Effects of homocysteine on intracellular nitric oxide and superoxide levels in the renal arterial endothelium

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Li, Ningjun, Fu-Xian Yi, Elizabeth Rute, David X. Zhang, Glenn R. Slocum, and Ai-Ping Zou. Effects of homocysteine on intracellular nitric oxide and superoxide levels in the renal arterial endothelium. Am J Physiol Heart Circ Physiol 283: H1237–H1243, 2002. First published May 23, 2002; 10.1152/ajpheart.00680.2001.—The present study was designed to test the hypothesis that homocysteine (Hcys) reduces intracellular nitric oxide (NO) concentrations ([NO]i) and stimulates superoxide (O2•−) production in the renal arterial endothelium, thereby resulting in endothelial dysfunction. With the use of fluorescence microscopic imaging analysis, a calcium ionophore, A-23187 (2 μM), and bradykinin (2 μM) were found to increase endothelial NO, in freshly dissected lumen-opened small renal arteries loaded with 4,5-diaminofluorescein diacetate (DAF-2DA; 10 μM). Preincubation of the arteries with L-Hcys (20–40 μM) significantly attenuated the increase in endothelial NO. However, L-Hcys had no effect on NO synthase activity in the renal arteries, as measured by the conversion rate of [3H]arginine to [3H]citrulline, but it concentration dependently decreased DAF-2DA-sensitive fluorescence induced by PAPA-NONOate in the solution, suggesting that L-Hcys reduces endothelial NO, by its scavenging action. Because other thiol compounds such as L-cysteine and glutathione were also found to reduce [NO]i, it seems that decreased NO is not the only mechanism resulting in endothelial dysfunction or arteriosclerosis in hyperhomocysteinemia (hHcys). By analysis of intracellular O2•− levels using dihydroethidium trapping, we found that only L-Hcys among the thiol compounds studied markedly increased O2•− levels in the renal endothelium. These results indicate that L-Hcys inhibits the agonist-induced NO increase but stimulates O2•− production within endothelial cells. These effects of L-Hcys on [NO]i and [O2•−] may contribute to endothelial injury associated with hHcys.

Risk factor of cardiovascular diseases; endothelium-derived relaxing factor, transnitrosation, reactive oxygen species, renal circulation

Hyperhomocysteinemia (hHcys) is recognized as a novel independent risk factor of thrombosis and arteriosclerosis involving coronary, cerebral, and peripheral arteries (3, 8–10, 30). Homocysteine (Hcys), a sulfur-containing amino acid, is not present in our regular diet and is primarily formed from methionine by demethylation in a variety of tissues or cells, and L-Hcys is the primary active form within the cells. On the basis of observations in patients with an inborn metabolic error (homocystinuria) and in animal models with experimental hHcys, Dr. McCully formulated the “homocysteine theory” of atherosclerosis to speculate that deranged Hcys regulation might cause cardiovascular diseases in the general population (25, 28). Recently, numerous clinical and epidemiological studies have demonstrated a positive correlation between plasma Hcys levels and cardiovascular diseases. It has been reported that plasma total Hcys levels are increased in many patients with essential hypertension, stroke, atherosclerosis, or end-stage renal disease (2, 8–10, 15). hHcys is also considered as a new crucial element in the pathogenesis of uremic cardiovascular complications (12).

Despite intensive clinical studies demonstrating the association of hHcys and cardiovascular diseases, the mechanisms by which hHcys produces cardiovascular dysfunction, atherosclerosis, and end-stage organ damage have not been fully elucidated. There is accumulating evidence indicating that endothelial injury or dysfunction is one of the important early pathological changes in the development of atherothrombotic vascular disease and some other end-stage organ damage (2, 8–10, 15). In this regard, animals with chronic hHcys exhibited an impaired endothelium-dependent vasodilator response (34). It has been suggested that Hcys produces endothelial dysfunction by decreasing NO production (23, 36). However, this conclusion was drawn based on only results obtained using pharmacological interventions or indirect measurements of NO metabolism (11, 14, 17, 28, 32, 35). In addition, oxidative stress or lipid peroxidation has been reported to be involved in the development of atherosclerosis or thrombosis in Hcys, but little is known about the direct effects of Hcys on the production of reactive oxygen species in the intact endothelium (7, 8).

