Effects of homocysteine on endothelial nitric oxide production

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Received 2 February 2000; accepted in final form 15 June 2000

Hyperhomocysteinemia (HHCy) is highly prevalent in patients with chronic renal failure (3) and mortality rate in patients with homocysteine (Hcy) levels above 20 μM is about sevenfold greater than in those with plasma Hcy below 9 μM. In a recent study of patients with end-stage renal disease on hemodialysis, HHCy was a universal finding, which was independently associated with the past history of cardiovascular events (19). In these patients HHCy has been demonstrated to confer a graded, independent, increased risk for thrombosis of vascular access, either graft or fistula (33). Therefore, understanding the mechanism(s) of HHCy-induced vascular complications represents a high-priority task.

Endothelial cell dysfunction is a common denominator for a variety of cardiovascular diseases (2, 5, 12, 13, 20, 22, 29, 30, 45). It has recently been appreciated that altered function of endothelial nitric oxide synthase (eNOS) and/or decreased availability of nitric oxide (NO) can account for a broad array of clinical manifestations in patients with endothelial dysfunction (8, 10, 14, 18, 28, 32, 35, 43). This notion points to eNOS as a potential target for HHCy. Indeed, some recent data strongly suggest that Hcy acts on eNOS. Controversy exists, however, as to the effects of HHCy on NO production; it has been shown that HHCy upregulates (38) and downregulates it (9). We revisited this problem by using amperometric electrochemical NO detection with a porphyrinic microelectrode and studied responses of microvascular endothelial cells, exposed to increasing concentrations of Hcy, to the stimulation with bradykinin, calcium ionophore, or L-arginine. Twenty-four-hour preincubation with Hcy (10, 20, and 50 μM) resulted in a gradual decline in responsiveness of endothelial cells to the above stimuli. Hcy did not affect the expression of endothelial nitric oxide synthase (eNOS), but it stimulated formation of superoxide anions, as judged by fluorescence of dichlorofluorescein, and peroxynitrite, as detected by using immunoprecipitation and immunoblotting of proteins modified by tyrosine nitration. Hcy did not directly affect the ability of recombinant eNOS to generate NO, but oxidation of sulfhydryl groups in eNOS reduced its NO-generating activity. Addition of 5-methyltetrahydrofolate restored NO responses to all agonists tested but affected neither the expression of the enzyme nor formation of nitrotyrosine-modified proteins. In addition, a scavenger of peroxynitrite or a cell-permeant superoxide dismutase mimetic reversed the Hcy-induced suppression of NO production by endothelial cells. In conclusion, electrochemical detection of NO release from cultured endothelial cells demonstrated that concentrations of Hcy >20 μM produce a significant indirect suppression of eNOS activity without any discernible effects on its expression. Folates, superoxide ions, and peroxynitrite scavengers restore the NO-generating activity to eNOS, collectively suggesting that cellular redox state plays an important role in Hcy-suppressed NO-generating function of this enzyme.

endothelial cells; nitric oxide synthase; peroxynitrite; nitrotyrosine

HYPERHOMOCYSTEINEMIA (HHCy) is an independent and graded cardiovascular risk factor, the strength of which is equal to that of hyperlipidemia (4, 15, 31).

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als (culture dishes, serological pipettes, and polypropylene conical tubes) were obtained from Becton Dickinson Labware (Lincoln Park, NJ); endothelial cell basal medium-2 was from Clonetics (Walkersville, MD); and basal medium Eagle, for electrode testing, was obtained from Life Technologies (Grand Island, NY).

Cell culture. Microvascular endothelial cells (RMVEC) were previously established and characterized by our laboratory (37). These SV-40-immortalized cells were established from explant cultures of microdissected rat renal resistance arteries and have been shown to express receptors for acetylated low-density lipoprotein, immunodetectable von Willebrand antigen, and are capable of capillary tube formation (37). Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics and used between passages 3 and 8. Both cell types were grown in endothelial cell basal medium-2 (Clonetics) containing 2% fetal bovine serum, 100 μg/ml penicillin, and 50 μg/ml streptomycin.

