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Homocyst(e)ine Decreases Bioavailable Nitric Oxide by a Mechanism Involving Glutathione Peroxidase*

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Hyperhomocyst(e)inemia is believed to injure endothelial cells *in vivo* through a number of mechanisms, including the generation of hydrogen peroxide (H₂O₂). Earlier *in vitro* studies demonstrated that homocyst(e)ine (Hcy) decreases the biological activity of endothelium-derived relaxing factor and that this decrease can be reversed by preventing the generation of hydrogen peroxide. Here we show that Hcy treatment of bovine aortic endothelial cells leads to a dose-dependent decrease in NO_x ($p = 0.001$ by one-way analysis of variance) independent of endothelial nitric-oxide synthase activity or protein levels and *nos3* transcription, suggesting that Hcy affects the bioavailability of NO, not its production. We hypothesized that, in addition to increasing the generation of H₂O₂, Hcy decreases the cell's ability to detoxify H₂O₂ by impairing intracellular antioxidant enzymes, specifically the intracellular isoform of glutathione peroxidase (GPx). To test this hypothesis, confluent bovine aortic endothelial cells were treated with a range of concentrations of Hcy, and intracellular GPx activity was determined. Compared with control cells, cells treated with Hcy showed a significant reduction in GPx activity (up to 81% at 250 μ M Hcy). In parallel with the decrease in GPx activity, steady-state GPx mRNA levels were also significantly decreased compared with control levels after exposure to Hcy, which appeared not to be a consequence of message destabilization. These data suggest a novel mechanism by which Hcy, in addition to increasing the generation of hydrogen peroxide, may selectively impair the endothelial cell's ability to detoxify H₂O₂, thus rendering NO more susceptible to oxidative inactivation.

Hyperhomocyst(e)inemia is a disease caused by an abnormality in either an enzyme (cystathionine β -synthetase or tem-

perature-sensitive methylenetetrahydrofolate reductase) or a cofactor (folate, vitamin B₁₂, or vitamin B₆) required for homocysteine metabolism. These abnormalities lead to elevations in plasma concentrations of homocyst(e)ine (Hcy)¹ and its precursor methionine as well as a reduction in plasma concentrations of cysteine (1–5). In its most severe form, hyperhomocyst(e)inemia confers a significant risk for thromboembolic complications that are often fatal (6). In contrast, the less severe form of the disease is commonplace and indolent, not presenting with clinical sequelae until the third or fourth decade of life. These individuals manifest atherosclerosis as well as recurrent episodes of acute arterial and venous thrombosis (6) with near normal levels of fasting plasma homocyst(e)ine; following a methionine challenge, however, levels rise significantly compared with normal levels. Many studies demonstrate that hyperhomocyst(e)inemia is an independent risk factor for atherosclerosis in the coronary, cerebral, and peripheral vasculature (7–11), and up to 40% of patients with coronary or cerebrovascular atherosclerosis have hyperhomocyst(e)inemia.

The mechanism by which Hcy damages the vessel wall and supports atherothrombosis is still unknown and may be multifactorial. Brattstrom *et al.* (12) demonstrated that endothelial cells are adversely affected by hydrogen peroxide generated during the oxidation of Hcy to homocystine and other mixed disulfides. Other prothrombotic effects induced by Hcy that may promote vascular disease include increased expression of tissue factor (13) and factor V (14) by endothelial cells, suppression of the anticoagulant activity of heparan sulfate on the endothelial cell surface (15), and decreased expression of thrombomodulin and activated protein C (16) as well as tissue-type plasminogen activator receptors (annexin II) on the endothelial cell surface (17). Earlier *in vitro* studies by our laboratory showed that Hcy treatment of vessel rings reduces the biological activity of endothelium-derived relaxing factor (18). In addition, we showed that the generation of hydrogen peroxide from Hcy is prevented by *S*-nitrosation of its thiol group and that, in contrast to Hcy, *S*-nitrosohomocyst(e)ine has potent vasodilator and antiplatelet effects (18).

In this study, we address the complex mechanisms for the decrease in endothelium-derived relaxing factor/NO by endothelial cells following Hcy treatment. Our data show that, in addition to increased hydrogen peroxide generation from Hcy oxidation, this biological thiol also uniquely decreases intracellular glutathione peroxidase (GPx). These results suggest a

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¹ The abbreviations used are: Hcy, homocyst(e)ine; GPx, glutathione peroxidase; β -MSA, β -mercaptosuccinic acid; DTPA, diethylenetriaminepentaacetic acid; HMVEC, human microvascular endothelial cells; BAEC, bovine aortic endothelial cells; eNOS, endothelial nitric-oxide synthase; kb, kilobase pair(s); HPLC, high performance liquid chromatography; ANOVA, analysis of variance.

unique mechanism by which Hcy decreases bioavailable NO and consequently produces an oxidative *in vivo* environment in the vasculature.