The present study was designed to test the hypothesis that Hcys reduces intracellular nitric oxide (NO) concentrations ([NO]i) and stimulates superoxide (O2•−) production in the renal arterial endothelium, thereby leading to endothelial dysfunction. [NO]i and intracellular O2•− concentrations ([O2•−]i) were measured by...
fluorescence microscopic imaging analysis using 4,5-diaminofluorescein diacetate (DAF-2DA) as a NO indicator and dihydroethidium as an $O_2^-$ probe. We also explored the mechanisms mediating the effect of Hcys on intracellular NO within the endothelium. These experiments provide direct evidence that Hcys decreases $[\text{NO}]_{i}$ but increases $[O_2^•]_{i}$ within the intact endothelium of small renal arteries. The effects of Hcys to reduce $[\text{NO}]_{i}$ and to stimulate $O_2^•$ production may be one of the important mechanisms resulting in endothelial dysfunction and arteriosclerosis in hHcys.

**MATERIALS AND METHODS**

**Preparation of small renal arteries.** Male Sprague-Dawley rats weighing between 250 and 300 g were anesthetized with pentobarbital sodium (80 mg/kg body wt ip), and the kidneys were rapidly removed and kept in ice-cold HEPES-buffered physiological saline solution (PSS) of the following composition (in mM): 140 NaCl, 4.7 KCl, 1.6 CaCl₂, 1.17 MgSO₄, 1.18 NaH₂PO₄, 5.5 glucose, and 10 HEPES; pH 7.4. Small renal arteries (250–300 μm inner diameter) were carefully dissected on ice and transferred to a 35-mm Sylgard-coated dissecting dish containing ice-cold PSS. The arterial segment was cut open along its longitudinal axis, and pinned onto the dish with lumen side upward. Care was taken not to disrupt the endothelium.

In an additional group of rats, the aorta below the left renal artery was isolated and cannulated. After the aorta was ligated at a site above the right renal artery, the kidneys were flushed with 10 ml ice-cold PSS after 60-ml air perfusion to remove the endothelium of the renal arteries. The small renal arteries were then dissected to measure agonist-induced NO production. These experiments were performed to confirm that NO was derived from the renal arterial endothelium (1, 19).

**Measurement of [NO] within the endothelium of small renal arteries.** A novel fluorescent NO indicator, DAF-2DA, which was recently developed by Kojima et al. (19), was used to measure NO within the endothelial cells of freshly isolated small renal arteries. DAF-2DA can readily enter the cell and be oxidized specifically by $O_2^•$ to yield ethidium, which binds to DNA to produce bright red fluorescence. The increase in ethidium/DNA fluorescence is suggestive of $O_2^•$ production within cells (22). Renal arteries with the endothelium side up were first incubated in PSS at 37°C for 30 min and then with 20 μM DAF-2DA at room temperature. The arteries were then incubated with vehicle, l-Hcys (40 μM), l-cysteine, and GSH (40 μM), respectively, for 4 h. The ethidium/DNA fluorescence images were acquired at 490-nm excitation and 510- to 560-nm emission and analyzed using a personal computer-controlled digital CCD camera by MetaMorph image analysis software. The average fluorescence intensity is presented as arbitrary units per pixel.

**Activity of NOS.** NOS activity was determined by measuring the conversion rate of $[^3H]\text{arginine}$ to $[^3H]\text{citrulline}$ (5) using an isotopic NOS detection kit (Calbiochem) according to the manufacturer’s instructions. Briefly, homogenates prepared from small renal arteries were incubated in 50 μl of reaction mixture containing (in mM) 25 Tris-HCl (pH 7.4), 0.6 CaCl₂, 1 β-NADPH, 0.003 tetrahydrobiopterin (BH₄), 0.001 FAD, 0.001 FMN, and 0.005 cold l-arginine and 1.0 μCi $[^3H]\text{arginine}$ in the absence or presence of l-Hcys. In this reaction mixture, a high concentration of Ca²⁺ was used to maximize the NOS activity. After incubation for 60 min at 37°C, the reaction was terminated by the addition of 400 μl of ice-cold stop buffer containing 50 mM HEPES (pH 5.5) and 5 mM EDTA. Equilibrated cation exchange resin was added to the samples, and they were then applied to spin columns. After centrifugation, the eluate (containing $[^3H]\text{citrulline}$) was collected, and the radioactivity was determined with a liquid scintillation counter. To determine the effect of l-Hcys on NOS activity, l-Hcys (100 μM) was added to the reaction mixture. EGTA (1 mM) was used as a positive control for NOS inhibition. In these experiments, the formation rate of $[^3H]\text{citrulline}$ represents NOS activity, which is expressed as femtomoles per milligram of protein per minute.