Measurement of eNOS activity with NO-selective microelectrodes in vitro. The NO concentration was monitored with porphyrin-electroplated, nafion-coated, carbon-fiber electrodes (30 μm OD), which were manufactured as previously detailed (36). The electrode oxidation current was low-pass filtered at 0.5 Hz and sampled every 2 s. The measurements were made by using constant potential amperometry (0.7 mV) utilizing a highly sensitive potentiostat (InterMedical, Nagoya, Japan). Calibration of the electrode was performed before each experiment by using dilutions of freshly prepared NO-saturated deionized water.

For the determination of NO production by the cultured cells, HUVEC were incubated in the presence of different concentrations of Hcy added to the culture medium for 24 h. Cells were placed on the stage of an inverted microscope equipped with a micromanipulator and enclosed in a Faraday chamber. After a stable baseline current was recorded, cells were stimulated with bradykinin, A-23187, or L-arginine, as specified in RESULTS. When necessary, L-NMMA, at the final concentration of 0.2 or 2 mM, was added to the solution to verify the NO dependence of a recorded electrode current. Alternatively, recordings were performed at 0.4-mV electrode holding potential, which is unfavorable for NO determination. Using these techniques, in the preliminary studies we found no evidence that any of the utilized agents interfered with the NO electrode function. Before and at the completion of experiments, electrode function was tested with different dilutions of NO-saturated deionized water.

Expression of eNOS and nitrotyrosine. Confluent HUVEC, treated with various concentrations of Hcy with or without 5-methyltetrahydrofolate (5-MTHF) (42) or eboslen (1) for 24 h, were lysed in a buffer containing (in mM) 150 NaCl, 1 sodium orthovanadate, 50 Tris-HCl (pH 8.0), 1 EDTA, 1 EGTA, and 1 dithiothreitol as well as 1% NP-40 and proteinase inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). Lysates were cleared by centrifugation (3,000 g for 10 min at 4°C). After protein determination using a bicinchoninic acid kit (Pierce, Rockford, IL), samples were denatured by boiling in 2.5% 2-mercaptoethanol for 3 min. Twenty micrograms of each sample were run on 4–20% Tris-glycine (Novex, San Diego, CA) gel. The proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) and blocked with 1% casein/PBS for 1 h. Immunoblotting was performed at room temperature for 2 h with monoclonal anti-eNOS antibody at 1:1,000 dilution (Transduction Laboratories, Lexington, KY), followed by peroxidase-conjugated sheep anti-mouse IgG at 1:2,000 dilution. Membranes were developed using SuperSignal chemiluminescence substrate (Pierce), and the intensity of staining was quantified by densitometry. Immunodetection of nitrotyrosine expression was performed according to manufacturer’s instructions (Upstate Biotechnology). Briefly, membranes were extensively washed, blocked in freshly prepared 1% casein/PBS at room temperature for 20 min, and incubated with 2 μg/ml of rabbit polyclonal anti-nitrotyrosine overnight at 4°C. After extensive washing, membranes were incubated with donkey anti-rabbit IgG (Amersham Life Science) at 1:3,000 dilution in freshly prepared 1% casein/PBS at room temperature for 1.5 h. Detection was performed as detailed above. For immunoprecipitation experiments, 500 μg of total lysate were precleared with 15 μl of protein A/G Plus-agarose beads (Santa Cruz Biotechnology) and the supernatant was incubated (overnight at 4°C) with 5 μg of monoclonal anti-nitrotyrosine antibody, followed by precipitation (2 h, 4°C) with 30 μl of protein A/G Plus-agarose beads, washed in lysis buffer and boiled with 2× sample loading buffer for 5 min, as described previously (23). The samples were separated by

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**Fig. 1.** Calibration of nitric oxide (NO)-selective microelectrode. A typical recording of electrode currents (A) elicited by the addition of known concentration of dissolved NO (amperometric titration) and linear regression analysis of the data (B; standard curve, r² = 0.998) are shown. The sensitivity of the NO electrode is defined as an increment in current per unitary change in NO concentration (in this particular case, it was 21.3 pA/10 nM NO).
4–20% Tris-glycine gel and immunoblotted with anti-nitrotyrosine antibody as detailed above.

