Role of homocysteinylation of ACE in endothelial dysfunction of arteries

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NUMEROUS CLINICAL STUDIES have demonstrated that homocysteine (Hcy), a nonproteinogenic amino acid derived from methionine (Met), is a strong predictor and independent risk factor for arterial ischemic events, such as coronary, cerebral, and peripheral vascular diseases (39, 40). In addition, epidemiological studies reported the association of elevated Hcy with vasculopathies, such as metabolic syndrome and diabetes (13, 18), Alzheimer and other cognitive dysfunctions (44), and osteoporosis-induced bone fracture (52). All of these conditions are thought to evolve as a result of systemic accumulation of Hcy (hyperhomocysteinemia, HHcy), defined as a plasma value greater than 15 \( \mu \)mol/L. HHcy has resulted from genetic mutations in protein function and/or aggregation of protein (21, 45). In all cases, homocysteinylation modified their capacity to regulate metabolic pathways. In a more wide-ranging consequence of posttranslational modifications of proteins, homocysteinylation of nascent protein can stall or hinder proper folding of proteins and can concomitantly activate the “unfolded protein response” (UPR) within the endoplasmic reticulum (14). Initiation of the UPR can adversely affect emerging proteins in endothelial cells (37), by extending or prolonging required activation of secreted proteins and subsequently evoking Hcy-induced oxidative stress.

The oxidative stress hypothesis seems to be the most widely accepted explanation for the deleterious effects of Hcy. Not only does Hcy itself have prooxidant properties, but also accumulating evidence from clinical and basic science studies indicate that the primary pathogenesis of diseases associated with HHcy is a concomitant generation of superoxide anion, which inactivates NO (7, 27, 28, 51, 56, 58). To date, several studies have suggested the existence of an interaction between Hcy and angiotensin II (ANG II) signaling as a pathway that contributes significantly to the potential oxidative stress in the vasculature (2, 31). Some studies have addressed the possibility of ANG II signaling-related proteins, such as angiotensin-converting enzyme (ACE), serving as a target of Hcy (4, 30, 34). However, these studies reported general correlations between HHcy and ANG II/ACE signaling in different disease models. The specific mechanism(s) responsible for the in situ interaction of vascular Hcy with ACE, followed by modifying ACE activity, still remain unknown.

To this end, we hypothesize that J) vascular synthesized/derived Hcy is primarily responsible for vascular/endothelial...
dysfunction; 2) vascular Hcy potentiates ACE activity via in situ homocysteinlation of the enzyme; and 3) increased ACE activity promotes ANG II-NADPH oxidase-dependent increase in oxidative stress to impair endothelial function.

METHODS

Animals and vessels. Twelve-week-old male Wistar rats were anesthetized with 60 mg/kg pentobarbital sodium. Plasma was collected, and the heart and mesentery were excised for the isolation of coronary and mesenteric arteries. All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conform to the guidelines of the National Institutes of Health and the American Physiological Society for the use and care of laboratory animals.

Vessel incubation. Isolated mesenteric arteries (~280 μm in diameter and ~10 mm in length) were cannulated in perfusion chambers. All side branches were carefully ligated. Intravascular pressure was maintained constant at 60 mmHg by an inflow pressure reservoir. Intraluminal flow was maintained at 100 μl/min by an outflow resistor. The perfusion solution in a total volume of 10 ml was recirculated from the perfusion chamber to pressure reservoir by a peristaltic pump. Isolated coronary arteries (~120 μm in diameter and ~0.5 mm in length) were cannulated in perfusion chambers (2 ml).

The intravascular pressure was maintained at 40 mmHg with an outflow hydrocylinder. An inflow syringe pump provided a 7 μl/min intraluminal flow, which generated ~5 dyne/cm² shear stress to the vessels. All perfusion chambers were autoclaved and the isolation and cannulation procedures were performed under sterile condition. Isolated arteries were perfused for up to 3 days with medium 199 (M199) containing 15 mmol/l HEPES (pH 7.4), without phenol red and serum, plus 1% antibiotics and 1% BSA. Perfusion solution was changed every 24 h. The chamber compartment was fully moisturized and gassed with 5% CO₂ plus air. Temperature of the chamber compartment was maintained constant at 37°C with a water jacket.