**Determination of the direct reaction of l-Hcys with NO.** Because Hcys contains a sulfhydryl group that can serve as an acceptor of transnitrosation, we hypothesized that Hcys may directly bind to and trap NO, thereby reducing $[\text{NO}]_{i}$. To test this hypothesis, NO release from PAPA-NONOate (100 μM) was determined in the presence or absence of l-Hcys. During the incubation of PAPA-NONOate with DAF-2, the fluorescence intensity was monitored every 5 min for 30 min using a fluorescence spectrometric microplate reader equipped with a 490-nm excitation and a 510- to 560-nm emission filter. To test the effect of Hcys on $[\text{NO}]_{i}$ in this reaction system with PAPA-NONOate, l-Hcys (10–40 μM) was added to the reaction mixture, and DAF-2 fluorescence was continuously monitored for 30 min. In an additional group of experiments, oxyhemoglobin (3 μM) was used to trap NO as a positive control.
Statistics. Data are presented as means ± SE. The significance of differences in mean values between and within multiple groups was examined using an ANOVA for repeated measures followed by a Duncan’s multiple-range test (Sigma-Stat). *P < 0.05 was considered statistically significant.

RESULTS

Blockade of the A-23187-induced increase in endothelial NO fluorescence by L-NAME. As shown in Fig. 1A, the addition of A-23187 (2 µM) to the bath solution produced a marked increase in endothelial green NO fluorescence of small renal arteries. In the presence of L-NAME, the A-23187-induced increase in NO green fluorescence was significantly attenuated, suggesting that L-NAME is capable of blocking the A-23187-induced increase in NO within the intact endothelium of these freshly isolated renal arteries. Figure 1B summarizes A-23187-induced alterations of [NO] measured by DAF-2T fluorescence intensity in the absence or presence of L-NAME (n = 6). A-23187 produced a significant increase in [NO] within the endothelium of renal arteries. In the presence of L-NAME, the A-23187-induced increase in [NO] was completely blocked. To confirm that the NO fluorescence in these arteries is within the endothelium, the arteries were denuded, and the A-23187 response of NO was then examined. In the denuded renal arteries, there was no detectable A-23187-induced increase in DAF-2T fluorescence.

Effects of L-Hcys on the A-23187-induced NO increase in renal arterial endothelium. L-Hcys was added to the incubation bath to determine the effect of Hcys on the A-23187-induced increase in endothelial [NO]. As shown in Fig. 2A, the A-23187-induced [NO] increase in the endothelium of the renal artery was significantly attenuated by L-Hcys (40 µM). The results of these experiments are summarized in Fig. 2B (n = 7). Consistent with the results in Fig. 1, A-23187 significantly increased [NO] in the renal arterial endothelium. L-Hcys blocked the A-23187-induced increase in [NO] in a concentration-dependent manner with a 78% blockade of this NO response at 40 µM. L-Hcys at 10 µM did not significantly attenuate the A-23187-induced NO increase in this preparation.

As shown in Fig. 3, two oxidized thiol compounds, homocysteine and GSSH, at 40 µM had no effect on the A-23187-induced NO increase in endothelial cells, but L-cysteine and GSH significantly attenuated this NO response to A-23187.

Effects of L-Hcys on the bradykinin-induced [NO] increase in renal arterial endothelium. Figure 4 presents the results obtained from these experiments. Incubation of the arteries with BK (2 µM) induced a significant NO increase within endothelial cells. In the presence of L-Hcys (40 µM), the BK-induced increase in endothelial [NO] was markedly inhibited. Figure 4A presents typical fluorescence microscopic images showing the BK-induced increase in [NO] in the renal arterial endothelium in the absence or presence of L-Hcys. As summarized in Fig. 4B, L-Hcys attenuated the BK-induced increase in [NO] by 58% (n = 6).