Measurement of reduced oxygen species. Reduced oxygen species (ROS) generation by endothelial cells was investigated by using a nonfluorescent probe 2',7'-dichlorofluorescin (DCFH), which acquires fluorescence properties on ROS-induced oxidation to DCFH. HUVEC were pretreated with increasing concentrations of Hcy in the presence of absence of the cell-permeable superoxide dismutase mimetic MnT-BAP (7). After 24 h incubation, HUVEC were washed with PBS and DCFH was added in its membrane-permeant diacetate form at a final concentration of 10 μM in the phenol red-free DMEM. ROS generation by the cells led to oxidation of DCFH, yielding the fluorescent product dichlorofluorescein (DCF), which was detected at an emission wavelength of 530 nm (excitation wavelength of 485 nm) by using a fluorescence plate reader, as described previously (44).

Recombinant eNOS studies. eNOS protein, a kind gift from Dr. S. S. Gross, was purified from Escherichia coli that had been transformed with independent vectors for expression of eNOS and GroELS, as previously described (24). Recombinant eNOS was prepared in 100-μl aliquots at a concentration of 167 μg/ml (Bradford assay). The activity of a 20-μl sample in 100 μl total volume was 0.416 optical density in the Griess/nitrite assay, confirming the authenticity of the product. For each experiment 25 pmol eNOS were used. To assess the effects of Hcy, eNOS activity was monitored with an NO-selective microelectrode in a stirred microcuvette at room temperature. The NO-selective and reference electrodes were equilibrated in an intracellular buffer at room temperature with constant stirring until a stable baseline current was obtained. The composition of the buffer was 50 mM Tris-HCl, pH 7.4, 500 μM NADPH, 5 μM FAD, 5 μM flavin adenine mononucleotide, 100 mM calmodulin, 0.01 mM CaCl₂, and 20 μM L-arginine. One micromolar tetrahydrobiopterin (BH4) was added to the eNOS aliquots 12–24 h before the measurements. After a stable baseline was obtained, 25 pmol eNOS were pipetted into the cuvette and the response was continuously recorded.

Fig. 2. Inhibition of bradykinin-stimulated NO release from endothelial cells by different concentrations of homocysteine (Hcy). Microvascular endothelial cells (RMVEC) preincubated in the presence of different concentrations of Hcy for 24 h, with and without 50 μM 5-methyltetrahydrofolate (5-MTHF; MTHF), were stimulated with 1 mM L-arginine, and NO release was detected with NO-selective microelectrode. *Statistically significant difference from control (CON), P < 0.001 (n = 5–9 experiments for each concentration of Hcy).

Fig. 3. Inhibition of L-arginine-stimulated NO release from endothelial cells by different concentrations of Hcy. RMVEC preincubated in the presence of different concentrations of Hcy for 24 h, with and without 50 μM 5-MTHF, were stimulated with 1 mM L-arginine, and NO release was detected with NO-selective microelectrode. *Statistically significant difference from CON, P < 0.001 (n = 6–8 experiments for each concentration of Hcy).

Statistical analyses. Statistical analyses comparing multiple variables were performed by using ANOVA followed by the Bonferroni correction. For comparisons between two variables, the unpaired Student’s t-test was used, with a P < 0.05 considered statistically significant. All values are presented as means ± SE.

RESULTS

NO release from endothelial cells stimulated by bradykinin, A-23187 or L-arginine. Twenty-four-hour preincubation with Hcy (10, 20, and 50 μM) did not cause any detectable phenotypic cell changes but resulted in a gradual decline in the resposiveness of endothelial cells to the employed standard stimuli for eNOS (Figs. 2–4). Responses to bradykinin (10 μM) were affected when endothelial cells were treated with 10, 20, and 50 μM Hcy producing 58 ± 7, 45 ± 8, and 27 ± 3 nM NO, respectively, compared with control 53 ± 4 nM NO (this represents −1.7, 21.8, and 52.9% suppression of NO release, respectively) (Fig. 2). L-Arginine addition (1 mM) to L-arginine-free medium resulted in a rapid release of 276 ± 38 nM of NO; incubation with 10 μM Hcy decreased this response by 9.8% (249 ± 30 nM) and 24.3% (188 ± 33 nM), and 50 μM reduced NO production by 83.8% to 44.4 ± 12.1 nM (Fig. 3). Similar results were obtained after 48-h incubation with Hcy (not shown). A-23187-stimulated release of NO from RMVEC or HUVEC was affected by 24-h preincubation with Hcy, resulting in a difference from control of 17.5 ± 5% (188.5 ± 57 vs. 190 ± 30 nM in control) and 44.5% (105.5 ± 18 vs. 190 ± 25 nM in control) suppression at 50 μM Hcy, respectively (Fig. 4, A and B).

