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Research Article

Elevated levels of homocysteine are associated with an increased risk of atherosclerosis and thrombosis. The reactivity of the sulfhydryl group of homocysteine has been implicated in molecular mechanisms underlying this increased risk. There is also increasingly compelling evidence that thiols react in the presence of nitric oxide (NO) and endothelium-derived relaxing factor (EDRF) to form S-nitrosothiols, compounds with potent vasodilatory and antiplatelet effects. We, therefore, hypothesized that S-nitrosation of homocysteine would confer these beneficial bioactivities to the thiol, and at the same time attenuate its pathogenicity. We found that prolonged (> 3 h) exposure of endothelial cells to homocysteine results in impaired EDRF responses. By contrast, brief (15 min) exposure of endothelial cells, stimulated to secrete EDRF, to homocysteine results in the formation of S-NO-homocysteine, a potent antiplatelet agent and vasodilator. In contrast to homocysteine, S-NO-homocysteine does not support H₂O₂ generation and does not undergo conversion to homocysteine thiolactone, reaction products believed to contribute to endothelial toxicity. These results suggest that the normal endothelium modulates the potential, adverse effects of homocysteine by releasing EDRF and forming the adduct S-NO-homocysteine. The adverse vascular properties of homocysteine may result from an inability to sustain S-NO formation owing to a progressive imbalance between the production of NO by progressively dysfunctional endothelial cells and the levels of homocysteine.

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Adverse Vascular Effects of Homocysteine Are Modulated by Endothelium-derived Relaxing Factor and Related Oxides of Nitrogen

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Abstract

Elevated levels of homocysteine are associated with an increased risk of atherosclerosis and thrombosis. The reactivity of the sulfhydryl group of homocysteine has been implicated in molecular mechanisms underlying this increased risk. There is also increasingly compelling evidence that thiols react in the presence of nitric oxide (NO) and endothelium-derived relaxing factor (EDRF) to form *S*-nitrosothiols, compounds with potent vasodilatory and antiplatelet effects. We, therefore, hypothesized that *S*-nitrosation of homocysteine would confer these beneficial bioactivities to the thiol, and at the same time attenuate its pathogenicity. We found that prolonged (> 3 h) exposure of endothelial cells to homocysteine results in impaired EDRF responses. By contrast, brief (15 min) exposure of endothelial cells, stimulated to secrete EDRF, to homocysteine results in the formation of *S*-NO-homocysteine, a potent antiplatelet agent and vasodilator. In contrast to homocysteine, *S*-NO-homocysteine does not support H₂O₂ generation and does not undergo conversion to homocysteine thiolactone, reaction products believed to contribute to endothelial toxicity. These results suggest that the normal endothelium modulates the potential, adverse effects of homocysteine by releasing EDRF and forming the adduct *S*-NO-homocysteine. The adverse vascular properties of homocysteine may result from an inability to sustain *S*-NO formation owing to a progressive imbalance between the production of NO by progressively dysfunctional endothelial cells and the levels of homocysteine. (*J. Clin. Invest.* 1993. 91:308–318.) Key words: thiols • nitric oxide • endothelial cells

Introduction

Elevated levels of the biological thiol homocysteine (HCYSH)¹ are an independent risk factor for atherosclerosis and are also associated with a variety of thrombotic complica-

tions (1, 2). Moderate hyperhomocysteinemia is found in 20–30% of patients with coronary and peripheral vascular disease, and levels of HCYSH may approach 200–250 μM in homocystinuria (2–5). High levels of oxidation products of HCYSH may further increase significantly the HCYSH-related burden in plasma and the cell cytosol, and thereby contribute to its pathogenicity (6–9). These species include homocysteine disulfide (homocystine), homocysteine mixed-disulfides, and the cyclized oxidation product of HCYSH, homocysteine thiolactone (HTL).

Marked platelet accumulation at sites of vascular injury and platelet-rich occlusive thrombi are distinctive pathologic features of both human and experimental hyperhomocysteinemia (10–12). To explain these pathologic findings, several groups have reported direct proaggregatory effects of HCYSH and HTL (13–15). These proaggregatory actions, however, have not been demonstrated uniformly (15–18), and supportive biochemical and molecular mechanisms have not been well elucidated. Moreover, the platelet activating effects of HCYSH, attributed to its reactive SH group, are difficult to reconcile with the known antiplatelet properties of other biological thiols with similar chemical and physical characteristics. In particular, glutathione, cysteine, and *N*-acetylcysteine have antiplatelet effects, albeit at millimolar concentrations (19, 20).

Harker and colleagues suggested that HCYSH-induced endothelial injury, by exposing subendothelial connective tissue, represents an alternative mechanism for platelet activation in vivo (11, 12). The endothelial cytotoxicity of HCYSH has since been confirmed (21–23), and attributed to H₂O₂ generated by way of the SH group (21, 22); direct toxic effects of HTL on endothelial cells have also been reported (24, 25). At the same time, however, our understanding of the role played by the endothelium in modulating platelet-vessel wall interaction has changed substantially. It is now widely appreciated that the endothelium plays a dynamic role in counteracting platelet activation by secreting products such as prostacyclin and endothelium-derived relaxing factor (EDRF), the latter having been identified as nitric oxide (NO [26, 27] or a closely related *S*-nitrosothiol [RS-NO] [28, 29]).

The disputed chemical nature of EDRF is important in this discussion in that oxides of nitrogen (NO_x) react readily with thiols to form *S*-nitrosothiols (30). The recent demonstration of RS-NO formation from endogenous NO (EDRF) (31) provides added evidence in favor of biological relevance for these compounds. *S*-nitrosothiols invariably possess EDRF-like vasodilatory and antiplatelet properties, and the same might be predicted of *S*-nitroso-homocysteine (SNOHO). Moreover, inasmuch as the reactivity of the SH group is central to the above proposed atherothrombotic mechanisms and the oxidative metabolism of HCYSH, *S*-nitrosation may represent a biological mechanism for altering favorably the metabolic fate of

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1. Abbreviations used in this paper: CZE, capillary zone electrophoresis; ECB, negatively charged spherical plastic beads; EDRF, endothelium-derived relaxing factor; GFP, gel-filtered platelets; HCYSH, homocysteine; HTL, homocysteine thiolactone; NO, nitric oxide; NO_x, oxides of nitrogen; SNOHO, *S*-nitroso-HCYSH; RS-NO, related *S*-nitrosothiol; PRP, platelet-rich plasma.

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HCYSH. Thus, in light of the potential regulatory importance of the S-NO functional group and the controversies regarding the thrombogenic mechanism(s) of HCYSH, we evaluated the effects of HCYSH on platelet-vessel wall interactions, and tested the hypothesis that its thrombogenicity is modulated by NO_x and EDRF. Our results show that HCYSH and HTL have no direct proaggregatory effects; that SNOHO forms under physiologic conditions and possesses EDRF-like properties; that HCYSH-induced thrombogenicity is a consequence of endothelial dysfunction; and that dysfunctional endothelium cannot sustain elaboration of NO sufficient to "detoxify" HCYSH by forming SNOHO. The data strongly support the notion that S-nitrosation of HCYSH represents an endogenous cytoprotective, antithrombotic cell regulatory mechanism.