EXPERIMENTAL PROCEDURES

Chemicals and Solutions—Trypan blue (0.4%), L-arginine, DL-homocysteine, L-cysteine, glutathione, the lactate dehydrogenase assay kit, horseradish peroxidase, scopoletin, catalase, 3-amino-1,2,4-triazole, β -mercaptosuccinic acid (β -MSA), pepstatin, leupeptin, phenylmethylsulfonyl fluoride, DTPA, GPx, perchloric acid, and disodium EDTA were purchased from Sigma. Hanks' balanced saline solution without calcium or magnesium, fetal bovine serum, newborn calf serum, penicillin G, streptomycin sulfate, trypsin, and media were purchased from Life Technologies, Inc. Phosphate-buffered saline, pH 7.4, consisted of 10 mM sodium phosphate and 150 mM NaCl. Protein concentrations were determined using the BCA protein assay reagent (Pierce). Human microvascular endothelial cells (HMVEC), used for the measurement of steady-state intracellular GPx mRNA, and CS-5.0 medium were purchased from Cell Systems Corp. (Kirkland, WA).

Cell Culture—Bovine aortic endothelial cells (BAEC) were isolated as described previously and were maintained in Dulbecco's modified Eagle's medium/nutrient mixture F-12 containing 20% newborn calf serum and antibiotics (100 units/ml penicillin G sodium and 100 μ g/ml streptomycin sulfate) (19). Culture plates were maintained in a humidified incubator at 37 °C with a 5% CO₂ atmosphere. Cells (passages 3–15) were subcultured after treatment with 0.05% trypsin and 0.53 mM disodium EDTA. BAEC were identified by their maintenance of density-dependent growth after serial passage, by their typical cobblestone configuration when viewed by light microscopy, and by a positive indirect immunofluorescence for von Willebrand factor (20–22).

BAEC were plated onto 20 $\times$ 100-mm Falcon 3003 tissue culture dishes and allowed to reach confluency over 5–7 days. All experiments were initiated with phenol red-free medium containing 1 mM L-arginine. Experimental groups consisted of cells treated with no additional thiols (controls) or with Hcy or Cys at concentrations ranging from 20 μ M to 5 mM.

Measurement of Nitric Oxide—The production of total NO_x (*S*-nitrosothiols plus free NO) was measured by photolysis/chemiluminescence as described previously (23) with *S*-nitrosoglutathione used as a standard. All measured NO_x is expressed as nmol of NO_x/g of cell protein.

Measurement of Nitric-oxide Synthase Activity—Nitric-oxide synthase activity was measured in endothelial cell lysates following 4-h incubations with or without Hcy using the method of Bredt and Snyder (24). Activity is expressed as fmol of L-citrulline/g of cell protein.

Measurement of Endothelial Nitric-oxide Synthase (eNOS) Protein Levels—Confluent BAEC treated with various concentrations of Hcy for 4 h were harvested by scraping and frozen at –70 °C. Upon thawing, the cell pellets were homogenized in a buffer containing 0.32 mM sucrose, 0.020 mM Hepes, 0.5 mM EDTA, 1 mM dithiothreitol, 2 μ M leupeptin, 1 μ M pepstatin A, and 1 μ M phenylmethylsulfonyl fluoride. Insoluble material was removed by centrifugation (1000 $\times$ *g* for 10 min at 2 °C), and a sample of each treatment group was used for total protein determination.

Prior to loading, each sample was denatured by boiling in the dithiothreitol-containing homogenization buffer for 2 min. Each sample was loaded onto a 4% stacking/7.5% separating gel at equal protein concentrations and electrophoresed at a constant current at 4 °C. After transferring the gel using standard Western blotting techniques, the blot was incubated in a 5% milk suspension for 2 h to block nonspecific binding sites. The blot was then exposed to a human monoclonal antibody to eNOS (1:2000 dilution; Transduction Laboratories, Lexington, KY). The ECL Western blot analysis system (Amersham Life Systems, Buckinghamshire, England) was used for detection of the primary antibody signal and included a peroxidase-labeled anti-mouse secondary antibody (1:1000). The immunoreactivity of eNOS was detected by changes in chemiluminescence. After transfer to an autoradiogram, the eNOS signal was quantified by densitometry.