Effects of Hcy on the expression of eNOS. Western analysis was performed in RMVEC and HUVEC lysates. (The latter cell type was used due to the poor immunodetection of rat eNOS with the available antibodies.) Twenty-four-hour incubation in the presence
of 10, 20, or 50 μM Hcy did not affect the expression of eNOS (Fig. 5). At neither concentration did Hcy induce iNOS expression (data not shown).

Lack of a direct effect of Hcy on recombinant eNOS.

To ascertain the possibility of Hcy acting directly on eNOS activity, in vitro studies on NO generation employing recombinant eNOS were performed, as previously described (33). The activity of eNOS was monitored with NO-selective microelectrode before and after addition of 50 μM Hcy (Fig. 6A). No differences in NO generation existed between two groups (159 ± 6 vs. 148 ± 43 nM after addition of Hcy; n = 5, P = no significant difference).

The same experimental setup was used to investigate any potential effect of ROS, normally generated in the process of Hcy oxidation (see below and Fig. 7), on eNOS activity. Oxidation of sulphhydryl groups of eNOS by diamide resulted in a dose-dependent decrease in NO generation by the recombinant enzyme in vitro (Fig. 6B). These data indicate that, although Hcy per se does not directly affect eNOS activity, ROS formed in the process of Hcy oxidation can decrease the NO-generating activity of eNOS.

Folate counteracts Hcy inhibition of eNOS.

Because 5-MTHF, a product of methylenetetrahydrofolate reductase, is required for the remethylation of Hcy to methionine, we next coincubated endothelial cells growing in high-Hcy medium with 50–100 μM 5-MTHF. After 24 h of coincubation, endothelial cells were stimulated by bradykinin or A-23187 and NO generation was detected with a NO-selective microelectrode. As shown in Figs. 2–4, coincubation of the cells with 100 μM 5-MTHF restored eNOS responses to the above agonists. This effect was not attributable to the change in eNOS expression, as combined Hcy and 5-MTHF treatment of endothelial cells did not result in any changes in the abundance of the immunodetectable eNOS (Fig. 5).

ROS and nitrotyrosine formation in response to Hcy.

To examine the mechanism of Hcy-induced suppression of NO release from stimulated RMVEC and HUVEC, we next tested its effect on the generation of ROS. ROS production was compared in cells pretreated with increasing concentrations of Hcy using a nonfluorescent probe, DCFH, oxidatively converted into the fluorescent probe DCF. Incubation of endothelial cells with 50 μM, but not with 10 or 20 μM, Hcy resulted in a statistically significant increase in DCF fluorescence (Fig. 7), thus confirming previous observations on Hcy-induced oxidative stress (21, 38, 39).

ROS could be also detected by the formation of nitrotyrosine, a marker of superoxide anion reaction with
NO, resulting in peroxynitrite formation (23). The level of nitrosylation of protein tyrosine residues was initially evaluated by using Western blot analysis. All tested concentrations of Hcy resulted in the formation of nitrotyrosine-modified protein with an apparent molecular mass of 66 kDa. Coincubation with various concentrations of 5-MTHF did not prevent nitrotyrosine formation (not shown). In a series of experiments employing immunoprecipitation with monoclonal antibodies against nitrotyrosine, the resolution of immunodetected proteins modified by tyrosine nitrosylation has improved over Western analysis (Fig. 8). This improved detection system allowed us to discern the previously undetectable dose-dependent increase in nitrotyrosine formation in the presence of escalating levels of Hcy. Again, 5-MTHF per se increased nitrotyrosine formation, thus implying that 5-MTHF may stimulate ROS formation. Alternatively, these findings can be attributable to the fact that, by restoring NO generation, 5-MTHF results in the increased supply of NO to participate in the reaction with superoxide anions to generate peroxynitrite. In contrast, HUVEC incubation in the presence of a scavenger of peroxynitrite, ebselen, resulted in the reduced nitrotyrosine formation.