Extraction of endothelial and smooth muscle lysates from isolated arteries. A detailed description of the extraction procedure has been reported (47). Briefly, isolated mesenteric arteries were cannulated on two glass pipettes in a perfusion chamber. A volume of 30 μl detergent-containing lysis buffer was continually perfused through the in-flow pipette into the vessel within 2 min, and endothelial (EC) lysates were collected from the outflow end. Endothelial cell lysates from 4–6 vessels from each rat were pooled as one sample. Proteins in lysates were extracted and determined by Bio-Rad protein assay. Following EC lysate collections, remaining parts of the vessels were pulverized in liquid N₂ and used for determinations of smooth muscle (SMC) proteins.

Detection of plasma and vascular Hcy by high-performance liquid chromatography (HPLC). Plasma (10 μl) and proteins extracted from vascular EC and SMC lysates, or from intact single mesenteric arteries (10 μg), were assayed for Hcy levels. Sample mixtures contained tris(2-carboxyethyl)phosphine (TCEP, 5 mmol/l), a disulfide bond reducing agent, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (0.1 mg/ml, a fluorescent labeling reagent of thiols), and 1 mmol/l EDTA in 100 mM borate buffer (pH 9.5) (total reaction volume, 40 μl). Following a 20-min incubation at 60°C, samples were chilled in ice and centrifuged (14,000 g) for 10 min at 4°C. Supernatant fractions (20 μl) were analyzed for Hcy concentrations using HPLC (PU-2080 Plus, Jasco) coupled with a C-18 column (Beckman Ultrasphere ODS, 5 μm, 4.6 × 250 mm) and a fluorescence detector (FP2020 Plus, Jasco). Samples were eluted with a running buffer composed of 0.6% methanol and 0.1 M acetic acid-acetate (pH 5) at a flow rate of 1 ml/min for 10 min. Fluorescent signals were detected at 385/515 nm (excitation/emission wavelengths, respectively). Deproteinization was performed by centrifuging samples at 14,000 g for 10 min with Microcon 3K molecular weight cutoff filters (YM-3, Millipore). Protein-bound Hcy was determined by measuring the difference between the total and unbound Hcy (Hcy in deproteinized samples). Total Hcy is defined as the levels of Hcy (reduced form) and homocysteine (Hcy-S-S-Hcy; oxidized form). Unbound Hcy was obtained by subtracting TCEP from deproteinized samples. Unbound Hcy-S-S-Hcy was calculated by subtracting the unbound Hcy from total unbound Hcy. Cysteamine was used as an internal standard.

Accordingly, cysteamine (10 μmol) was added to each sample as well as to Hcy standards prior to incubation. Hcy formation in arterial vessels was induced by preincubation of vessels with Met, the amino acid precursor to Hcy. Specifically, isolated arteries were incubated with either MOPS-buffered physiological salt solution (MOPS-PSS; without Met), M199 (containing 0.1 mmol/l Met), or M199 plus an additional amount of Met (0.3 mmol/l in total Met). After incubation, EC and SMC lysates or intact vessels were analyzed for Hcy. Final content of Hcy in the endothelium or in SMC and that within intact vessels were normalized to total protein and the internal surface area of vessels, respectively.

Shear stress-induced vasodilation (SSID). Isolated coronary arteries were incubated for 3 days in control media (M199) and in the presence of Met (0.3 mmol/l), or Met plus losartan (10 μmol/l, a blocker of ANG II type 1 receptor). After incubation, M199 was replaced with MOPS-PSS; and Met and losartan were correspondingly provided. After vessels developed spontaneous tone (~65% of their passive diameter) in response to 60 mmHg of intravascular pressure under a no-flow condition, initial values of shear stresses (5, 10, 15 and 20 dyn/cm²) were applied to vessels, and changes in vessel diameter were recorded. Wall shear stress was increased by increasing perfusate flow via a syringe pump. Flow rates were calculated based on basal diameter of vessels recorded before the onset of flow and the sequential diameter after each selected value of shear stress. Shear stress was calculated using the modified Hagen-Poiseuille equation, \( \tau = 4\eta Q/r^2 \), where \( \eta \) is the perfusate viscosity (0.007 poise at 37°C), \( Q \) is the perfusate flow (ml/s), and \( r \) is the vessel radius in centimeters.

At the conclusion of experiments, vessels were incubated in a calcium-free MOPS-PSS for 10 min. Passive diameters (PD) of arteries at 60 mmHg were obtained. Changes in diameter of vessels were normalized to their passive diameter and expressed as %PD.