Northern Analysis of eNOS (*nos3*) and Glutathione Peroxidase mRNA—Cell monolayers of $\sim 1.1 \times 10^7$ cells were grown to subconfluency. Total RNA was isolated from monolayers of BAEC treated for 4 h with or without exogenous Hcy. Total RNA was extracted using the Oncogene Science RNA purification system, which is based on a guanidinium thiocyanate/phenol/chloroform extraction method.

nos3 mRNA was detected by Northern analysis using a full-length cDNA derived from a bovine clone kindly provided by Drs. Thomas Michel and Santiago Lamas (GenBank™ accession number M89952) (25). Ten μ g of total RNA were loaded in each lane and electrophoresed

on a denaturing 1.2% agarose gel containing 2.2 M formaldehyde. The gel was blotted onto a Nytron-N⁺ membrane by capillary action, hybridized with the ³²P-radiolabeled probes for *nos3* and bovine β -actin (generous gift of Dr. David R. Morris), and then exposed on Kodak X-Omat film for 3 days at –70 °C. For quantitative evaluation of *nos3* and β -actin transcripts, phosphoimage analysis was performed using a PhosphorImager SF (Molecular Dynamics, Inc., Sunnyvale, CA). The blots were repeated and analyzed in triplicate for each of the experimental groups.

In separate experiments, subconfluent HMVEC were exposed to control medium, to medium containing Hcy, or to medium containing Cys, each for 4 h. HMVEC were used in these experiments because the human GPx probe did not recognize the bovine intracellular GPx mRNA. Total cellular RNA was extracted from HMVEC, and 10 μ g of total RNA were electrophoresed on a 1.5% denaturing agarose gel and blotted as described above. The full-length human GPx cDNA probe (1.1 kb) was the generous gift of Dr. Peter Newburger (University of Massachusetts, Worcester, MA). Hybridization was performed according to the method described by Chada *et al.* (26) with phosphoimage data obtained after overnight exposure. GPx transcription was normalized by comparison with β -actin as described above.

In separate experiments, HMVEC were treated with or without Hcy for 4 h in the presence of actinomycin D (5 μ g/ml). Total mRNA was collected at 0, 1, 2, and 4 h, and Northern analyses using the GPx and β -actin probes were performed as described above.

Effect of β -Mercaptosuccinic Acid on NO_x Production—In these experiments, β -MSA, an inhibitor of intracellular GPx (27), was incubated with BAEC in the presence of Hcy. BAEC were incubated with 100 μ M Hcy, 100 μ M β -MSA, both, or neither for 4 h, and media were collected and assayed for NO_x as described above.

Determination of Glutathione Peroxidase Activity—Intracellular GPx activity was determined by an assay that couples the reduction of peroxides and the oxidation of glutathione with the reduction of oxidized glutathione by glutathione reductase using NADPH as a cofactor. Hydroperoxide reduction was followed by a decrease in NADPH absorbance at 340 nm (28). Briefly, BAEC were treated with control medium or with medium containing Hcy. After 4 h, the cells were washed with Hanks' balanced saline solution and treated with phosphate-buffered saline containing 1% Triton X-100. Hydroperoxide reduction was monitored as a decrease in light absorbance with oxidation of NADPH at 340 nm. Results are given in absorbance units/min/mg of protein and represent the averages of seven experiments, each performed in duplicate.

Intracellular Reduced Glutathione Levels—Intracellular GSH was measured by high performance liquid chromatography (HPLC) with electrochemical detection (23). The technique reliably separates and detects thiols in the nM range and readily distinguishes the various biological thiols from one another. In these experiments, cells were exposed to control medium or to medium containing Hcy. After 4 h, BAEC were washed with Hanks' balanced saline solution and treated with 0.4 N perchloric acid containing 2 mM DTPA. Intracellular glutathione levels were determined by HPLC/electrochemical detection and normalized to total cell protein.

Cell Viability—Cell viability of confluent monolayers was determined by one of three methods. At the end of each treatment, cells were examined by light microscopy for vacuolation and other signs of cell death. Treated cells were also compared with control cells using trypan blue exclusion. Total lactate dehydrogenase in the medium was quantified spectrophotometrically. Using light microscopy, trypan blue, and lactate dehydrogenase, no significant differences were appreciated at 4 h between the control group and cells treated with up to 5.0 mM Hcy.

Statistical Analyses—Statistical analysis was performed using Student's *t* test or an analysis of variance (ANOVA) with either a Bonferroni or a Newman-Keuls *post hoc* test. Data are presented as the means $\pm$ S.E. *p* values < 0.05 were considered statistically significant.

