays a key physiologic role in the control of renal hemodynamics (2,42). Although hyperhomocysteinemia induces vascular endothelial dysfunction and the renal vasculature is very sensitive to changes in endothelial function (25), no studies reporting the effects of
hyperhomocysteinemia on renal function are available. Thus, the present work is the first to show that hyperhomocysteinemia induces renal oxidative stress and promotes renal vasoconstriction.

Our results show that hyperhomocysteinemia induces higher $O_2^\bullet$ production and greater levels of TBARS and CD denoting an increased renal oxidative stress and lipid peroxidation. This is supported by strong evidence of increased production of superoxide anion radical and markers of lipoperoxidation being involved in reduced NO bioavailability (43,44). In addition, it is known that the association of higher NO synthesis and a greater $O_2^\bullet$ production induces rapid formation of $OONO^\bullet$ (45), which is responsible for nitrating the tyrosine residues of proteins. This is consistent with the increased renal nitrotyrosinated proteins observed in HYC rats. Reduced NO synthesis or increased NO inactivation decreases RPF and GFR and increases RVR (46); therefore, our results suggest that hyperhomocysteinemia is able to alter the L-Arg-NO pathway.

Higher NO synthesis and cGMP production suggest that the endothelial dysfunction induced by hyperhomocysteinemia is not related to reduced NO synthesis or production of the second messenger responsible for NO effects. However, the magnitudes of increase of NO synthesis (185%) and cGMP production (88%) were dissimilar. This suggests that NO synthesis increased to compensate the NO inactivation induced by reactive oxygen species (ROS), with the aim of maintaining a normal cGMP production.

With the exception of microalbuminuria, neither clinical nor laboratory variables can be reliably used to differentiate patients with progressive renal injury from those who follow a more benign evolution (47). Using low doses of L-Arg and L-NAME, several investigators have been able to avoid
changes of arterial BP, and thus could dissociate the direct (renal) effects of NO from the indirect effects (46) resulting from changes in systemic BP. L-Arg has been shown to induce renal vasodilation by promoting NO formation in the kidney. Despite the sparsity of evidence about the real importance of the altered renal vasodilatory response to L-Arg, it could be proposed that the L-Arg study is sensitive enough to detect subtle endothelium-related renal hemodynamic changes (47). Thus, the attenuated response to L-Arg observed in HYC rats suggests that a higher superoxide anion radical production induced by hyperhomocysteinemia favors a greater NO inactivation by sequestration, which, in turn, reduces the ability of NO to produce renal vasodilation. Therefore, higher NO synthesis does not suffice to preserve normal renal vasodilation. On the other hand, it is known that under oxidative stress conditions, tonically produced NO plays a pronounced role in the maintenance of normal renal function, suggesting that synthesis and/or release of NO is enhanced to compensate the increased NO inactivation (48). This is consistent with some reports (49,50) in which both acute and chronic NO inhibition in rats causes exaggerated renal vasoconstriction. Thus, the functional response to NO inhibition allows evaluation of the degree of NO involvement in maintaining renal function. Accordingly, our results showed an exaggerated vasoconstrictor response to L-NAME, suggesting enhanced sensitivity to NO inhibition. This is consistent with an increased renal vascular dependence on endogenous NO, reflecting a physiologic adaptation to increased NO inactivation. The altered responses to L-Arg and L-NAME suggest that NO plays a critical role in the maintenance of renal hemodynamic function and that the adaptive NO increase might mask the real magnitude of renal hemodynamic dysfunction. On the other hand, it is also possible that the alteration of NO bioavailability induced by oxidative stress could not be the only mechanism involved in homocysteine-induced renal hemodynamic dysfunction. Thus, amplification and/or activation of other vasoconstrictor systems might also be involved and must be further investigated (3).

In conclusion, the present study provides the first evidence that hyperhomocysteinemia produces renal oxidative stress and renal hemodynamic dysfunction involving altered L-Arg-NO pathway. In addition, our results suggest that follow-up of renal function should be taken into account in all subjects with high homocysteine levels, because renal failure may aggravate the vascular disease. Finally, oxidative stress plays a major role on homocysteine-induced endothelial dysfunction, antioxidant therapy may be an alternative clinical approach to prevent and/or treat the vascular damage.

Acknowledgments
We kindly acknowledge Dr. Alberto Crottogini and Dr. Peter Willshaw for their collaborative assistance in the correction of this manuscript. This study was supported by Favaloro University, Id # 008-01 and 009-01.

References