Detection of peroxynitrite. Nitrite concentrations were determined as previously described (17). Briefly, cannulated mesenteric arteries were incubated in control buffer and in the presence of supplemental Met (0.3 mmol/l) for 3 days. A 100 μl/min flow was applied to the vessels, which generated ~5 dyne/cm² shear stress in vessels with a diameter of ~280 μm. Perfusion sample was collected every 24 h and centrifuged to remove protein using molecular weight cutoff filters. Nitrite formation in the perfusate was assessed by using 2,3-diaminonaphthalene (DAN) and an HPLC/fluorescence detector-based assay to detect 1-(H)-naphtho-triazole, a fluorescent product formed by interaction of nitrite and DAN under acidic conditions. Fluorescent signals were detected at 375/415 nm. Standard curves of sodium nitrite (0–640 μmol/l) were generated using M199 as the solvent vehicle. Nitrite formation in vessels was expressed as picomoles per millimeter squared of the internal surface of vessels per 24 h.

Detection of vascular superoxide. Superoxide production in mesenteric arteries was assessed by using dihydroethidium (DHE) and an HPLC/fluorescence detector-based assay to detect 2-hydroxyethidium (2-EOH), a superoxide-induced oxidative product of DHE (15, 55). Isolated mesenteric arteries were incubated in control media and in the presence of 0.3 and 1 mmol/l Met, and Met (1 mmol/l) plus enalapril (100 μmol/l, ACE inhibitor), losartan (10 μM), or 3-benzyl-7-[2-benzoxazolyl]-thio-1,2,3-triazolo(4,5-d)-pyrimidine (VAS2870, 5 μM, a selective inhibitor of NADPH oxidase; Enzio Life Sciences) for 24 h, respectively. VAS2870 has been used in cultured endothelial cells and isolated vessels and has a high specificity as an inhibitor of vascular NADPH oxidase (16, 17, 53). After the incubation, vessels were cut longitudinally, removed from the perfusion chamber, and...
treated with DHE (10 μmol/l) at 37°C for 1 h. Vessels were then washed to remove extra DHE, pulverized in liquid N2, and homogenized in 100 μl acetonitrile/water (1:1). After centrifugation (14,000 g for 10 min), supernatant fractions were collected and saved for detection of 2-EOH by HPLC analysis. Fluorescent signals were detected at 480/580 nm, excitation/emission, respectively. Centrifuged pellets were dissolved in 1 N NaOH for protein quantification. A standard curve of 2-EOH (0.3–10 picomol) was generated and used to standardize superoxide generation of vessels. Values were expressed as picromoles per microgram protein in response to 1-h incubation with 10 μmol/l DHE.

Native gel electrophoresis of ACE. A search of the protein sequence of rabbit ACE revealed a total of 15 cysteine residues which would make available at least one free cysteine residue for a disulfide linkage with Hcy. Thus the presence of Hcy-ACE was assessed by protein native gel electrophoresis. Purified rabbit lung ACE (100 ng) was incubated with Hcy (0.1 mmol/l) in phosphate-buffered saline (PBS, 10 mmol/l and pH 7.4) at 37°C for 1 h, and then mixed with 2X native sample buffer. A final volume of 10 μl mixture was carefully placed on 7.5% native polyacrylamide gel without stacking gel. Tris-glycine running buffer (pH 8.4) was used for the electrophoresis. The gel was run at 150 V at 4°C for 5 h. Protein bands were visualized by silver staining of gels. No SDS or reducing agents (dithiothreitol) were used in either the sample preparation or during gel electrophoresis.

Dot-blot assay of ACE homocysteinilation. EZ-Link maleimide-PEG2-biotin (Thermo Scientific) is a sulfhydryl-reactive biotinylation reagent. Maleimide reacts specifically with free sulfhydryl moieties to form stable thioether bonds (46). In a similar fashion, EZ-Link NHS-biotin (Thermo Scientific) reacts with primary amino moieties (-NH2) to form stable amide bonds. Accordingly, anti-ACE polyclonal antibody (5 μl, 1 μg/ml, Santa Cruz) was placed in the center of wells of high binding 96-well microplates (black/clear bottom, 360 μl, Costar) and incubated at 37°C for 1 h, followed by blocking with 1% BSA. Purified rabbit lung ACE (1 picomol) was incubated with maleimide-PEG2-biotin or NHS-biotin (20 picomol) in PBS at 37°C for 60 min. Extra biotin was removed from ACE-biotin mixture by 3-kDa protein cutoff filters. The biotinylated ACE reconstituted in 100 μl PBS was added into ACE antibody-coated wells. The immunoreaction between biotinylated ACE and immobilized ACE antibody was detected by horseradish peroxidase-linked avidin (NeutrAvidin-HRP, Thermo Scientific) and exposed on X-ray film with enhanced chemiluminescence (ECL). In separate experiments, TCEP was used to reduce disulfide bonds of ACE. Specifically, ACE was incubated with 5 mmol/l TCEP at 37°C for 30 min. TCEP was removed by cutoff filters, followed by immediately performing the ACE and maleimide-PEG2-biotin reactions as described above. In other experiments, Hcy and HCTL were incubated with ACE to compete with ACE binding with maleimide-PEG2-biotin or NHS-biotin. Either Hcy or HCTL at final concentrations of 0.1 and 1 mmol/l were incubated with ACE and maleimide-PEG2-biotin, or ACE and NHS-biotin for the same period of time and then dot-blot assays were performed.

