Endothelial Dysfunction

Impaired Hydrogen Sulfide–Mediated Vasodilation Contributes to Microvascular Endothelial Dysfunction in Hypertensive Adults

Jody L. Greaney,* Jessica L. Kutz,* Sean W. Shank, Sandeep Jandu, Lakshmi Santhanam, Lacy M. Alexander

Abstract—Reductions in hydrogen sulfide (H\textsubscript{2}S) production have been implicated in the pathogenesis of vascular dysfunction in animal models of hypertension; however, no studies have examined a functional role for H\textsubscript{2}S in contributing to microvascular dysfunction in hypertensive (HTN) adults. We hypothesized that endogenous production of H\textsubscript{2}S would be reduced, impaired endothelium-dependent vasodilation would be mediated by reductions in H\textsubscript{2}S-dependent vasodilation, and vascular responsiveness to exogenous H\textsubscript{2}S (sodium sulfide) would be attenuated in HTN compared to normotensive adults. Fifteen normotensive (51±2 years; blood pressure, 116±3/76±3 mm Hg) and 14 HTN adults (57±2 years; blood pressure 140±3/89±2 mm Hg) participated. H\textsubscript{2}S biosynthetic enzyme expression (Western blot) and substrate-dependent H\textsubscript{2}S production (amperometric probe) were measured in cutaneous tissue homogenates. Red cell flux (laser Doppler flowmetry) was measured during graded perfusions of acetylcholine (ACh; 10\textsuperscript{-6}–10\textsuperscript{-3} mol/L) and sodium sulfide (10\textsuperscript{-5}–10\textsuperscript{-1} mol/L) using intradermal microdialysis; the functional role of H\textsubscript{2}S was determined using pharmacological inhibition with aminoxyacetic acid (0.5 mmol/L). H\textsubscript{2}S biosynthetic enzyme expression and substrate-dependent H\textsubscript{2}S production were reduced in HTN adults (all \textit{P}<0.05). ACh-induced endothelium-dependent vasodilation was blunted in HTN adults (\textit{P}=0.012). Aminoxyacetic acid attenuated ACh-induced vasodilation in normotensive adults (ACh, 1.31±0.13 versus ACh+a aminoxyacetic acid, 1.07±0.09 flux/mm Hg; \textit{P}=0.025) but had no effect on vasodilation in HTN adults (ACh, 1.16±0.10 versus ACh+a aminoxyacetic acid, 1.37±0.11 flux/mm Hg; \textit{P}=0.47). Sodium sulfide–induced vasodilation was not different between groups. Collectively, these findings indicate that while the microvasculature maintains the ability to vasodilate in response to exogenous H\textsubscript{2}S, reductions in endogenous synthesis and H\textsubscript{2}S-dependent vasodilation contribute to endothelial dysfunction in human hypertension. (\textit{Hypertension}. 2017;69:902-909. DOI: 10.1161/HYPERTENSIONAHA.116.08964.) • Online Data Supplement

Key Words: 3-mercaptopyruvate sulfurtransferase ■ cystathionine γ-lyase ■ endothelial dysfunction ■ endothelium-dependent dilation ■ microdialysis ■ nitric oxide

Hydrogen sulfide (H\textsubscript{2}S) is one of 3 gasotransmitters, along with nitric oxide (NO) and carbon monoxide, critical for cardiovascular homeostasis.\textsuperscript{1} It is increasingly apparent that dysregulation of the enzymatic production and function of H\textsubscript{2}S play an important role in the pathogenesis of hypertension-associated vascular dysfunction.\textsuperscript{2-4} In the vasculature, H\textsubscript{2}S is enzymatically synthesized by cystathionine γ-lyase (CSE)\textsuperscript{5} and 3-mercaptopyruvate sulfurtransferase (3-MPST).\textsuperscript{6} Mice lacking endogenous CSE exhibit reduced cholinergic vasorelaxation and hyperpolarization,\textsuperscript{5,7} resulting in the development of pronounced hypertension,\textsuperscript{5} comparable to that observed in endothelial NO synthase (NOS)–deleted mice.\textsuperscript{8} These data suggest that impairments in H\textsubscript{2}S-mediated regulation of vascular function contribute to elevations in blood pressure (BP).