RESULTS

Homocysteine Decreases Bioavailable NO_x—BAEC were incubated with media containing a range of concentrations of Hcy for 4 h, the media were collected, and NO_x concentrations were determined. Fig. 1 shows the average of five experiments, each performed in triplicate. The data demonstrate that BAEC incubated with increasing concentrations of Hcy sustained a dose-dependent decrease in NO_x (*p* = 0.001 by ANOVA). Compared with control cells (106 $\pm$ 8 nmol of NO_x/g of protein), treatment of BAEC with Hcy led to a maximum 86% reduction (15 $\pm$ 3

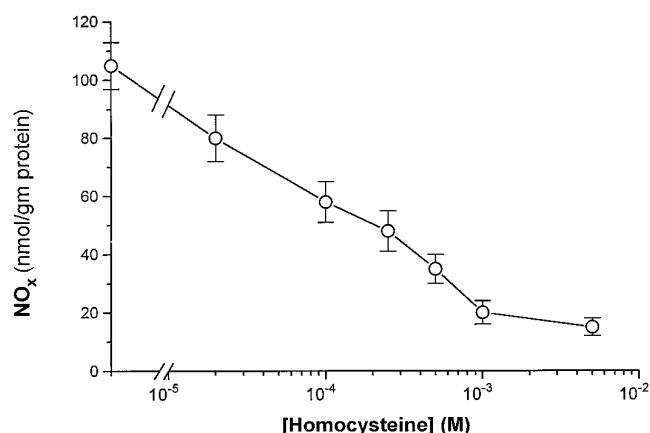


FIG. 1. **Effect of increasing concentrations of Hcy on NO production by endothelial cells.** Confluent bovine aortic endothelial cells were treated for 4 h with media containing the indicated concentrations of Hcy. Media were collected, and NO_x concentrations were determined by photolysis/chemiluminescence as described under "Experimental Procedures." Results are expressed for NO_x in nmol/g of protein as the means $\pm$ S.E. of five experiments, each performed in triplicate ($p < 0.001$ by ANOVA).

nmol of NO_x/g of cell protein) in NO_x. Cys, at similar concentrations, failed to produce a statistically significant decrease in NO_x (data not shown).

Hcy Treatment of BAEC Does Not Alter eNOS Activity, eNOS Protein Levels, or Steady-state *nos3* mRNA—Having shown a maximum 86% decrease in NO_x after treatment of BAEC with Hcy, we next attempted to determine if this decrease in NO_x was the result of an alteration in eNOS activity or protein levels or steady-state *nos3* mRNA. Lysates of BAEC following a 4-h incubation with 0, 0.05, or 5 mM Hcy showed no significant change in eNOS activity (138 ± 22 , 150 ± 40 , and 155 ± 35 fmol of L-citrulline/g of cell protein, respectively; p = not significant). Western analysis was performed on BAEC incubated with medium containing 0, 0.05, 0.5, or 5 mM Hcy for 4 h. Following a 4-h incubation, BAEC eNOS protein levels were determined as described under "Experimental Procedures." In contrast to NO_x, cells incubated with increasing concentrations of Hcy manifested no detectable reduction in eNOS protein compared with control cells (Fig. 2A).

We also examined steady-state *nos3* mRNA in BAEC after cell treatment either with control medium (Fig. 2B, CON) or with medium containing 5.0 mM Hcy (Hcy) for 4 h. Fig. 2B is one of three Northern blots of BAEC total RNA probed with a bovine full-length *nos3* cDNA and a bovine β -actin cDNA (4.5 and 2 kb, respectively). Quantitative results from densitometric analysis of Northern phosphorimages show that, in the presence of 5.0 mM Hcy, steady-state *nos3* mRNA was not statistically different from control levels after normalizing for β -actin (p = not significant; $n = 3$).

Inhibition of GPx in the Presence of Hcy Decreases NO_x—Recent evidence from our laboratory suggests that GPx potentiates the action of NO produced by BAEC and does so by a mechanism that requires glutathione as a cosubstrate (28). Based on this observation, which suggests that a decrease in intracellular GPx may cause a decrease in bioavailable NO, we tested the hypothesis that Hcy causes a reduction in NO by impairing the reduction of peroxides by endothelial GPx. In initial experiments, we tested the hypothesis that inhibition of GPx potentiates the decrease in endothelial NO_x produced by Hcy oxidation. In these experiments, BAEC were incubated with 100 μ M Hcy, 100 μ M β -MSA, both, or neither for 4 h, and total NO_x production was determined. The results show that cells treated with β -MSA and Hcy sustained a 88% reduction in