Detection of ACE activity. ACE activity was measured spectrophotometrically by detecting hippuric acid generated from enzymatic reaction of hippuryl-L-histidyl-L-leucine (HHL) and ACE (6). Purified rabbit lung ACE (0.5 mU) or 10 μg protein from mesenteric or coronary arteries were treated with control media or with 0.5 mmol/l Hcy-S-S-Hcy or HCTL at 37°C for 1 h. The reaction was initiated by addition of HHL. Each assay mixture (25 μl) contained 5 mmol/l HHL in 50 mmol/l HEPES buffer with 300 mmol/l NaCl at pH 8.3. The mixture was incubated for 15 min at 37°C. The reaction was stopped by adding 25 μl 1 N HCl. Hippuric acid hydrolyzed from HHL was extracted into ethyl acetate. Half volume of ethyl acetate was transferred to a new tube and evaporated to dryness on a heating block. Hippuric acid was reconstituted in water and measured spectrophotometrically at 228 nm. Background control was set in the absence of the enzyme. For the calculation, hippuric acid has an extraction efficiency in ethyl acetate of 91% and a molar absorbitivity of ε228 = 9.8 M-1·cm-1. ACE activity was expressed as nanomoles of hippuric acid generated per minute per milligram protein.

Statistical analysis. Data are expressed as means ± SE. N refers the number of rats. Statistical analysis was performed using repeated-measures ANOVA followed by the Tukey-Kramer post hoc test and Student’s t-test. Statistical significance was accepted at a level of P < 0.05.

RESULTS

Plasma and vascular Hcy. To provide evidence for the presence of local Hcy generation in blood vessels, EC and SMC protein were extracted from isolated and cannulated mesenteric arteries, and Hcy was measured by HPLC-fluorescent detection. Figure 1A shows the chromatographic separation of cysteine, Hcy, and glutathione using cysteamine as an internal control; summarized data of Hcy in vascular EC and SMC are shown in Fig. 1B and reveal a higher total Hcy level in EC than in SMC. Analysis of protein-bound and free forms of Hcy in vascular endothelium and plasma of rats revealed a higher percentage of Hcy-S-S-Hcy (oxidized form) in the endothelium (43.9% of total Hcy) than in plasma (26.5%). The protein-bound and reduced forms of Hcy were 50.5% and 5.5% vs. 71.0% and 2.5% in the endothelium and plasma, respectively. To confirm that the Hcy detected from freshly isolated vascular EC and SMC (Fig. 1) was synthesized strictly by the vessels, and not taken up from plasma, vascular Hcy production was manipulated in separate experiments by incubation of isolated vessels with MOPS-PSS without methionine (Met) or M199 (with 0.1 mmol/l Met) alone, or M199 plus additional (0.2 mmol/l) Met for 2, 4, and 24 h, respectively, followed by determination of total Hcy in endothelial cell lysates. As shown in Fig. 2, when isolated arteries were exposed to Met-free MOPS-PSS, a time-dependent decline in endothelial Hcy lev-
eties from normal rats were isolated and exposed to control (SSID) of arteries. Accordingly, mesenteric and coronary arteries released of endothelial NO, and shear stress-induced dilation relationship existed among the production of vascular Hcy, the mediator of endothelial dysfunction, we examined whether a endogenously synthesized Hcy plays a primary role as hypothesis of Hcy by the endothelium.