Methods

Materials

HCYSH, homocystine, HTL (free base and hydrochloride), homocysteic acid, cupric sulfate, ferrous sulfate, methylene blue, Sepharose 2B-300, acetylsalicylic acid, epinephrine, ADP, acetylcholine, *N*^w-nitro-L-arginine, and calcium ionophore (A23187) were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine thrombin was obtained from ICN ImmunoBiologicals (Lisle, IL). Sulfanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride were purchased from Aldrich Chemical Co. (Milwaukee, WI). Calf skin collagen was obtained from Worthington Biochemical Corp. (Freehold, NJ). Sodium nitrite was purchased from Fisher Scientific Co. (Fairlawn, NJ). NO gas was obtained from Matheson Gas Products (Secaucus, NJ). Radioimmunoassay kits for the determination of cyclic GMP levels were purchased from New England Nuclear (Boston, MA). HEPES-buffered saline consisted of 140 mM NaCl, 6 mM HCl, 6 mM *N*-[2-hydroxyethyl]-piperazine-*N*-[2-ethane-sulfonic acid], 2 mM Na₂HPO₄, 2 mM MgSO₄, 0.1% dextrose, and 0.4% BSA. PBS consisted of 140 mM NaCl, 10 mM sodium phosphate, pH 7.4. Krebs's buffer consisted of 140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 12.5 mM NaHCO₃, and 11 mM D-glucose.

Microcarrier endothelial cell culture

Endothelial cells were isolated from bovine aorta by established techniques (32) and cultured on a microcarrier system of negatively charged spherical plastic beads (ECBs), according to the method of Davies and colleagues (33).

EDRF Generation from cultured cells

In this method, ECB are stimulated by high shear force to secrete EDRF. The details of this system have been published previously (34). In all experiments, prostanoïd synthesis was inhibited with acetylsalicylic acid according to established protocol (34, 35). For the purposes of this study, ECB were placed in chambers containing a total volume of 1 ml PBS (pH 7.4) at a density of 5×10^3 cells/ μ l, and exposed to a shear stress of 0.43 dyn/cm² for 15 min, as we have previously described (34).

Endothelial cell viability

Assessments of cell growth and degree of endothelial cell confluence on microcarrier beads were routinely studied by phase-contrast microscopy. During the course of experiments, 25- μ l aliquots of incubation medium were removed at hourly intervals for lactate dehydrogenase measurements, determined spectroscopically (with use of an LD-1 assay kit, Sigma Chemical Co.). At similar intervals, cell viability was assessed by vital dye exclusion with trypan blue (36).

Platelets

Venous blood was obtained from volunteers who had not consumed nonsteroidal antiinflammatory drugs for at least 10 d, and was anticoag-

ulated with 3.4 mM trisodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation at 150 *g* for 10 min at 25°C, and platelet-poor plasma was prepared by centrifugation at 800 *g* for 10 min. Gel-filtered platelets (GFP) were obtained by passing PRP over a Sepharose-2B column in Tyrode's-Hepes buffer, as previously described (37). Platelet counts were measured using a Coulter counter (model ZN; Coulter Electronics, Hialeah, FL) and were adjusted to 150,000/ μ l by the addition of platelet-poor plasma or Hepes-buffered saline.

Platelet aggregation

Aggregation of PRP and GFP was monitored using a standard nephelometric technique (38) in which 0.3 (GFP) or 0.5 (PRP) ml aliquots of platelets were incubated at 37°C and stirred at 1,000 rpm in a PAP-4 aggregometer (Biodata Corp., Hatboro, PA). Aggregations were induced by the addition of varying concentrations of ADP (0.75, 5, or 15 μ M), thrombin (0.025 U/ml), and collagen (0.016, or 0.16 mg/ml), and changes in light transmittance recorded. In typical experiments, platelets were incubated with HCYSH and its derivatives for 3 min at 37°C before the addition of agonist. In selected experiments, however, preincubations lasted for as long as 1 h. Our methods for examining the effects of EDRF generated from ECBs on platelet aggregation have been published elsewhere in detail (35). Notably, in these experiments, ECBs were preincubated in the presence and absence of HCYSH (5 mM) in DMEM for 6 h, during which time EDRF-mediated inhibition of platelet aggregation was assayed at hourly intervals. ECBs were washed with PBS to remove HCYSH before their incubation with platelets, and EDRF release stimulated for 3 min by the effects of continuous stirring (flow) in the aggregometer (35). Aggregations were then induced with 5 μ M ADP. In all cases, aggregation was quantitated by measuring either the maximal rate or extent of change in light transmittance.

Vessel bioassay

The details of this standard bioassay have been reported previously (39). Briefly, the descending thoracic aortae of New Zealand white female rabbits weighing 3–4 kg were isolated and cleaned of adherent connective tissue. In certain experiments, the endothelium was removed by gently rubbing with a cotton-tipped applicator inserted into the lumen. The rings were mounted on stirrups, suspended in oxygenated (95% O₂/5% CO₂) glass chambers containing 7 ml Krebs's buffer at 37°C, and connected to force transducers (model FTO3C, Grass Instruments, Quincy, MA) which recorded changes in isometric tension. Sustained contractions were induced with 1 μ M epinephrine after which the direct effects of HCYSH and SNOHO were tested. Concentrations of thiol were confirmed with Ellman's reagent (40). In some cases, methylene blue (100 μ M) was preincubated with vessel rings for 30 min before initiation of contraction.

Hydrogen peroxide generation

Scopoletin serves as a hydrogen donor in the catalyzed reduction of H₂O₂ by horseradish peroxidase. In this oxidative reaction, scopoletin fluorescence decreases in direct proportion to H₂O₂ concentration in the medium (41). The following assay conditions, designed to detect H₂O₂ production from HCYSH, have been published previously (22). Reactions were performed in cuvettes containing 4 μ M scopoletin in 2.5 ml Krebs's buffer, and were initiated by the addition of 2.2 μ M horseradish peroxidase. Fluorescence measurements were performed using a spectrofluorimeter (Fluorolog 2 model F11; Spex Industries, Inc., Edison, NJ) with sample excitations at 360 nm and emission at 460 nm.

Cyclic nucleotide assays

Measurements of cyclic GMP (cGMP) were performed by radioimmunoassay of 400- μ l aliquots of PRP and processed as described previously (31, 35). Incubations of HCYSH, SNOHO, and other compounds of interest with cells were conducted for 60 s, and reactions were terminated by the addition of 10% TCA which was subsequently removed by ether extraction.

Analytical methods

Saville reaction. The method of Saville was used for detection of *S*-nitrosothiols (42). The method, which assays NO_x free in solution, involves a diazotization reaction with sulfanilamide and subsequent coupling with the chromophore *N*-(1-naphthyl)ethylenediamine. The specificity for RS-NOs derives from assay determinations performed in the presence and absence of HgCl₂, the latter reagent displacing NO equivalents for colorimetric reaction in the formation of RSHg (42).

Ultraviolet/visible spectroscopy. The determination of RS-NO absorption was performed using a Gilford Response UV/VIS spectrophotometer.

Nuclear magnetic resonance spectroscopy. Measurements of RS-NOs were made according to the method of Bonnett and colleagues (43). ¹⁵N-NMR spectra were recorded with a 500 MHz spectrometer (Bruker Instruments, Inc., Billerica, MA). Deuterium lock was effected with [D]₂O and the spectra referenced to a [¹⁵N] natural abundance spectrum of a saturated solution of NaNO₂ at 587 ppm. Spectra were recorded at 50.68 MHz and the nine transients of 16 K data points collected with a 30°-pulse width and a 10-s relaxation delay. Data were multiplied by a 2-Hz exponential line broadening factor before Fourier transformation.

Chemiluminescence. Chemiluminescence analyzers operate on the principal that the reaction of NO with ozone results in the emission of detectable light. We have, recently, adapted a highly sensitive new analyzer (TEA model 543; Thermedics, Inc., Woburn, MA) for the purpose of specific RS-NO detection. The details of this method are published elsewhere (44). Briefly, this instrument first uses ultraviolet (UV) photolysis to cleave homolytically NO from parent *S*-nitroso-compounds (in place of chemical reduction used in standard methodology), after which the released NO is separated from other nonvolatile substances in cold traps and carried by He into the chemiluminescence detector. *S*-nitroso-compounds are readily identified and distinguished from authentic NO by requisite UV photolysis for signal detection. *S*-nitrosothiols are further distinguished from other nitroso-adducts by sample pretreatment with excess HgCl₂ under aerobic conditions. By binding to SH groups selectively, mercuric ion displaces NO equivalents from RS-NOs (42), with the rapid formation of undetectable higher oxides of nitrogen. Accordingly, RS-NOs are identified by: (a) the requirement of UV photolysis for detection of signal, and (b) the successful elimination of signal by sample pretreatment with HgCl₂. The sensitivity of this method for SNOHO detection approaches 0.1 pM and the reproducibility is within 2%.