Scavengers of peroxynitrite or superoxide restore NO production in Hcy-treated cells. The above findings are indicative of the formation of superoxide and peroxynitrite by Hcy-treated endothelial cells. Therefore, in the next series of experiments attempts were made to suppress their effect by scavenging either superoxide anion or peroxynitrite. Endothelial cells were incubated with escalating concentrations of Hcy in the presence of either a superoxide dismutase mimetic, MnTBAP, or a peroxynitrite scavenger, ebselen, and

Fig. 6. Lack of the direct effect of Hcy on NO-generating capacity of a recombinant eNOS (A) and suppression of NO production by diamide (B). A: in vitro detection of NO generation by the recombinant eNOS in the presence and absence of 50 μM Hcy (n = 4–5 experiments for each condition). eNOS activity was monitored with an NO-selective microelectrode in a stirred microcuvette at room temperature, as detailed in METHODS. B: in vitro detection of NO generation by the recombinant eNOS is suppressed by the diamide-induced oxidation of sulphydryl groups in a dose-dependent manner.

Fig. 7. Reduced oxygen species (ROS) production in the Hcy-pre-treated HUVEC. Treatment with Hcy (10, 20, and 50 μM for 24 h) resulted in a gradual increase in ROS production, which reached statistical significance at 50 μM Hcy and was completely abolished by coincubation with the cell-permeant superoxide dismutase mimetic manganese(III)tetrakis(4-benzoic acid) porphyrin (MnTBAP; MnT; 40 μM). *P < 0.05 vs. CON, n = 6.

Fig. 8. Immunodetection of nitrotyrosine in endothelial cells cultured in the presence of different concentrations of Hcy. HUVEC were coincubated with Hcy with or without 5-MTHF (100 μM) or ebselen (50 μM) as indicated. After immunoprecipitation (IP) of proteins modified by tyrosine nitrosylation, selected material was immunoblotted (IB) with antibodies to nitrotyrosine (NT), as detailed in METHODS. Several unidentified bands of molecular mass ~60–70 kDa are readily visible. Coadministration of 5-MTHF did not affect Hcy-induced formation of nitrotyrosine. However, coincubation with ebselen inhibited Hcy-induced nitrotyrosine formation. These experiments were performed twice with the same results. L, position of IgG light chains on the blot; MW, molecular weight marker.
compared with the effect of 5-MTHF. NO release from the endothelial cells stimulated with 5 \( \mu \)g/ml A-23187 was studied 24 h later by using NO-selective microelectrodes. As summarized in Fig. 9, A-C, neither of these supplements per se affected NO responses to the calcium ionophore in the intact endothelial cells, but each was capable of restoring NO responsiveness to Hcy-treated cells.

**DISCUSSION**

The direct measurement of NO release from cultured microvascular endothelial cells, employing an NO-selective amperometric electrode detection system, demonstrated that 50 \( \mu \)M Hcy produced a significant suppression of NO release from endothelial cells stimulated with various receptor-dependent, transporter-dependent, and receptor-independent eNOS stimuli. This effect was not mimicked in the in vitro system where NO generation by a recombinant eNOS was found to be unaffected by Hcy, indicating that the effect of Hcy is indirect. However, oxidation of sulphydryl groups in eNOS reduced NO-generating activity of the enzyme. Increased levels of nitrotyrosine, an indicator of the concomitant NO and superoxide radical formation, explains decreased NO detection as a result of decreased bioavailability, rather than suppressed production. This conclusion is further supported by the finding that scavengers of superoxide anions and peroxynitrite, MnTBAP and ebselen, reduced oxidant stress in endothelial cells, decreased the formation of nitrotyrosine-modified proteins, and ultimately improved endothelial cell NO responsiveness to a nonselective stimulus, the calcium ionophore A-23187. This is the first demonstration of an indirect inhibitory effect of Hcy on receptor-mediated, non-receptor-mediated, and L-arginine-stimulated NO release by endothelial cells. Considering the long-debated role of Hcy in the development of atherosclerosis (26, 27), our data suggest that the reduced availability of NO for its physiological targets, owing to the formation of peroxynitrite and inhibition of eNOS activity, may lead to endothelial cell dysfunction, thus explaining development of cardiovascular complications.