A significant reduction of endothelial Hcy, as a function of incubation period (n = 5). The level of endothelial Hcy was maintained in M199-incubated vessels and increased when additional Met was administered (n = 8). A significant difference from freshly isolated vessels (time 0). A significant difference from all others.

**Vascular Hcy and endothelial dysfunction.** To determine whether endogenously synthesized Hcy plays a primary role as a mediator of endothelial dysfunction, we examined whether a relationship existed among the production of vascular Hcy, the release of endothelial NO, and shear stress-induced dilation (SSID) of arteries. Accordingly, mesenteric and coronary arteries from normal rats were isolated and exposed to control media as well as media containing Met or Met plus losartan for 3 days. As shown in Fig. 3, Met-loading significantly increased Hcy production (Fig. 3A), which was associated with a concomitant time-dependent reduction in endothelial release of NO (Fig. 3B) and an impairment of SSID (Fig. 3C). This latter parameter was prevented when Met-treated vessels were incubated with losartan. Conversely, vessels exposed to Met for 3 days exhibited dilator responses to sodium nitroprusside (SNP; an endothelium-independent dilator agent) comparable to those observed in control vessels, implying normal smooth muscle reactivity (Fig. 3D). These results indicate that endogenously produced Hcy does not significantly impair vascular smooth muscle function, but rather induces endothelial dysfunction, mediated by ANG II/AT1R signaling.

**Target of ACE by Hcy.** We determined whether the deleterious effects of vascular (EC/SMC)-derived Hcy was associated with enhanced ACE activity caused by homocysteinylation of critical sulfhydryl moieties on the protein. These results are summarized in Fig. 4. Figure 4A shows positive immunodots involving a recombination of maleimide-PEG2-biotin with purified ACE. The specificity of this reaction is supported by negative results shown in *column B* when ACE was eliminated from the reaction. A reduction of disulfide bonds with TCEP on ACE led to a marked increase in the densitometry of dots (Fig. 4B). The increased intensity of these images substantiates the specificity of sulfhydryl-dependent binding between maleimide-PEG2-biotin and cysteine moieties on ACE. Moreover, a dose-dependent reduction of maleimide-PEG2-biotin signal by Hcy (Fig. 4C) suggests a competitive interaction between maleimide-PEG2-biotin and Hcy for available sulfhydryl moieties on ACE. When NHS-biotin was used in the presence of HCTL to characterize the availability of lysyl moieties on ACE for N-homocysteinylation, we found that HCTL competed with the NHS-biotin signal in a dose-dependent fashion (Fig. 4D).

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assess whether the activity of ACE is modified by homocysteine redox. Furthermore when ACE was preincubated with Hcy for 1 h prior to native protein electrophoresis, a drag shift band of homocysteinylated and N-homocysteinylated ACE (Fig. 4E) as well as chronic incubation of vessels with methionine (Fig. 5D). Thus vascular ACE can indeed be targeted and activated in situ by endogenous production of Hcy-S-S-Hcy and/or HCTL within the same vessels. Our results, in their entirety, obtained using purified ACE enzyme and isolated arteries exposed acutely and chronically to Hcy metabolites and precursors provide strong evidence for homocysteinylation-dependent increases in ACE activity within blood vessels.

Vascular Hcy and superoxide. In view of our observations that losartan can block Met-mediated endothelial dysfunction (Fig. 3) and that Hcy-S-S-Hcy/HCTL can covalently bind to and activate vascular ACE (Figs. 4 and 5), we tested whether ACE/ANG II-dependent production of vascular superoxide can also be regulated based on findings illustrated in Figs. 3–5. ACE activity was significantly increased in these vessels (Fig. 5, B and C). These results provide additional evidence for a greater percentage of Hcy-S-S-Hcy being present in the endothelium than in the plasma. Our results also highlight an important role of Hcy-S-S-Hcy relative to that of Hcy in vascular dysfunction. Significant increases in vascular ACE activity can result from both acute exposure (1 h) of vessels to Hcy-S-S-Hcy and HCTL (Fig. 5, B and C) as well as chronic incubation (3 days) of vessels with methionine (Fig. 5D). Thus vascular ACE can indeed be targeted and activated in situ by endogenous production of Hcy-S-S-Hcy and/or HCTL within the same vessels. Our results, in their entirety, obtained using purified ACE enzyme and isolated arteries exposed acutely and chronically to Hcy metabolites and precursors provide strong evidence for homocysteinylation-dependent increases in ACE activity within blood vessels.