High performance capillary zone electrophoresis (CZE). This chemical method for separating HCYSH from its various oxidized and *S*-nitrosated derivatives has been detailed in a recent publication (45). Briefly, isotachopheretic analyses were performed on a capillary system (HPE-100; Bio-Rad Laboratories, Richmond, CA) fitted with a silica-coated capillary (20 cm × 25 cm). Electrophoretic separations were detected on-line and recorded with a model 1321 single-pen strip chart recorder (model 1321; Bio-Rad) at a chart speed of 1.0 cm/min with a rise time of 1 s. Samples were diluted in 0.01 N HCl, 0.01 M sodium phosphate (pH 2.3), and injected using Hamilton syringes. Analyses were performed at 11 kV with the polarity of the internal power supply set for the migration of cations towards the detector (+ polarity). Eluted volumes were monitored at 200 nm for optimal sensitivity. Confirmatory evidence for *S*-nitrosothiol detection was obtained at 320 nm.

Synthesis and chemical characterization of SNOHO

The synthesis of SNOHO, to our knowledge, has not been previously described. The compound was prepared by a commonly used method of *S*-nitrosation (31, 46–48) in which HCYSH is treated with an equivalent of acidified NaNO₂ (0.5 N HCl) at 25°C. Under these conditions, the general reaction of thiol with oxide of nitrogen is reported to be complete and stoichiometric (47, 48). For HCYSH, however, its acid-catalyzed conversion to a lactone derivative (49) is a potential competing reaction that requires examination. Characteristic of other *S*-nitro-

sothiols, the above solutions turned red rapidly upon product formation, and displayed distinct absorption maxima at ~ 250 nm, 340 nm, and 550 nm (30, 31, 46–48); Fig. 1 A). The calculated absorptivity of this compound at 547 nm is 16.7 M⁻¹·cm⁻¹, and agrees well with the published values of 16.6 and 16.1 for *S*-nitroso-cysteine and *S*-nitroso-glutathione, respectively (46). Using ¹⁵N-NMR (see above Methods), solutions of HCYSH treated with acidified Na[¹⁵N]O₂ exhibited a chemical shift at 750 ppm relative to an internal [¹⁵N] standard, indicative of S-NO bond formation (31, 43; Fig. 1 B). In the Saville reaction (42), the SH/S-NO stoichiometry was ~ 1.0. CZE analysis of samples revealed a loss of the peak of HCYSH (5.94±0.15 min; Fig. 1 C) with coincident generation of a new peak of slower electrophoretic mobility (7.74±0.18 min; Fig. 1 D). The peak is identified as SNOHO based on its theoretical predicted elution time of 7.2 min determined from its charge-to-mass ratio (45). Importantly, HTL was not present in reaction solutions (elution time of standard, 2.53±0.08 min, Fig. 1 E). Taken together, these chemical data provide evidence for stoichiometric *S*-nitrosation of HCYSH.

In further support of the potential biological relevance of this reaction, SNOHO was synthesized under physiologic conditions. The methods used are reported here; however, other details are discussed within context elsewhere in this paper. In one approach (50), *S*-nitrosation of HCYSH was achieved in saturated solutions of HYSCH (40 mM) containing 0.5 M sodium phosphate (pH 7.4) by brief exposure to authentic NO gas bubbled into solution. In other studies, HCYSH (1 mM) was incubated with endothelial cells stimulated to secrete EDRF in response to high shear stress, as we have previously published (31). These experiments were conducted in 1 ml PBS at an endothelial cell density of 5 × 10³ cells/μl. Cells were exposed to a shear stress of ~ 0.43 dyn/cm² for 15 min (34).

Results

Homocyst(e)ine and platelet aggregation. The effects of HCYSH on platelet aggregation induced by ADP (15 μM) and collagen (0.16 mg/ml) are illustrated in Fig. 2. Experiments were performed in PRP taken from eight different volunteers, and unanimously exhibited dose-dependent inhibition of platelet aggregation with an IC₅₀ of ~ 10 mM. Based on theoretical mechanisms proposed to explain the aggregation of platelets by synthetic thiols (19, 51, 52), specifically, oxy-radical generation and nonspecific surface membrane disulfide reduction, the effects of HCYSH were further examined during incubations for up to 1 h after additional supplementation with Cu²⁺ (1–10 μM) and Fe²⁺ (1–10 μM) to facilitate SH-dependent superoxide and H₂O₂ generation (vide infra). Again, agonist-induced aggregations were inhibited at millimolar concentrations of HCYSH. To preclude possible inactivation of oxy-radicals by plasma reactants, similar experiments were also performed using GFP; and, in view of the potential proaggregatory influence of H₂O₂ on primary wave aggregation (52), the effects of HCYSH were also studied using low doses of agonists (see Methods). In both instances comparable antiplatelet effects of thiol were demonstrated. In addition, spontaneous aggregations were not observed under any of the above conditions (data not shown).

The effects of HTL (free base and hydrochloride) on ADP- and collagen-induced platelet aggregation are also shown in Fig. 2. The compound, in both forms, demonstrates weak antiplatelet activity. In these studies, stock solutions of the HTL free base were dissolved in 1 N HCl, and similar results were demonstrated for the compound dissolved in DMSO (data not shown). Platelet aggregation induced by HTL (free base) in chloroform was shown recently (15) and attributed to the