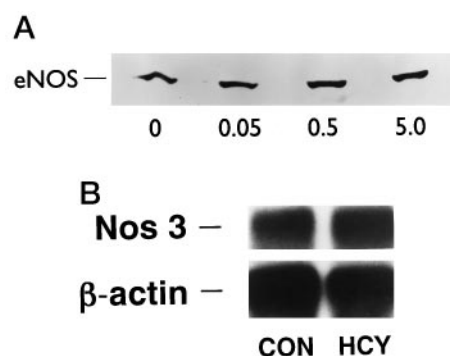


FIG. 2. A, Western analysis of BAEC exposed to 0, 0.05, 0.5, or 5.0 mM Hcy for 4 h. After exposure to a monoclonal eNOS antibody, a single band corresponding to eNOS was identified by autoradiography. This blot demonstrates that BAEC treated with Hcy did not decrease eNOS levels compared with control cells as determined by densitometric scanning. B, nitric-oxide synthase (*nos3*) mRNA after Hcy treatment as determined by Northern analysis. Endothelial cells were treated with control medium (CON) or with medium containing 5.0 mM Hcy (Hcy) for 4 h. This blot is one of three probed with a full-length *nos3* cDNA (4.5 kb) and a β -actin cDNA (2.0 kb); after normalization for β -actin, no significant difference in steady-state *nos3* mRNA was detected.

NO_x compared with control cells (Fig. 3).

Intracellular Glutathione Peroxidase Activity and Transcription Are Decreased following Treatment of BAEC with Hcy—After showing that the addition of β -MSA to BAEC in the presence of Hcy potentiated the decrease in NO_x observed when cells were treated with Hcy alone, we next attempted to determine if Hcy, a known generator of H₂O₂, might lead to a decrease in intracellular GPx activity. Intracellular endothelial GPx activity was measured following a 4-h incubation with increasing concentrations of Hcy. Compared with control levels (0.16 ± 0.04 absorbance units/min/mg of protein), pathophysiological concentrations of Hcy (50–250 μ M) led to a 41–81% decrease (0.095 ± 0.03 to 0.03 ± 0.01 absorbance units/min/mg of protein at 50 and 250 μ M Hcy, respectively; $p < 0.001$) in GPx activity (Fig. 4). Higher concentrations of Hcy (1 mM) reduced GPx activity by 91% compared with control levels (0.014 ± 0.005 absorbance units/min/mg of protein; $p < 0.001$).

In separate experiments in which a known concentration of exogenous GPx (200 units/ml) was added to the medium for 4 h, increasing concentrations of Hcy did not alter the enzyme activity compared with control levels ($p = 0.64$ by ANOVA) (data not shown). This finding suggests that the effect of Hcy on endogenous GPx is not direct and instead occurs either through an attenuation of GPx expression or through reduced availability of cosubstrate GSH.

In an attempt to determine if Hcy modified GPx activity by altering transcription of GPx, Northern analysis was performed on total cellular mRNA after HMVEC were treated with control medium or with medium containing either 5.0 mM Hcy or Cys. Steady-state GPx mRNA in cells treated with 5.0 mM Hcy was decreased significantly (by 90%) compared with control cells (Fig. 5). By contrast and importantly, cells treated with 5.0 mM Cys showed no change in steady-state GPx mRNA compared with control cells, possibly explaining in part why Cys treatment of BAEC induces no change in NO_x production despite the similar generation of H₂O₂ during Cys oxidation.

After demonstrating a decrease in steady-state GPx mRNA following Hcy incubation, we next attempted to determine if the effect of Hcy on GPx mRNA affected transcription or message stability. The results from actinomycin D treatment of HMVEC concomitant with Hcy incubation are shown in Fig. 6 and demonstrate no change in steady-state GPx mRNA over the time course of this experiment. These results suggest that