Vascular Hcy and increased ACE activity. Consequently, to assess whether the activity of ACE is modified by homocysteinylation, we exposed both purified ACE and isolated arteries to excess Hcy. We identified a positive relationship between the extent of ACE homocysteinylation and ACE activity. These results are summarized in Fig. 5. Figure 5A demonstrates that incubation of rabbit lung ACE with Hcy-S-S-Hcy and HCTL for 1 h significantly increases the activity of the enzyme. This response is consistent with the definition for an altered activity of ACE in response to homocysteinylation. Also noteworthy is the finding that Hcy-S-S-Hcy rather than Hcy elicits significant increases in ACE activity. This is consistent with the ability of Hcy-S-S-Hcy to form mixed disulfides with sulfhydryl moieties of cysteine residues on proteins as shown in the equation below:

$$2 \text{Hcy} + \text{H}_2\text{O}_2 \rightarrow \text{Hcy-S-S-Hcy} + 2\text{H}_2\text{O}$$

Hcy-S-S-Hcy + protein-SH $\rightarrow$ protein-S-S-Hcy + Hcy

Next, proteins extracted from isolated mesenteric and coronary arteries were incubated with Hcy-S-S-Hcy or HCTL for 1 h. ACE activity was significantly increased in these vessels (Fig. 5, B and C). These results provide additional evidence for a greater percentage of Hcy-S-S-Hcy being present in the endothelium than in the plasma. Our results also highlight an important role of Hcy-S-S-Hcy relative to that of Hcy in vascular dysfunction. Significant increases in vascular ACE activity can result from both acute exposure (1 h) of vessels to Hcy-S-S-Hcy and HCTL (Fig. 5, B and C) as well as chronic incubation (3 days) of vessels with methionine (Fig. 5D). Thus vascular ACE can indeed be targeted and activated in situ by endogenous production of Hcy-S-S-Hcy and/or HCTL within the same vessels. Our results, in their entirety, obtained using purified ACE enzyme and isolated arteries exposed acutely and chronically to Hcy metabolites and precursors provide strong evidence for homocysteinylation-dependent increases in ACE activity within blood vessels.

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of superoxide. This response was markedly attenuated when vascular ACE, AT1R, and NADPH oxidase activities were blocked by cotreatment of vessels with enalapril, losartan, and VAS2870, respectively (Fig. 6A). In view of vascular function, Met-loading for 3 days impaired shear stress-induced vasodilation, which was normalized by VAS2870 (Fig. 6B). These findings further suggest that endogenous Hcy-induced endothelial dysfunction is mediated by vascular NADPH oxidase/superoxide. Our data profile a specific pathway responsible for the local Hcy-initiated endothelial dysfunction of blood vessels. Figure 7 is a diagrammatic representation of our findings which shows that endothelium-derived Hcy and its metabolites can covalently bind to cysteiny1 (S-homocysteinylation) and lysyl (N-homocysteinylation) moieties to enhance ACE activity. This posttranslational modification of ACE leads to increased activation of the ANG II/AT1 receptor/NADPH oxidase signal cascade, potentiating oxidative stress and impairing endothelial function.

**DISCUSSION**

Our data demonstrate the following: 1) Increased local/vascular production of Hcy evokes endothelial dysfunction.

This implies that modulating vascular Hcy synthesis may mitigate endothelial dysfunction. In addition, targeting local/vascular production of Hcy should diminish vascular contribution to the development of systemic HHcy. 2) Vascular production of Hcy and covalent attachment of its metabolites Hcy-S-S-Hcy and/or HCTL to ACE enhances ACE activity. Increased ACE activity potentiates ANG II signaling within vascular endothelium, which, in part, provides evidence for an autocrine- or paracrine-based ANG II signaling with the vasculature. This is the first demonstration that covalent targeting of vascular ACE by endogenously-produced Hcy and its metabolites upregulates ACE activity in single arteries. 3) As a function of posttranslational homocysteinylation, ACE/ANG II can mediate NADPH oxidase-dependent synthesis of superoxide that impairs endothelial NO bioavailability. Moreover, the endothelial dysfunction caused by enhanced Hcy production in the vasculature is reversible in response to blunting vascular ANG II signaling. In its entirety, our studies provide a mechanistically based paradigm for the cytotoxic action of Hcy and its metabolites in triggering ANG II signaling coupled with oxidative injury in the vasculature.