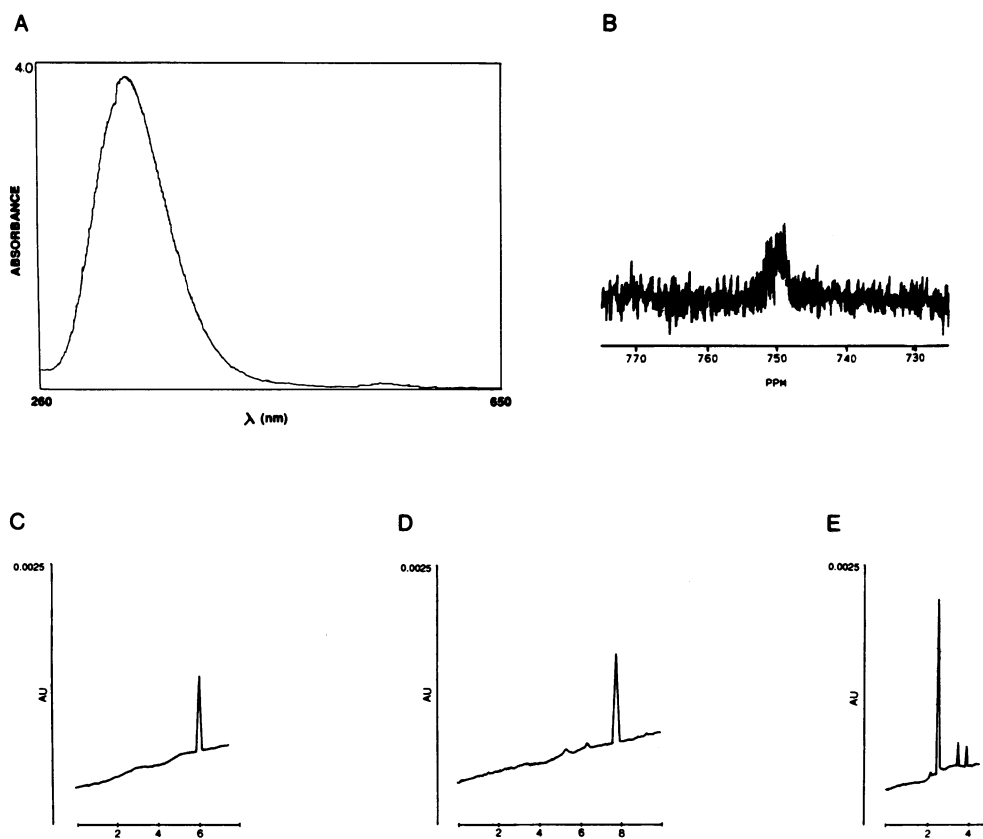


Figure 1. Spectroscopy and chemical characterization of SNOHO. (A) The ultraviolet absorption spectrum of SNOHO ($5 \mu\text{M}$) reveals the typical absorption maximum of an *S*-nitrosothiol at 338 nm, with a secondary peak at 546 nm. (B) The ^{15}N -NMR spectrum demonstrates a chemical shift for SNOHO (referenced to a secondary $\text{Na}[^{15}\text{N}]\text{O}_2$ standard at 749.5 ppm). No other chemical shifts were noted. (C) Isotachopheric analysis (CZE) of HCYSH ($167 \mu\text{M}$), which elutes at 5.94 min. (D) *S*-nitrosation of HCYSH ($167 \mu\text{M}$) was performed as described in Methods followed by isotachopheric analysis (CZE). The electropherogram illustrates essentially stoichiometric conversion of HCYSH (C, 5.94 min) to SNOHO (D, 7.74 min). There is no evidence for formation of HTL, which elutes at 2.5 min ($167 \mu\text{M}$, E) under identical conditions.

membrane solubility of the compound in this solvent. In our hands, however, control incubations with chloroform alone induced platelet aggregation at a vol % half that used by these investigators in the absence of HTL. Moreover, the added presence of HTL did not increase the aggregation response compared to chloroform alone. In other studies, we induced acid-catalyzed conversion of HCYSH to HTL (6 N HCl) (49) and tested the effects of HTL generated in this manner on platelet aggregation. The effects observed were analogous to those shown in Fig. 2. Homocystine and homocysteic acid had no effect on ADP-induced platelet aggregation in PRP at concentrations up to 10 mM, confirming previous reports (13, 15, 16).

SNOHO and platelet aggregation. SNOHO was prepared by treatment of HCYSH with acidified nitrite as described in Methods. The solutions were neutralized by dilution in PBS (pH 7.4) and then incubated in PRP. As depicted in Fig. 3, SNOHO markedly inhibited platelet aggregation. In control experiments using equivalent concentrations of NaNO_2 , no significant inhibition of platelet aggregation was observed (normalized extent of aggregation for NaNO_2 [$100 \mu\text{M}$] = 1.02 ± 0.12 ; $n = 12$).

SNOHO and cyclic GMP. Inasmuch as the antiplatelet effects of EDRF, NO, and other nitroso-compounds are mediated through increases in intracellular cyclic GMP (53), the effects of SNOHO on platelet cyclic GMP were studied. Incu-

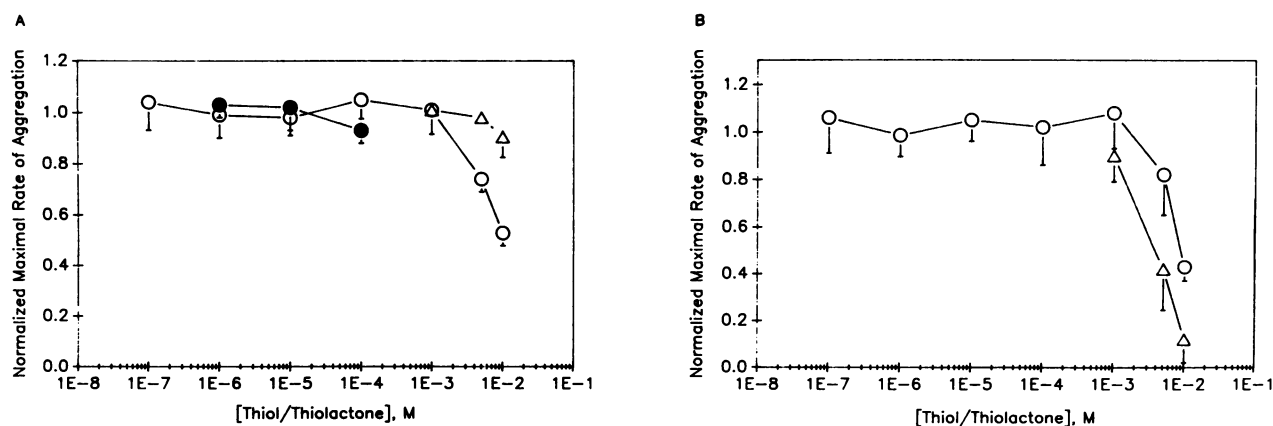


Figure 2. Platelet inhibition of HCYSH and HTL. Dose-effect curves for platelet inhibition by HCYSH (\circ), HTL (free base) (\bullet), and HTL (hydrochloride) (Δ) plotted as normalized maximal rate of aggregation relative to control. Platelet aggregation in PRP was induced with (A) $15 \mu\text{M}$ ADP or (B) 0.16 mg/ml collagen. Data are presented as mean \pm SD. ($n =$ eight different subjects).

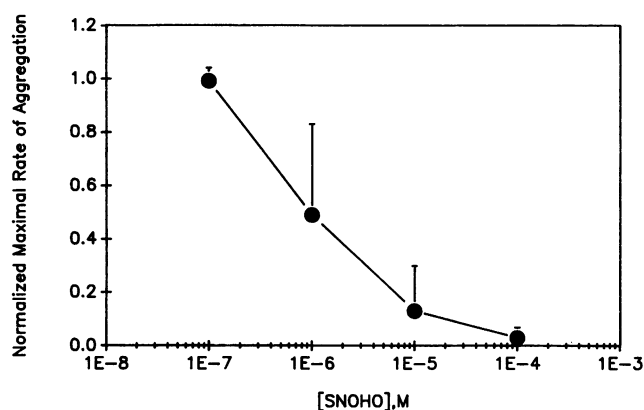


Figure 3. Dose-dependent inhibition of platelet aggregation by SNOHO. Dose-effect curves for platelet inhibition, plotted as normalized maximal rates of platelet aggregation relative to control, were generated in response to platelet aggregation by 0.16 mg/ml collagen. Data are presented as mean \pm SD. ($n =$ six different subjects).

bation of SNOHO (100 μ M) in PRP for 60 s resulted in a 3.6-fold increase in intracellular platelet cyclic GMP above basal levels ($P < 0.05$). Equivalent concentrations of NaNO₂ and HCYSH had no significant effect on platelet cyclic GMP (Table I).