The mechanism of development of endothelial cell dysfunction in hyper-Hcy deserves analysis. Endothelial cells challenged with all three eNOS stimuli displayed reduction in NO release. This finding argues that elevated levels of Hcy do not selectively inhibit receptor-mediated responses (bradykinin); rather, Hcy inhibits stimulation of eNOS by increased cytosolic calcium level independently of receptor function (A-23187) and suppresses the L-arginine “paradox.” This indicates a global dysfunction of eNOS. However, Hcy does not suppress the enzyme directly. Studies with the recombinant eNOS and in vitro detection of NO production in the presence of Hcy clearly reject this possibility. Collectively, these findings would suggest that the reduction in NO release at high levels of Hcy,
which do not suppress the expression of eNOS, is a result of either decreased bioavailability of NO through formation of peroxynitrite or decreased activity of the enzyme per se. The fact that peroxynitrite formation, judged by nitrotyrosine fingerprinting, was found with all tested concentrations of Hcy, even those that did not suppress endothelial cell ability to generate NO, casts doubt on the predominant role of the former mechanism.

An understanding of the mechanism(s) of high Hcy-induced suppression of NO release from endothelial cells can be derived from the results of studies on combined effects of Hcy and scavengers of superoxide and peroxynitrite or the active metabolite of folic acid, 5-MTHF. This latter compound has clearly improved the amplitude of NO responses to all stimuli, while failing to change the abundance of eNOS or, even more importantly, the degree of peroxynitrite formation. This finding strongly suggests that the improved NO release associated with 5-MTHF is not due to the reduced peroxynitrite formation and improved bioavailability of NO, thus implying that folate therapy acts by improving eNOS activity. The following observations support this conclusion, as schematically summarized in Fig. 10.

Hcy is a well-known mediator of oxidative stress in endothelial cells (21). Upchurch et al. (39) have demonstrated that Hcy does not affect eNOS expression or its gene transcription; rather, it decreases the activity and steady-state mRNA levels of glutathione peroxidase, leading to the enhanced generation of reduced oxygen intermediates. Clinical experience demonstrates that supplementation with folic acid reduces only marginally the level of Hcy in hemodialysis patients or patients with HHcy (16, 19, 40), despite dramatic increases in the level of folate. In patients with familial hypercholesterolemia and normal levels of Hcy, folate supplementation also improved endothelial dysfunction (41, 42). Collectively, these data suggest that the mode of folate’s action transcends its ability to accelerate the metabolism of Hcy. It has been shown that folates stimulate regeneration of endogenous BH4 from dihydopterin (BH2) (25). In this vein, recent studies have revealed that eNOS binding of BH2, instead of BH4, converts eNOS from a NO-generating to a superoxide-generating enzyme (17). Collectively, these data indicate that the mechanism whereby Hcy reduces NO release is through oxidative stress in endothelial cells, which could result in the conversion of BH4 to BH2 and suppression of the NO-generating activity of eNOS. The potential ability of folates to promote the regeneration of BH4 can explain, at least in part, its observed restoration of the NO-generating capacity of the enzyme.

The authors are grateful to Dr. S. S. Gross (Cornell Medical College, Dept. of Pharmacology) for providing recombinant eNOS used in these studies and Dr. L. Moore (SUNY Stony Brook, Dept. of Physiology and Biophysics) for help with manufacturing NO electrodes.

These studies were supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-45462 and DK-52783 (M. S. Goligorsky). X. Zhang was a PhD student in the Department of Physiology. Z. Ebin was a high school student participating in the Intel Project.

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