**Increased vascular Hcy initiates endothelial dysfunction in a local ANG II signaling-dependent manner.** Our studies are based on the rationale that local production of Hcy is the cause of endothelial dysfunction and are consistent with epidemiological findings that fail to support a relationship between plasma levels of Hcy (HHcy) and the pathogenesis of cardiovascular diseases. Additionally, intervention protocols which lower Hcy levels in the general population with non-genetically based HHcy fall short in lowering cardiovascular risk as well as preventing endothelial dysfunction (49, 59). HHcy is the result of a systemic production of Hcy and a subsequent cellular export to dispose of nonproteinogenic Hcy which did not accumulate within cells (5). Hcy in plasma can react with plasma proteins forming mixed disulfides of proteins (38). For example, due to its abundance and an accessible cysteine residue at position 34, albumin is the major S-homocysteinylated protein in the circulation (10). Not adequately addressed in this process is that endogenous production of Hcy coupled with its more reactive metabolites, Hcy-S-S-Hcy and HCTL, may exert effects within local tissues that are more deleterious than those produced by Hcy in the circulation. Thus the molecular targeting of critical intracellular proteins by Hcy and...
the formation of stable mixed disulfide complexes would be anticipated to provoke serious cellular damage.

From these observations it is plausible to speculate that cellular dysfunction may precede cellular export of Hcy into plasma. Our data support the notion that the vascular endothelia produce levels of Hcy that are higher than that produced in smooth muscle cells (Fig. 1). In view of this finding, an Hcy gradient may be operational between the endothelium and smooth muscle cells as well as into plasma. A rapid de novo biosynthesis of Hcy in vessels (Fig. 2) is evident by the vast dependence of vascular endothelium on Met necessary for synthesis of S-adenosylmethionine, one-carbon metabolism as well as for methylation of DNA and proteins (8, 26). Furthermore, the positive correlation between vascular Hcy production and endothelial dysfunction is proportional to the Met loading (Fig. 3). In support of these findings, other investigators have demonstrated that Met-loaded endothelial cells exhibit increased accumulation and enhanced extracellular transport of Hcy metabolites, factors that are associated with an upregulation of inflammatory molecules, oxidative stress, and an increased susceptibility to coronary artery disease (35, 43).

Furthermore, studies of minipigs on a methionine-rich caseinate-based diet (42) and in patients with prevalent cardiovascular disease (50) show that Met intake correlates positively with hypertension.

Our studies collectively support the hypothesis that vascular (local) production of Hcy, a pathological process that takes significant precedence over increases in circulating Hcy, is more likely the primary contributor to vascular injury. We also found a higher percentage of Hcy-S-S-Hcy in the endothelium than in plasma. Hcy-S-S-Hcy has a greater ability to form stable disulfide bonds with protein cysteine residues at physiological pH than does Hcy even in the presence of other thiols (29). Due to the electron/proton donating properties of histidyl and tyrosyl moieties in proteins, the sulfhydryl group of cysteinyl moieties typically exhibit lower $pK_a$ values than that of the free amino acid (~5–6 vs. 8.3) (33). This creates a thiolate anion near or below the relevant physiological pH, which facilitates a nucleophilic attack at the disulfide of Hcy-S-S-Hcy. In this manner, thiolates are ~3–4 orders of magnitude more nucleophilic than their thiol counterparts (24, 25). Indeed, we have shown this phenomenon where Hcy-S-S-Hcy displays a much greater potential to activate ACE than does Hcy (Fig. 5A).

Homocysteinylation of ACE activates the enzyme, followed by recruitment of NADPH oxidase in blood vessels. The crucial role of oxidative stress in the mediation of Hcy-dependent cardiovascular dysfunction has been well documented; however, the causality of oxidation vs. HHcy has remained controversial. Due to the paradox created by the failure of clinical trials to show the benefits of Hcy-lowering therapy, it has been proposed that oxidative stress is a primary initiator of HHcy, which in turn potentiates oxidation (12). In this context, an increase in plasma Hcy is a marker/indicator of systemic or vascular oxidative stress and, as such, may play a secondary role in exacerbating oxidative injury. Our studies, however, challenge this notion by showing that vascular production of superoxide is proportional to Met loading (Fig. 6A) and results in impaired vasodilation and reduced release of endothelial NO (Fig. 3, B and C; Fig. 6B), which was secondary to the initial vascular Hcy formation (Fig. 3A). As a potential mechanism for Hcy-induced vascular pathogenesis, our studies suggest that Hcy-S-S-Hcy and HCTL can target proteins at critical sulfhydryl and ε-amino moieties, respectively (Fig. 4), and thus provide the impetus to initiate oxidative injury within the vessels (Fig. 6) by causing both S- and N-homocysteinylation of intracellular proteins, including ACE (Fig. 4) (19).