HCYSH: Oxidative metabolism and reactivity towards oxides of nitrogen. Further studies were undertaken to elucidate the reactivity of sulfur in oxidized derivatives of HCYSH toward NO_x. To recall, S-nitrosation of HCYSH in acidified nitrite was verified by several analytical methods (see Methods). Synthesis of SNOHO was also achieved in phosphate buffer (0.5 M, pH 7.4) into which authentic NO gas was bubbled. Solutions turned red upon product formation, and the characteristic spectroscopic absorption maxima for SNOHO (Fig. 1 A) were observed. Using both of these S-nitrosation methods, it was determined that homocysteine does not react to form an S-nitrosothiol. Similarly, as determined by CZE and in complementary Saville assays, the ring sulfur of HTL is not amenable to electrophilic attack by NO_x. Importantly, however, S-nitrosation of HCYSH prevented ring closure (thiolactone formation) in the acid-catalyzed reaction: incubations of HCYSH in acidic milieu (1–12 N HCl) resulted in acid- and time-depen-

Table I. S-NO-Homocysteine and Platelet Cyclic GMP

	Cyclic GMP
	pmol/10 ⁸ platelet
Control	2.14 \pm 0.64
NaNO ₂	2.78 \pm 1.07
HCYSH	2.34 \pm 0.25
SNOHO	7.80 \pm 0.92*

Platelet cyclic GMP production and SNOHO. Exposure of platelets (PRP) to 100 μ M SNOHO for 60 s resulted in significant elevations in intracellular platelet cyclic GMP. Neither HCYSH (unmodified) nor NaNO₂ (used as the source of NO equivalents for synthesis of SNOHO) exhibited any significant effect on platelet cyclic GMP at equivalent (100 μ M) concentrations. Results are expressed as mean \pm SD for four experiments each performed in duplicate.

dent formation of HTL as previously reported (49), which did not occur in the added presence of equimolar NaNO₂. Under these circumstances, SNOHO formed to the exclusion of HTL and subsequent conversion to HTL was not observed over the time course of several hours as determined by CZE and by the Saville reaction. These data indicate that a free sulfhydryl group is a requirement for S-nitrosation of HCYSH, and suggest that SNOHO formation and the oxidative metabolism of HCYSH are reactions that occur exclusively of one another.

EDRF, SNOHO, and platelet inhibition. Effluent from ECB stimulated by high shear force to secrete EDRF in the presence and absence of HCYSH (1 mM) were analyzed by the chemiluminescence and Saville methods for the formation of S-nitrosothiol. Quantitative concordance between the two methods was observed with SNOHO measuring 0.81 \pm 0.24 μ M by chemiluminescence (Fig. 4) and 0.92 \pm 0.43 μ M by Saville (mean \pm SD; $n = 5$); the greater variation among measurements by the method of Saville being accounted for by its lower sensitivity (100 nM). To assay the bioactivity of these samples, 100- μ l aliquots of ECB effluent were incubated with PRP (200 μ l) for 10 min, and aggregations induced with ADP (5 μ M). Platelet inhibition induced by ECB stimulated to secrete EDRF in the presence of HCYSH is illustrated in Fig. 5. The treatment of ECB with the NO synthase inhibitor N^w-nitro-L-arginine (200 μ M) entirely prevented the formation of S-nitrosothiol (i.e., below limits of detection) and attenuated the degree of platelet inhibition by 81 \pm 15% (mean \pm SD; $n = 3$; $P < 0.001$). As further evidence that SNOHO is the active species in these studies, the degree of platelet inhibition observed corresponded with that predicted from the SNOHO dose-response relationship based on the S-NO concentration of the solutions (Fig. 5 B). Moreover, it is notable in the context of an EDRF half-life of 5–30 s (26–29, 31, 34) that the time delay in ECB-effluent transfer to PRP in these studies was \sim 5 min, and that the bioactivity of samples was fully preserved with a 30-min time delay, as well ($n = 2$); in the absence of added HCYSH, effluent from stimulated ECB did not significantly inhibit platelet aggregation ($P =$ NS). These findings are consistent with the stability of SNOHO under physiologic conditions (Fig. 6). Taken together these experiments are interpreted as follows: (a) exposure of HCYSH to endogenous NO_x results in the formation of SNOHO; (b) brief exposure of endothelial cells to HCYSH engenders antiplatelet activity through formation of SNOHO; and (c) HCYSH preserves (stabilizes) NO and EDRF-like bioactivity by forming SNOHO.

EDRF and HCYSH-related platelet aggregation. In the absence of a detectable direct, proaggregatory effect of HCYSH, we tested the hypothesis that HCYSH-mediated endothelial cell dysfunction leads secondarily to platelet activation. For this purpose, ECB were incubated with HCYSH (5 mM, Weymouth's medium) for up to 6 h; HCYSH was excluded from the medium in time-control experiments. At regular intervals, ECB (1 \times 10⁶ cells) were removed, washed in PBS, and added to PRP. EDRF secretion was stimulated by stirring in the aggregometer for 3 min (35), and aggregation induced with ADP (5 μ M). The results depicted in Fig. 7 reveal that with prolonged (4 h) exposure to HCYSH, ECB lose their innate capacity to inhibit platelet aggregation through secretion of EDRF. Measurements of lactate dehydrogenase released into the medium revealed modest (< 20%/6 h) increases over time and no difference between HCYSH-exposed and control ECBs. Similarly, no difference in cell detachment from microcarriers or in

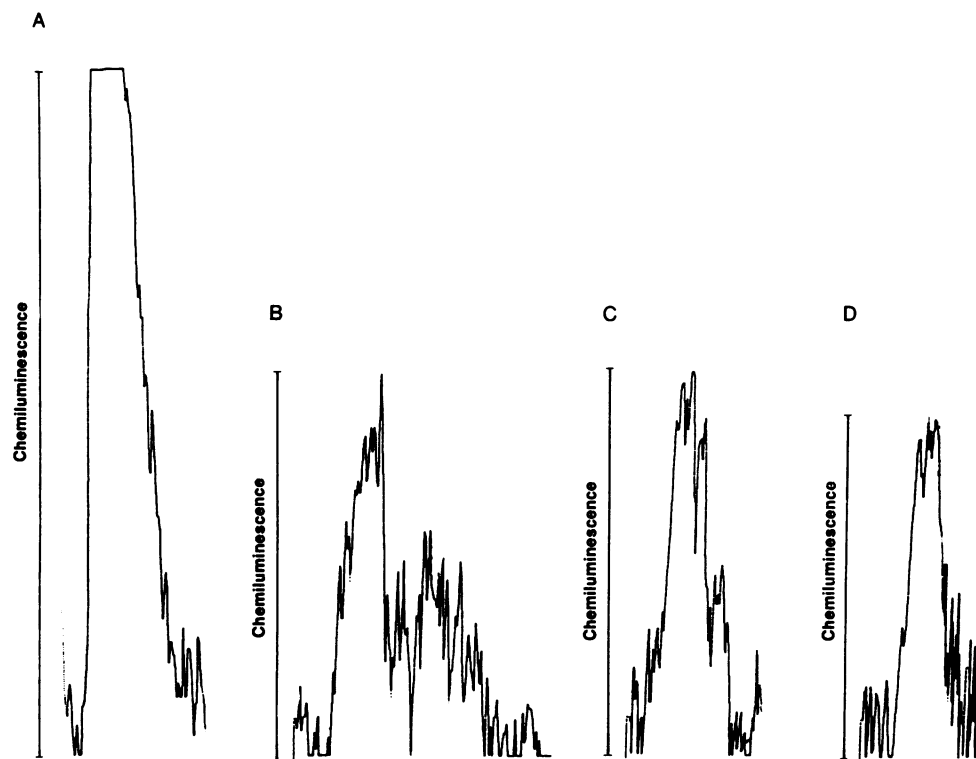


Figure 4. Detection of SNOHO formation from endogenous NO_x . Endothelial cells were stimulated by high shear forces to secrete EDRF for 15 min (as described in Methods) in the presence (*A, B*) or absence (*C, D*) of 1 mM HCYSH. Effluent was then transferred within 10 min for analysis by chemiluminescence. (*B*) and (*D*) are paired samples of (*A*) and (*C*), respectively, after their pretreatment with HgCl_2 , which displaces NO selectively from thiol adducts. HgCl_2 is shown to attenuate significantly the signal from HCYSH-exposed cells (*A* vs. *B*) but has little effect on signal in the absence of HCYSH (*C* vs. *D*). The signal is also greatly reduced (seven-fold) in the absence of added thiol (*A* vs. *C*), which is compatible with the oxidation of free NO to higher oxides which are not detected sensitively by chemiluminescence. Note that the relative gain in *B, C*, and *D* is twice that in *A*. RS-NO-derived NO was only detected after photolytic cleavage of the S-NO bond; signal was not detected when the photolysis lamp was turned off. Quantification of RS-NO in these experiments is provided in the text.

trypan blue exclusion was detectable in the presence of HCYSH under these experimental conditions.