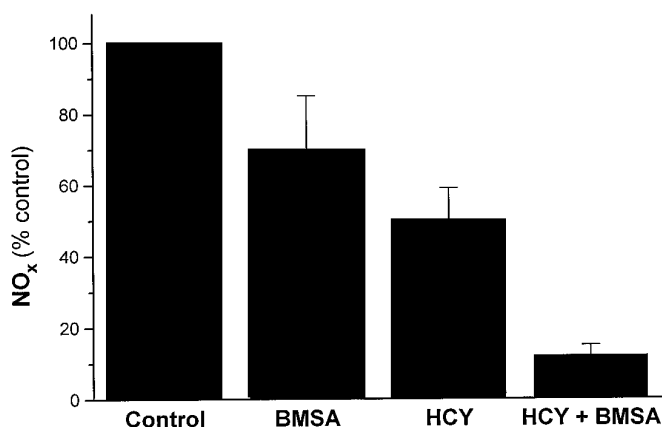


FIG. 3. Effect of β -MSA on NO_x in the presence of Hcy. Confluent BAEC were exposed for 4 h to control medium or to media containing 100 μ M β -MSA, 100 μ M Hcy, or both (HCY + BMSA). Media were collected, and NO_x levels were determined by photolysis/chemiluminescence as described under "Experimental Procedures." Results are expressed as percent control NO_x (mean $\pm$ S.E., respective experiment) with $p = 0.01$ compared with β -MSA or Hcy alone.

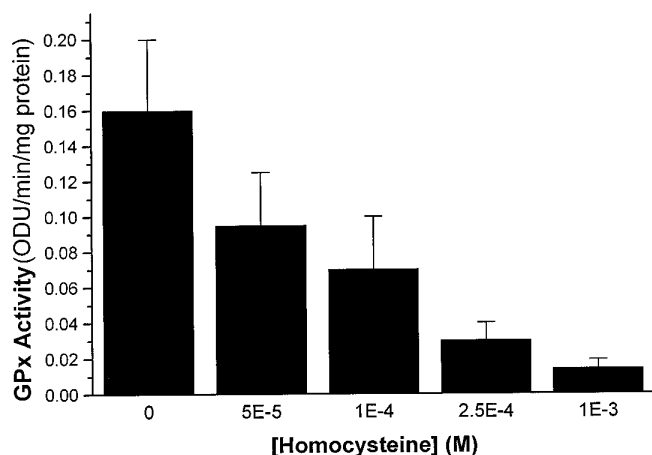


FIG. 4. Effect of increasing concentrations of Hcy on intracellular endothelial GPx activity. Confluent bovine aortic endothelial cells were treated for 4 h with media containing 0–1 mM Hcy, and GPx activity was determined as described under "Experimental Procedures." GPx activity is expressed in absorbance units (ODU)/min/mg of protein, with each point representing the average of seven experiments, each performed in duplicate ($p < 0.001$ by ANOVA).

Hcy treatment of HMVEC alters GPx mRNA transcription and does not destabilize mRNA.

Treatment of Endothelial Cells with Hcy Does Not Decrease Intracellular Glutathione—Having demonstrated a decrease in intracellular GPx activity as well as GPx transcription following Hcy exposure, we next addressed the issue of whether or not the reduction in GPx activity might also be secondary to a limited cosubstrate (GSH). We found that BAEC respond to increasing concentrations of Hcy by increasing GSH. As shown in Table I, BAEC exposed to 50 μ M Hcy or 5.0 mM Hcy showed increases in intracellular GSH of 32 and 132%, respectively, compared with control cells ($p < 0.05$).

Lactate Dehydrogenase and Total Protein Determination—Lactate dehydrogenase released from BAEC treated for 4 h with control medium or with medium containing 5.0 mM Hcy was next measured and compared with total cell lactate dehydrogenase (0.1% Triton X-100). Compared with control medium, BAEC treated with medium containing 5.0 mM Hcy for 4 h showed no increase in lactate dehydrogenase release (511 ± 46 units/ml for control *versus* 513 ± 62 units/ml for 5.0 mM Hcy;

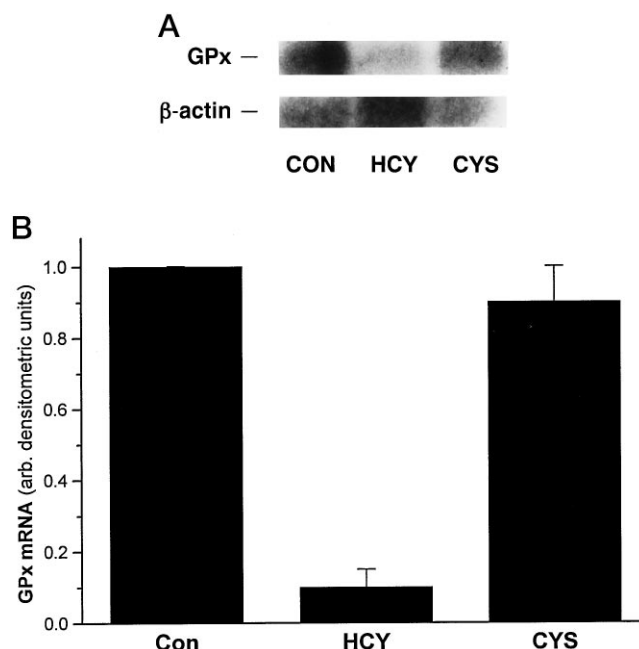


FIG. 5. Endothelial steady-state GPx mRNA after Hcy treatment as determined by Northern analysis. Confluent HMVEC were treated with control medium (Con), with medium containing 5.0 mM Hcy (HCY), or with medium containing 5.0 mM Cys (CYS) for 4 h. The blots were probed with a full-length human GPx cDNA (1.1 kb) and β -actin cDNA (2 kb). A, a representative Northern blot; B, results of three experiments.