The presence of a Hcy-generating system (Fig. 1) and the ANG II signaling pathway (36) in blood vessels as well as abundant expression of ACE (3) in the endothelium provide the biophysiological basis for these interactions. Although the activation of NADPH oxidase by ANG II signaling has been demonstrated to be responsible for exacerbation of vascular oxidative stress in isolated arterioles (15, 16), the specific action of local Hcy in promoting ANG II-dependent activation of NADPH oxidase is still unknown. Our studies show that AT$_1$R and ACE are active participants in Hcy-driven, superoxide-mediated endothelial dysfunction, as evidenced by the fact that inhibition of vascular ACE and AT$_1$R fully prevents the Met/Hcy-induced impairment of SSID and NO release, as well as the NADPH oxidase-dependent production of superoxide (Figs. 3 and 6). ACE reacts with both Hcy-S-S-Hcy and HCTL and causes a dose-dependent formation of homocysteinylated ACE (Fig. 4). These results are consistent with other findings, seen in cultured endothelial cells and arteries exposed to Met. Our studies show that the physiological concentration of Hcy is sufficient enough to enable timely synthesis of Hcy-S-S-Hcy and HCTL and that these metabolites exhibit a greater capacity to S- and N-homocysteinylated proteins than their parent compound (23). Accordingly, homocysteinylated-ACE, produced either by exposure of the enzyme (Fig. 5A) or isolated single arteries (Fig. 5, B–D) to different forms of Hcy and Met, exhibits significantly greater activity than that of the native enzyme. Thus it appears that endogenously synthesized Hcy causes transduction of ACE/ANG II/AT$_1$R signaling, which is followed by activation of NADPH oxidase.

Studies by other investigators show that homocysteinylation of a variety of endothelial proteins may underlie endothelial dysfunction associated with the multiple diseases affiliated with elevated Hcy (11, 32). The property of homocysteinylation of endothelial proteins in Hcy-dependent endothelial damage (23) inspired our investigations into the specific candidate(s) that would be particularly targeted by Hcy in vessels. The novel findings of the present study unravel the molecular basis of cytotoxic actions of Hcy to the vasculature, by demonstrating that in freshly isolated arteries, ACE is a specific target of Hcy-S-S-Hcy/HCTL. As such, Hcy produced endogenously by vascular endothelium can either be oxidized to Hcy-S-S-Hcy or converted to its thiolactone metabolite. Subsequent posttranslational modification of ACE either by $N$-homocysteinylation of lysyl moieties or $S$-homocysteinylation through thiol-disulfide exchange reactions may result in an exacerbation of ACE-stimulated NADPH oxidase in arterial vessels. Enhanced production of vascular superoxide anion impairs nitric oxide synthesis and exerts negative effects on endothelial function.

Subsequent studies are needed to address several limitations of the current investigation. For example, what is the causal relationship between homocysteinylation of ACE and endothelial dysfunction? Although our studies examined the cotreatment of Met/Hcy-loading vessels with inhibitors of AT$_1$R, ACE and NADPH oxidase, respectively, to prevent endothelial
dysfunction, further investigation is needed to address whether agent(s) that react directly with Hcy are able to prevent ACE homocysteinylation in the vessels and, thus, reverse vascular dysfunction. A study of this type could provide greater insight into identifying a correlation between covalent modification of ACE activity and altered endothelial function, thus defining a pathophysiological relevance. Also, use of isolated vessels does not allow determination of ACE being covalently modified within the cytoplasmic or membrane regions of the cell. Further studies are needed to provide greater mechanistic insights into covalent modification of ACE by Hcy.

Collectively, our findings reveal novel mechanisms that regulate bioactivity of NO and contribute specifically to our understanding of the pathogenesis of hypertension associated with homocysteinylation of ACE. Importantly, during the pathological procedure of Hcy-mediated cardiovascular disorders, such as fructose-induced metabolic syndrome and diabetes, increasing local/vascular Hcy may take precedence over the changes in plasma/circulating Hcy (our unpublished data). To this end, ACE inhibitors can be used in combination with traditional Hcy-lowering therapies, to prevent local Hcy-initiated recruitment of vascular ANG II signal cascade before development of HHcy.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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