HCYSH, SNOHO, and H_2O_2 generation. There is strong evidence and agreement among studies that HCYSH injures

endothelial cells by generating H_2O_2 , and that its toxicity is prevented by H_2O_2 elimination (21, 22). As part of elegant work supportive of this biochemical mechanism, it was shown that the thiol autooxidizes in a two-electron transfer to O_2 with

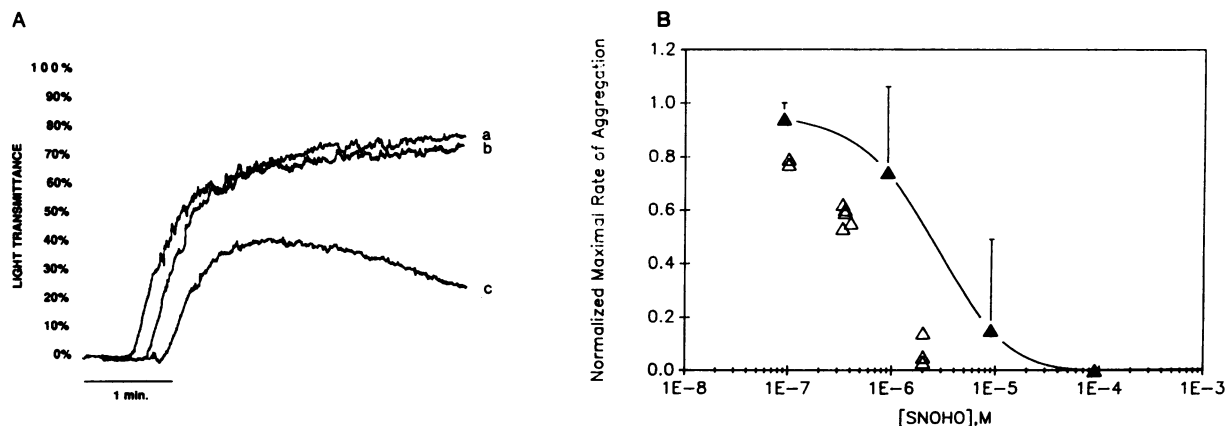


Figure 5. Stabilization of NO bioactivity through formation of SNOHO with HCYSH, and bioactivity of endogenously derived SNOHO in platelet aggregation studies. SNOHO was synthesized by incubation of HCYSH (1 mM) with endothelial cells stimulated to secrete EDRF for 15 min as described in Methods and Fig. 6. Effluent (100 μl) was then transferred to PRP (200 μl) within 5 min and aggregations induced with 5 μM ADP. Concomitantly, chemical quantification of RS-NO in samples was performed. (*A*) demonstrates platelet inhibition by 350 nM RS-NO (*c*) relative to a control aggregation (*a*). HCYSH, exposed to unstimulated endothelial cells for 15 min, had little effect on platelet aggregation (*b*), and RS-NO formation was not detectable chemically. In the absence of added thiol, effluent from cells stimulated to secrete EDRF had no significant effect on platelet aggregation (normalized rate of aggregation = 0.98 compared to control, $n = 2$). (*B*) shows the dose-effect relationship for platelet aggregation by SNOHO synthesized from acidified NaNO_2 and EDRF (\blacktriangle). The open triangles are each representative of single experiments in which SNOHO was synthesized by exposure of HCYSH to endothelial cells as described above. The degree of inhibition by endogenously derived RS-NO is seen to correlate well with that predicted from the dose-response relationship for chemically synthesized SNOHO.

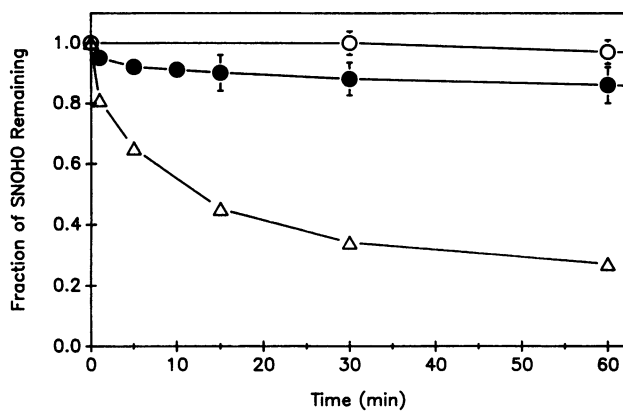


Figure 6. Stability of SNOHO. SNOHO was synthesized with acidified NaNO_2 and then diluted in 10 mM phosphate buffer or plasma. The stability of SNOHO was determined over time at pH 7.4 (●) and pH 2.5 (○) by monitoring the absorbance at 336 nm. In human plasma (△), SNOHO was measured after protein precipitation according to the method of Saville (42).

formation of H_2O_2 (22). We therefore tested the hypothesis that S-nitrosation, by blocking the SH group with NO, would limit H_2O_2 generation. Confirmatory results are shown in Fig. 8. HCYSH induced a rapid loss of scopoletin fluorescence indicative of H_2O_2 generation. In marked contrast, H_2O_2 production from SNOHO is negligible, even in the presence of a metal catalyst. Thus, the cytotoxic mechanism of HCYSH through H_2O_2 production is attenuated by S-nitrosation.

SNOHO and vessel relaxation. The effects of SNOHO were further examined in the vessel bioassay. As illustrated in Fig. 9 and Table II, the compound is an active vasodilator with an IC_{50} of 250 nM. Indicative of the potency of this S-NO adduct, the IC_{50} of S-nitroso-L-cysteine in this system is 4 μM . Consistent with a cyclic GMP-dependent mechanism of action, relax-

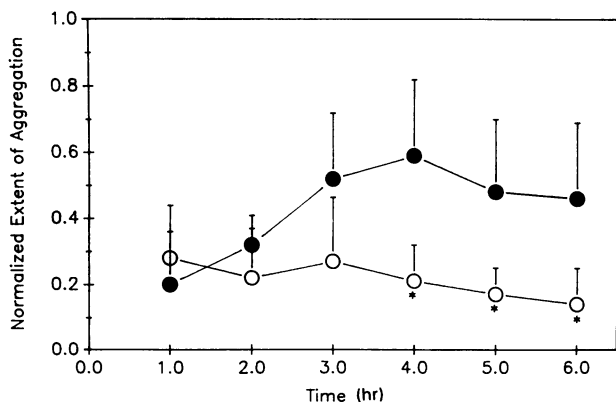


Figure 7. HCYSH-mediated attenuation of platelet inhibition by EDRF. Endothelial cells (ECB) were incubated in the presence (●) or absence (○) of 5 μM HCYSH for up to 6 h in Weymouth's culture medium. At hourly intervals, 8.8×10^5 cells were removed, washed, and incubated with platelets for 3 min during which time EDRF secretion was stimulated by stirring in the aggregometer. Aggregations were then induced with 5 μM ADP, and the normalized extent of aggregation plotted for $n = 3$ experiments as mean \pm SD. These curves are significantly different from one another by ANOVA to $P < 0.01$, and the 4–6-h time points are different from one another to $P < 0.01$ by Newman-Keul's comparison.