H\textsubscript{2}S elicits vasodilation by directly hyperpolarizing vascular smooth muscle cells predominantly through ATP-sensitive and calcium-dependent potassium channels (K\textsubscript{ATP} and K\textsubscript{Ca}, respectively).\textsuperscript{7,9} H\textsubscript{2}S also modulates vascular function via extensive cross talk with the NO signaling pathway at multiple regulatory points,\textsuperscript{7} and multiple lines of evidence suggest that the vascular effects of H\textsubscript{2}S and NO are interdependent. In this regard, H\textsubscript{2}S administration increases vascular NO\textsuperscript{10} and, at low concentrations, potentiates the vasodilatory effect of NO.\textsuperscript{11} Further, CSE silencing attenuates NO-dependent vasodilation,\textsuperscript{11} and conversely, inhibition of NOS attenuates the vasodilatory response to H\textsubscript{2}S.\textsuperscript{11} Taken together, these data demonstrate a functional synergistic interaction between the H\textsubscript{2}S and NO signaling pathways in the control of vascular function.
function and suggest that each is obligatory for the maintenance of vascular homeostasis.

In animal models of hypertension, expression and activity of \( \text{H}_2\text{S} \)-synthesizing enzymes, as well as plasma \( \text{H}_2\text{S} \) concentrations, are reduced and associated with impairments in endothelium-dependent dilation.\(^{1,12,13} \) Further, systemic treatment with \( \text{NaHS} \) improves endothelial function, at least in part, via increased NO bioavailability.\(^{12,14,15} \) In humans, plasma \( \text{H}_2\text{S} \) concentration appears to be negatively correlated with BP.\(^{16} \) However, despite the compelling preclinical evidence, no studies have mechanistically examined the role of \( \text{H}_2\text{S} \) in contributing to vascular dysfunction in hypertensive (HTN) adults.

The aim of the present study was to examine a functional role for \( \text{H}_2\text{S} \) in contributing to cutaneous microvascular dysfunction in middle-aged HTN adults. The human cutaneous circulation is a validated model to assess mechanisms underlying microvascular dysfunction\(^{17,18} \) because the pathogenesis of hypertension-associated microvascular dysfunction occurs simultaneously in multiple vascular beds.\(^{17,19,20} \) We hypothesized that \( \text{CSE} \) and 3-MPST expression, as well as endogenous production of \( \text{H}_2\text{S} \), would be reduced in HTN compared with that in normotensive (NTN) adults. We additionally hypothesized that \( \text{H}_2\text{S} \)-mediated endothelium-dependent vasodilation would be attenuated in HTN adults. Finally, we hypothesized that vascular responsiveness to exogenous \( \text{H}_2\text{S} \) would be blunted in HTN adults.

**Methods**

A complete description of the Materials and Methods is provided in the online-only Data Supplement.

**Subjects**

The Institutional Review Board at The Pennsylvania State University approved all experimental procedures. Verbal and written consent was obtained voluntarily from all subjects prior to participation and according to guidelines set forth by the Declaration of Helsinki. Fifteen adults with normal BP and 14 adults with untreated stage 1 essential hypertension participated. All subjects underwent a complete medical screening, including a resting 12-lead ECG, physical examination, 24-hour BP monitoring, and 12-hour fasting blood chemistry (Quest Diagnostics, Pittsburgh, PA). Consistent with JNC7 guidelines,\(^{21} \) and assessed in accordance with American Heart Association standards,\(^{22} \) HTN adults had a resting seated systolic BP >140 mm Hg or a diastolic BP >90 mm Hg, and NTN adults had a resting seated systolic BP <120 mm Hg and a diastolic BP <80 mm Hg. Ambulatory BP monitoring (Ambulo 2400; Mortara Instrument Inc, Milwaukee, WI) was used to confirm the diagnosis of hypertension. Subjects were nonobese (body mass index <30 kg/m\(^2 \)), did not use tobacco products, were recreationally active, and were not taking any over-the-counter or prescription medications with primary or secondary cardiovascular effects (eg, statins, anticoagulants, antidepressants, etc).