Effects of L-Hcys on NOS activity in renal arterial homogenates. To address whether Hcys decreases [NO] in renal arteries through inhibition of NOS, we examined the effects of L-Hcys on NOS activity by measurement of the conversion rate of L-[3H]arginine to L-[3H]citrulline. As shown in Fig. 5, the formation rate of [3H]citrulline in the homogenates prepared from renal arteries was found to be 59.7 ± 12 fmol-mg protein⁻¹ min⁻¹. The addition of EGTA (1 mM) to decrease Ca²⁺ concentrations in the reaction mixture largely inhibited the formation of [3H]citrulline, to 21.6 fmol-mg protein⁻¹ min⁻¹. However, L-Hcys even at a high concentration of 100 µM had no significant effect on the activity of NOS in the arterial homogenates (n = 6).

Trapping effect of L-Hcys on NO in solution. With the use of a microtiter plate reader with a fluorescence spectrometer, PAPA-NONOate was demonstrated to time-dependently release NO, as monitored by the DAF-2T fluorescence increase. This time-dependent NO increase in the solution was abolished by oxyhemoglobin, a classical NO trapping or scavenging re-
Similar to the effect of oxyhemoglobin, the addition of 20 or 40 \( \mu \)M L-Hcys significantly blocked the NO increase in solution with PAPA-NONOate (Fig. 6). L-Hcys at 10 \( \mu \)M had no significant effect on NO concentrations in the PAPA-NONOate solution (Fig. 6).

**DISCUSSION**

In the present study, we directly measured \([NO]_i\) in the endothelium of small renal arteries using DAF-2.
fluorescence microscopic imaging analysis and monitored the production of NO within renal arterial endothelial cells in response to the endothelium-dependent vasodilators A-23187 and BK. In the intact endothelium of freshly dissected small renal arteries loaded with DAF-2DA, both A-23187 and BK were found to stimulate the production of a strong green fluorescence, which represents increases in [NO] within endothelial cells. The NOS inhibitor L-NAME or the removal of the endothelium completely blocked A-23187- and BK-induced increases in [NO] in this preparation, suggesting that detected NO increases in response to both compounds are derived from the endothelium of these arteries.

In the last decade, numerous studies have been performed to examine the regulation of NO production in the arterial endothelium and to explore the mechanisms of NO-mediated endothelial dysfunction under different pathological conditions, such as hypertension, atherosclerosis, and myocardial ischemia and reperfusion. In the kidney, endothelium-derived NO has been found to play a critical role in the regulation of renal vascular tone and renal function. However, most of those studies used pharmacological interventions to block or enhance NOS activity and then observed the changes in endothelial function such as endothelium-dependent vasodilation or endothelium-dependent alterations of blood perfusion in different vascular beds. There were few studies to address these issues by directly measuring endothelial NO.

With the use of this direct measurement of NO in the intact endothelium of freshly dissected small renal arteries, we examined the effects of Hcys on the NO response of these small arteries to A-23187 and BK.
was found that L-Hcys at concentrations of 20 and 40 μM markedly attenuated A-23187- or BK-induced increases in [NO] within renal arterial endothelial cells. To our knowledge, this provides the first direct evidence that Hcys decreases [NO] in the renal arterial endothelium and inhibits the endothelial NO response to A-23187 and BK. These results support the view that hHcys may produce endothelial dysfunction through NO-mediated mechanisms (20, 34, 36). In previous studies, Hcys has been reported to impair endothelium-dependent vasodilation (20). In animals with chronic hHcys, acetylcholine (ACh)-induced, NO-mediated vasodilation was significantly blunted (21, 34). With the use of isolated arterial preparations, the addition of L-Hcys to the bath solution produced a significant concentration-dependent impairment of the relaxation response to both ACh and a Ca2+ ionophore, A-23187 (20, 23). Taken together, these results indicate that the impairment of NO-mediated endothelial function may be one of the important mechanisms mediating the detrimental effects of elevated plasma Hcys levels (17, 32, 35). Endothelial dysfunction may induce the growth of arterial smooth muscle cells, ultimately resulting in alterations of the extracellular matrix and sclerotic plaques on the artery wall (2–4, 8, 15, 26a–26c).