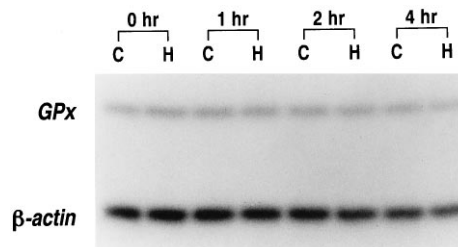


FIG. 6. Endothelial GPx transcription after Hcy treatment in the presence of 5 μ g/ml actinomycin D as determined by Northern analysis. Confluent HMVEC were treated with control medium (C) or with medium containing 5.0 mM Hcy (H) for 4 h in addition to 5 μ g/ml actinomycin D. Total RNA was collected at 0, 1, 2, and 4 h following treatment, and Northern analysis was performed. The blot was probed with a full-length human GPx cDNA (1.1 kb) and β -actin cDNA (2 kb) and demonstrates no change in GPx signal, suggesting that the effect of Hcy on GPx mRNA was at the transcriptional level.

TABLE I
Homocysteine and endothelial cell GSH

Treatment	GSH μ mol/g protein	GSH % control
Control	2.96 $\pm$ 0.51	
Hcy 50 μ M	3.91 $\pm$ 0.61	32
5.0 mM	6.87 $\pm$ 0.61 ^a	132

^a $p < 0.05$ compared with control and 50 μ M Hcy.

$p =$ not significant). Cells treated with 0.1% Triton X-100 served as a positive control.

DISCUSSION

We first showed that Hcy, a thiol known to generate H₂O₂, decreases bioavailable NO independent of eNOS protein expression and steady-state *nos3* mRNA. In this study, we also demonstrate that inhibition of intracellular GPx can potentiate the deleterious effects of Hcy by leading to a further decrease in

bioactive NO. Subsequently, we examined the effects of increasing concentrations of Hcy on intracellular endothelial GPx and thiol pools (GSH). In this study, Hcy treatment of BAEC reduced intracellular GPx activity and transcription in a manner that is not the consequence of a direct effect on GPx activity or a consequence of limited cosubstrate (GSH). These observations suggest that Hcy, in addition to causing endothelial cell injury by promoting the formation of peroxides, also reduces intracellular levels of GPx and thereby potentiates inactivation of NO by peroxides.

Earlier work by Starkebaum and Harlan (29) and Wall *et al.* (30) demonstrated *in vitro* that Hcy undergoes oxidation to homocystine, and in the process, H_2O_2 is generated. In these experiments, catalase prevented Hcy-induced endothelial cell injury. These authors concluded that Hcy-induced endothelial cell injury *in vitro* was largely secondary to H_2O_2 generation. Harker *et al.* (31) postulated that Hcy-induced H_2O_2 -mediated endothelial cytotoxicity led to the exposure of the smooth muscle cell-containing vascular medium. Once exposed, the vascular smooth muscle cells begin to proliferate and to evoke other deleterious effects, including the activation of platelets and leukocytes.

In addition to the generation of H_2O_2 , Hcy may also play a role in inducing endothelial cytotoxicity by producing other reactive oxygen species. Superoxide anion radical (O_2^-), a potent reactive oxygen free radical generated following the oxidation of Hcy, has been shown to cause lipid peroxidation (32).

Hcy is not unique in its ability to generate H_2O_2 or superoxide, as other thiols can generate these oxidative by-products as well. Heinecke *et al.* (33) have also shown that supplementation of the biological thiol Cys leads to an increase in superoxide production by smooth muscle cells. In distinct contrast to Hcy, we found that Cys was unable to decrease bioavailable NO or to cause a significant decrease in steady-state GPx mRNA in this system. We further speculate that variations in the reduced state of the various thiols may be important in determining the selective toxicity of Hcy compared with other biological thiols. Andersson *et al.* (34) showed that Hcy, similar to GSH, has a $t_{1/2}$ in plasma of ~ 14 min, while the $t_{1/2}$ of Cys is almost three times longer (37 min). The differences in the $t_{1/2}$ of the structurally similar reduced biological thiols, which are both capable of being oxidized, suggest that the mechanism behind the respective oxidation of each thiol must differ in some manner.