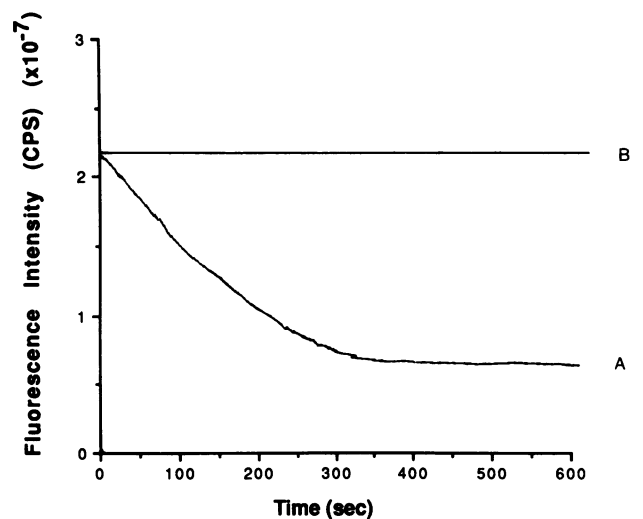


Figure 8. H_2O_2 generation by HCYSH and its prevention by S-nitrosation. The generation of H_2O_2 was detected by extinction of scopoletin fluorescence during its oxidation by horseradish peroxidase over a 10-min period. The maximal rate of fluorescence extinction is proportional to the concentration of H_2O_2 in solution; HCYSH (500 μM)-mediated H_2O_2 generation (A) is prevented by S-nitrosation (B). The initial rates ($\mu\text{M}/\text{min}$) of H_2O_2 generation by HCYSH in the presence and absence of 5 μM Cu^{2+} are 0.16 ± 0.013 and 0.11 ± 0.04 , respectively (mean \pm SD; $n = 3$). The initial rates were 0 $\mu\text{M}/\text{min}$ for SNOHO and were unchanged by the addition of 5 μM Cu^{2+} ($n = 3$).

ations by SNOHO were prevented by the guanylate cyclase inhibitor, methylene blue (data not shown). Thus, these relaxation responses to SNOHO are reminiscent of NO and EDRF (26, 31).

Discussion

Thrombosis is a well recognized complication of hyperhomocysteinemia (1, 2, 5). The thrombogenic mechanism(s) of this disorder, however, are not well understood, and the related literature is often controversial. In particular, reports on the direct effects of HCYSH and its derivatives on platelet aggregation are conflicting and inconclusive. McDonald and colleagues first demonstrated increased platelet "stickiness" in the blood of homocystinurics, and by the addition of homocystine to plasma (13). Changes in platelet adhesiveness, however, have not been confirmed subsequently by other groups (54–56). Several groups have also failed to demonstrate aggregation

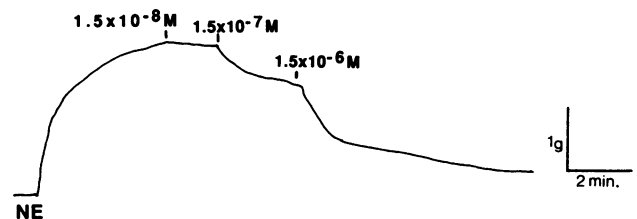


Figure 9. SNOHO-induced vasorelaxation. Deendothelialized rabbit aortae were contracted with epinephrine (1 μM) and relaxations induced with SNOHO in a dose-dependent manner.

Table II. Vasorelaxation by S-NO-Homocysteine

	Percent relaxation		
	NaNO ₂	HCYSH	SNOHO
15 nM	0	0	6±4
150 nM	0	0	33±10
1.5 μM	7±7	0	85±3

S-NO-homocysteine (SNOHO) was synthesized from acidified NaNO₂ and HCYSH at equimolar concentrations as described in Methods. Values are expressed as mean±SD. *n* = 3.

of platelets by homocystine (13, 15, 16). The effects of HCYSH have not been well studied. HCYSH has been reported to increase thromboxane production in platelets (14), and proaggregatory properties of the thiol have been suggested (14, 57). In a contrasting report, however, McCully and Carvalho were unable to demonstrate HCYSH-induced platelet aggregation (15). Notwithstanding the negative findings of the latter report (15), it should be noted that these conclusions were based on studies in only two subjects.

The effects of HTL on platelet function are particularly controversial. Early studies testing the thrombogenic properties of HTL showed that the compound had little effect on platelet aggregation (16). More recently, the free base of HTL was reported to cause platelet aggregation, and its prothrombotic properties clearly distinguished from those of the inactive polar salt (hydrochloride) (15). The authors reasoned that the lipid soluble properties of the free base were responsible for platelet activation. In an entirely different context, HTL has been shown to act synergistically with other methyl transferase inhibitors to inhibit platelet aggregation (58, 59).

In this study, we demonstrate that HCYSH does not cause platelet aggregation directly. Moreover, our findings of dose-dependent inhibition of platelet aggregation by HCYSH are in keeping with the inhibitory properties of other low molecular weight biological thiols, notably cysteine and glutathione (19). The remarkable similarity in the inhibitory potency among these thiols further bespeaks their similar physiochemical properties and a common mechanism of action. Inasmuch as plasma levels of acid-soluble thiol do not achieve concentrations sufficient to inhibit platelet aggregation (58), the biological relevance of this effect is in question. We also demonstrate weak platelet inhibitory properties of HTL. We did not find the platelet bioactivity of the HTL free base to be significantly different from that of the salt. Thus, we attribute the previous findings of HTL (free base)-induced platelet activation (15) to the effects of the chloroform solvent employed. In light of our results, we now conclude that there is internal consistency as well as general agreement among studies: HTL has little effect on platelet aggregation at concentrations less than millimolar (16, 58); at higher concentrations (Fig. 2) or in the presence of a methylation inhibitor with which HTL acts synergistically (59, 60), platelet inhibition is observed. Platelet inhibition by HTL might be explained in part by hydrolytic cleavage of the lactone under physiologic conditions (61) (by our estimate, *t*_{1/2} = 10 min at pH 7.4, data not shown) with generation of inhibitory concentrations of HCYSH. Finally, we confirm the work of many others that homocystine does not affect platelet aggregation (13, 15, 16). Taken together, our data provide no

evidence that the thrombotic tendencies associated with hyperhomocyst(e)inemia derive from a direct effect of HCYSH or its oxidized derivatives on platelets.

Endothelial injury is an alternative explanation for thrombogenicity associated with hyperhomocyst(e)inemia. Several groups have reported that HCYSH and HTL are toxic to endothelial cells in culture (21–23, 62), and by continuous infusion in animal models (11, 24, 25, 63). We defined cytotoxicity (and consequent thrombogenicity) in terms of functional bioassay responses to EDRF, finding an inverse relationship between the capacity to secrete EDRF and a thrombogenic mechanism through platelet activation. Specifically, during prolonged exposure to HCYSH, we observed progressive impairment in the capacity of the endothelium to inhibit maximally platelet aggregation. Thus, we have shown for the first time a causal relationship between HCYSH-mediated endothelial dysfunction and a predisposition to platelet activation. At the same time, our data are in keeping with previous observations that the thrombotic tendencies in hyperhomocysteinemia do not necessarily impart a supranormal aggregation response (11, 12).

The interaction between EDRF (or a derivative thereof) and HCYSH was further explored in detail. We (34, 35) and, more recently, others (64) have reported that biological thiols potentiate the bioactivity of EDRF. There is ample evidence in this regard that thiols react with NO_x to form S-nitrosothiols (28, 29, 46, 53). In support of biological relevance for SNOHO, we have now demonstrated HCYSH-dependent S-nitrosothiol formation from endogenously derived NO_x. Moreover, this S-NO adduct possesses potent antiplatelet and vasodilatory properties, mediated through cyclic GMP reminiscent of EDRF. The unusual stability of SNOHO under physiologic conditions vis a vis the half-life of NO is a noteworthy distinguishing characteristic of this compound and suggests a mechanism by which the endogenous bioactivity of NO is preserved. Thus, we propose that the endothelium can counteract the thrombogenic mechanism(s) of HCYSH through secretion of EDRF, and that the formation of SNOHO is fundamental to this counterregulatory pathway. These data are also supportive of the hypothesis that the endogenous formation of SNOHO may play an integral role in the hemostatic process through platelet inhibition and vasorelaxation.