However, it seems that the Hcys-induced decrease in the NO response of endothelial cells does not necessarily predict the occurrence of arteriosclerosis, because the NO response of the arterial endothelium was also found to be abolished by other thiol compounds such as cysteine and glutathione. These compounds contain a free sulfhydryl group and are generally not considered as sclerotic factors. It remains unknown why elevations of plasma Hcys but not cysteine or glutathione produce arteriosclerosis, despite the fact that they all can decrease intracellular NO levels in the endothelium. In the present study, we examined the effects of Hcys and its thiol analogs on O2·− production. It was found that L-Hcys markedly increased O2·− levels in the intact renal arterial endothelium, as measured by the oxidation of DHE. However, L-cysteine and glutathione had no effect on O2·− levels in the endothelium in these renal arteries. This difference in the actions of Hcys and other thiol compounds may be one of the important determinants of endothelial dysfunction and arteriosclerosis. In this regard, L-Hcys may decrease NO levels, increase O2·− production, and thereby accelerate oxidative stress in arterial wall, resulting in arteriosclerosis. Other thiol compounds such as L-cysteine and glutathione do not stimulate O2·− production, which may not induce arteriosclerosis, despite the fact that they reduce NO levels in endothelial cells. These results indicate that, although it may impair the endothelium-dependent vasodilator response, decreases in NO levels in the endothelium may not necessarily contribute to a sustained endothelial injury and arteriosclerosis associated with hHcys. On the basis of recent studies, hHcys-induced arteriosclerosis and thrombosis are associated with many pathological processes such as endothelial damage, proliferation of vascular smooth muscle cells, increased lipid peroxidation, hemostatic imbalance, DNA methylation, and accumulation of collagens (7, 8, 13, 29, 35). Among many factors that activate or promote these pathological processes, therefore, decreased NO levels in the endothelium may only be one of the important factors. Other factors or mechanisms such as local oxidative stress, reduced adenosine and PGI2, increased angiotensin II and thromboxane A2, and hypomethylation may also importantly participate in the sclerotic effect of Hcys (6–8, 13, 27, 29, 35, 40).

To explore the mechanisms by which Hcys reduces [NO], we determined the effects of L-Hcys on NOS activity in renal arterial homogenates. By measuring the conversion rate of L-arginine to L-citrulline, we surprisingly demonstrated that L-Hcys had no effect on the conversion of L-arginine to L-citrulline, suggesting that Hcys does not inhibit NOS activity to reduce [NO] in the renal arteries. Because Hcys contains a sulfhydryl group that may bind to NO to form nitrosohomocysteine and thereby decrease NO levels within the endothelium, we tested the hypothesis that Hcys serves as an acceptor of transnitrosation to trap NO and thereby decreases free [NO] within cells. With the use of a microtiter plate reader with a fluorescence spectrometer, the NO donor PAPA-NONOate was found to produce a time-dependent increase in [NO]. Similar to the effect of a classical NO scavenger, oxyhemoglobin, the addition of L-Hcys to the reaction mixture completely abolished the increase in NO production due to the reaction mixture completely abolished the increase in NO production due to the reaction mixture completely abolished the increase in NO production. As discussed above, hHcys has been found to increase local oxidative stress in arterial wall, which may result in NO uncoupling, decreasing NO production. Because we determined NOS activity under a condition with standard cofactor concentrations, this indirect effect of Hcys on NOS activity cannot be detected. Therefore, our results importantly suggest that Hcys had no direct effect on NOS activity and that NO trapping through its sulfhydryl group represents one of the mechanisms mediating the Hcys-induced reduction of endothelial NO levels.

In summary, the present study directly detected the production of NO in the intact endothelium of small renal arteries in response to endothelium-dependent vasodilators, A-23187 and BK. L-Hcys was found to decrease [NO] in the arterial endothelium through a direct trapping effect on NO. We conclude that Hcys may reduce [NO]3, abolish the NO release response of the vascular endothelium to stimuliators, and consequently result in endothelial or vascular dysfunction. This reduction of endothelial NO levels was not a specific effect of L-Hcys to induce arteriosclerosis, however, because nonsclerotic thiol compounds such as L-cysteine and glutathione also decreased NO levels in
these endothelial cells. L-Hcys, but not L-cysteine and glutathione, stimulated O2− production, indicating that L-Hcys-induced oxidative stress in combination with reduced NO may be importantly involved in arteriosclerosis associated with hHcys.

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