The cell protects itself against oxidative damage from H_2O_2 by two separate intracellular enzyme systems, GPx and catalase. GPx, containing a selenocysteine moiety in its catalytic center, is an antioxidant enzyme that reduces both lipid and hydrogen peroxide to their respective alcohols. In contrast, catalase is a ubiquitous enzyme that scavenges H_2O_2 exclusively. Compared with GPx, catalase is found in lower concentrations intracellularly and has a higher K_m for hydrogen peroxide (35), further implicating GPx as the major intracellular detoxifying mechanism for H_2O_2 and lipid peroxides.

A number of studies have documented a link between differences in GPx and an increased incidence of atherosclerosis. Buczynski *et al.* (36) have shown that patients with coronary artery disease have lower platelet and plasma levels of GPx. Other investigators have shown that a selenium-deficient diet leads to a decrease in the GPx level (37) and consequently an increase in the incidence of coronary artery disease (38). In addition, certain diets containing $\omega 3$ -polyunsaturated fatty acids, which have been shown to reduce cardiovascular disease, are also rich in selenium, further implying a role for GPx in preventing atherosclerosis (39).

A recent study by our laboratory has suggested that GPx

might also be important in the regulation of NO, a potent inhibitor of platelet activation (28). Our data support a dual role for GPx: first, by reducing lipid peroxides, GPx prevents the inactivation of NO; and second, GPx also preserves the antiplatelet effects of an endogenous NO donor, *S*-nitrosoglutathione. These data suggest that this latter effect of GPx is secondary to the ability of *S*-nitrosoglutathione to serve as an effective cosubstrate for GPx in place of GSH. Based on these observations, we hypothesized that if GPx protects bioactive NO from oxidative attack, then the corollary might also be true, *viz.* a decrease in GPx might lead to a decrease in bioavailable NO.

The thrombogenic mechanism(s) involved in hyperhomocyst(e)inemia are not well understood, yet data from our laboratory and others suggest that the ability of Hcy to damage the endothelium and to support atherothrombosis may be multifactorial, with different and specific effects on both endothelial and vascular smooth muscle cells. For example, Hcy at doses of 0.1–1.0 mM markedly inhibits endothelial cell growth over time *in vitro*; in contrast, vascular smooth muscle cells respond to similar concentrations of Hcy with an increase in cyclin D1 and cyclin A mRNA expression and a resulting marked increase in cell proliferation (40). It has been proposed that Hcy first produces severe endothelial cell injury and that this injury, in turn, leads to platelet activation (induced by reducing endothelium-derived relaxing factor/NO production), smooth muscle cell proliferation, and subsequent thrombosis. These effects stand in direct contrast to the known effects of NO on smooth muscle and endothelial cell growth: NO (produced by *nos2* in vascular smooth muscle cells) is cytotoxic to smooth muscle cells, yet leads in co-culture to endothelial cell proliferation, perhaps by release of basic fibroblast growth factor (41) and/or vascular endothelial cell growth factor from smooth muscle cells.

Our data support this proposed mechanism of Hcy toxicity and suggest that Hcy-induced attenuation of bioavailable NO compromises the antithrombotic properties of the endothelium and predisposes to platelet activation as well as thrombin generation. Recent observations from our laboratory suggest that, through enhanced NO release, the endothelium can modify the toxicity of Hcy for a limited time (19). However, as GPx expression decreases and the oxidative by-products of Hcy begin to accumulate, endothelial dysfunction occurs, leading to subsequent attenuation of NO production. Eventually, a progressive imbalance between NO production by an increasingly dysfunctional endothelium and an increase in Hcy and its oxidative by-products develops. As recently demonstrated by Lentz *et al.* (42), this deficiency of bioactive NO produced by dysfunctional endothelium leads to impaired endothelium-dependent vasorelaxation in a monkey model.

These data suggest that hyperhomocyst(e)inemia limits the bioavailability of endothelium-derived relaxing factor/NO through a mechanism that involves the increased production of reactive oxygen species coupled to an impaired ability to detoxify peroxides. Future therapeutic strategies designed to stimulate endogenous NO production, to provide exogenous NO donors, or to improve the antioxidant profile of the vasculature may help to ameliorate endothelial cell injury evoked by hyperhomocyst(e)inemia.

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**Homocyst(e)ine Decreases Bioavailable Nitric Oxide by a Mechanism Involving
Glutathione Peroxidase**

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