HCYSH-induced endothelial injury in cell culture derives in large part (if not entirely) from H₂O₂ generated by way of the SH group (21, 22). Inasmuch as biochemical measurements of H₂O₂ generation correlate directly with the cytotoxicity of HCYSH in these studies (22), it is noteworthy that S-nitrosation of HCYSH effectively inhibits sulfhydryl-dependent H₂O₂ generation. Thus, these observations, taken together with our findings of SNOHO formation in the presence of EDRF, suggest a novel mechanism by which the endothelium can modify the toxicity of sulfur-containing amino acids. In this context, the atherogenicity of HCYSH imparted by its oxidative metabolism to a thiolactone (15, 24, 25) may be accounted for, at least in part, by the inaccessibility of lactone sulfur (in an ester linkage) to electrophilic attack by NO_x. Additional experiments are in order to determine the regulation of the apparently competing pathways of S-nitrosothiol synthesis and HCYSH oxidation to HTL.

Our findings introduce several theoretical issues worthy of consideration. The concept that NO may modulate the pathogenicity of HCYSH raises the possibility that the toxic manifes-

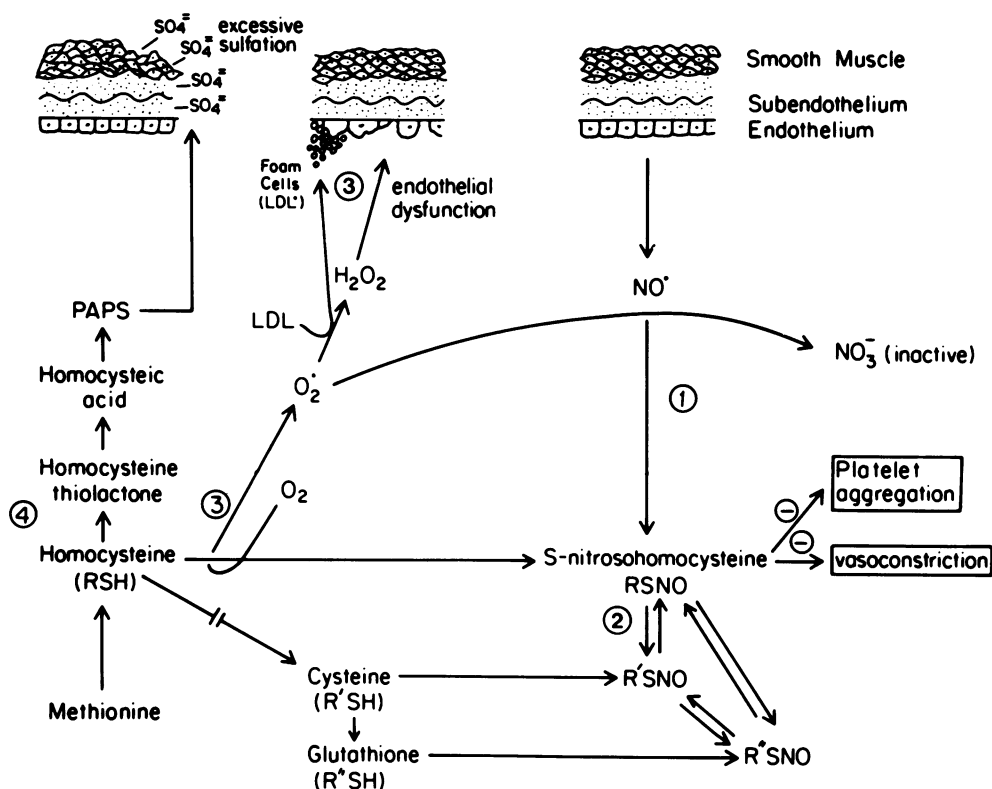


Figure 10. Proposed mechanism of HCYSH-mediated atherothrombosis and its modulation by NO. (1) HCYSH reacts with NO equivalents derived from endothelium to form a vasodilatory, antiplatelet S-nitrosothiol adduct. (2) SNOHO is one of several potential biological RS-NOs which may react through thiol-nitrosothiol exchange to serve as a pool of molecules active for relaxation and platelet inhibition in plasma and the cell cytosol. (3) In disease states in which HCYSH levels are elevated or NO release is compromised, HCYSH (free SH) may damage endothelium through generation of H_2O_2 , thereby embarrassing NO production; by a similar oxy-radical-dependent mechanism, HCYSH may facilitate oxidation of LDL and its recognition by the scavenger receptor. (4) With a relative NO deficit, HCYSH is shunted toward oxidative metabolism to HTL; this pathway leads to further vessel wall damage at least,

in part, through excessive sulfation of connective tissue (9, 25). In this paradigm, HCYSH plays an indirect causal role in platelet activation and vasoconstriction by disarming the endogenous counterregulatory mechanisms of EDRF generation and RS-NO formation. (// = classic enzymatic block in homocystinuria [cystathionine beta-synthetase deficiency]).

tations of HCYSH reflect an imbalance between NO availability and HCYSH levels. Accordingly, an NO-free thiol index may provide a more accurate measure of the pathogenic potential of HCYSH than the absolute level of "thiol excess." Defined as such, hyperhomocysteinemia may indicate a relative deficiency of NO (as a result of endothelial injury). The mechanism of hyperhomocysteinemia notwithstanding, the resultant endothelial damage would predictably embarrass NO production and, consequently, set a cycle in motion in which the anti-thrombotic cytoprotective mechanisms of S-nitrosation are increasingly compromised at the expense of a predisposition to atherosclerosis and thrombosis (Fig. 10). We also entertain the possibility that the cytoprotective mechanism of S-nitrosation may play a more general role in modulating the atherogenicity of other sulfur-containing amino acids. Cysteine, for example, is highly toxic to cells in culture (65), also supports the generation of reactive oxygen species (66, 67), and has been strongly implicated in atherogenesis through modification of low density lipoproteins to a form recognized by the scavenger receptor (66, 67). The little appreciated toxicity of cysteine is especially pertinent in this context in light of recent attention paid to its S-nitrosated derivative, S-nitroso-L-cysteine. The vasodilatory and antiplatelet properties of this molecule have been important considerations in the claims for its identity as EDRF (28). Thus, S-nitrosation may represent one such cytoprotective cell regulatory mechanism that simultaneously confers upon biological thiols EDRF-like bioactivity.

In summary, the data presented show the following: (a) HCYSH and HTL are, in fact, weak platelet inhibitors and do

not directly promote platelet aggregation; (b) HCYSH-mediated endothelial injury, with consequent attenuation of EDRF/NO_x production, attenuates endothelial-mediated platelet inhibition and predisposes to platelet activation; (c) HCYSH reacts with NO_x under physiologic conditions to form a stable S-nitrosothiol (SNOHO); (d) with oxidative metabolism of HCYSH, the potential for SNOHO formation is lost; (e) the mechanism of HCYSH-mediated endothelial injury through generation of reactive oxygen species is not supported by SNOHO; and (f) SNOHO possesses intrinsic EDRF-like antiplatelet and vasodilatory properties mediated through cyclic GMP.

These data provide new insight into the mechanism of HCYSH-induced atherosclerosis and thrombosis, and highlight the larger potential of the S-NO functional group in regulation of cellular thiol biochemistry and metabolism. In proposing that NO availability may be of central importance to the thrombogenic and cytotoxic potential of HCYSH, our findings may have important pharmacological implications, as well. Denitrication of organic nitrates, for example, is facilitated by thiols, including HCYSH, and S-nitrosothiols are proposed reaction intermediates (51, 68). Thus, the provision of NO equivalents may be of therapeutic value in disease states in which HCYSH plays a pathogenic role.

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