**Protocol 1: Assessment of Endogenous Expression and Activity of \( \text{H}_2\text{S} \) Biosynthetic Enzymes**

In a subset of participants (NTN, \( n=5 \); HTN, \( n=7 \)); cutaneous tissue samples were obtained via punch biopsy.\(^{23,24} \) Using sterile technique, two 3-mm diameter samples were obtained after anesthetization (2% lidocaine without epinephrine), immediately snap frozen in liquid nitrogen, and stored at \(-80^\circ \text{C} \) until analysis. CSE and 3-MPST expression were determined by Western blot, and \( \text{H}_2\text{S} \) production was measured by amperometry (Apollo 4000 Free Radical Analyzer detector with a 3-mm \( \text{H}_2\text{S} \)-selective electrode; World Precision Instruments, Sarasota, FL) as previously described\(^{25} \) (online-only Data Supplement).

**Protocol 2: Assessment of \( \text{H}_2\text{S} \)-Dependent Cutaneous Vasodilatory Responsiveness**

Four intradermal microdialysis probes (CMA Linear 30 probe, 6 kDa; Harvard Apparatus, Holliston, MA) were inserted into the forearm skin\(^{3,24} \) for the local delivery of pharmacological agents: lactated Ringer solution (control), 20 mmol/L \( \text{N}^\circ \)-nitro-L-arginine methyl ester (L-NAME; Calbiochem, EMD Millipore, Billerica, MA) to nonselectively inhibit NOS, 0.5 mmol/L \( \text{a} \)-aminooxycetic acid (AOAA; Sigma-Aldrich Corp, St Louis, MO) to inhibit \( \text{H}_2\text{S} \) biosynthesis,\(^{26} \) or 20 mmol/L L-NAME+0.5 mmol/L AOAA to inhibit both NO and \( \text{H}_2\text{S} \) vasodilatory pathways concurrently. Pilot studies using an in vitro preparation confirmed the efficacy of this concentration of AOAA to inhibit \( \text{H}_2\text{S} \) production (online-only Data Supplement). After microdialysis fiber insertion, 60 to 90 minutes were allowed for hyperemia resolution, during which site-specific pharmacological solutions were perfused (2 \( \mu \)L/min; Hive controller and microinfusion pumps; BASi, West Lafayette, IN). Thereafter, progressively increasing concentrations of acetylcholine (ACH; 10\(^{-6}\)–10\(^{-1}\) mol/L; USP, Rockville, MD) were coperfused with the site-specific pharmacological agent. After the ACh dose–response protocol, 28 mmol/L sodium nitroprusside (USP) was perfused and local temperature increased to 43°C to elicit maximal dilation.\(^{27,28} \) Cutaneous red blood cell flux, an index of cutaneous blood flow, was continually measured directly over each microdialysis site with an integrated laser Doppler flowmetry probe placed in a local heating unit (Temperature Monitor SH02; Moor Instruments, Devon, United Kingdom). Brachial BP (Cardiocap; GE Healthcare, Milwaukee, WI) was measured every 5 minutes during the protocol.

**Protocol 3: Assessment of Cutaneous Vascular Sensitivity to Exogenous \( \text{H}_2\text{S} \)**

As described above, 2 intradermal microdialysis probes were inserted into the forearm skin. Sites were perfused with lactated Ringer solution or 20 mmol/L L-NAME (Calbiochem) for 60 to 90 minutes after probe placement. An index of cutaneous blood flow was obtained directly over the microdialysis site during perfusion (2 \( \mu \)L/min) of progressively increasing doses of the \( \text{H}_2\text{S} \) donor sodium sulfide (Na\(_2\text{S} \); 10\(^{-5}\)–10\(^{-1}\) mol/L; Sigma-Aldrich Corp).\(^{24} \) Using this model, we have previously demonstrated no difference in cutaneous vasodilation between the 2 commonly used \( \text{H}_2\text{S} \) donors NaHS and Na\(_2\text{S} \).\(^{24} \) Na\(_2\text{S} \) was dissolved in lactated Ringer solution and titrated with hydrochloric acid (0.5 mol/L HCl) to pH 7.0. Solutions were mixed immediately before use, wrapped in foil to prevent photodegradation, and sealed from air to maintain pH 7.0 throughout the experiment. At the conclusion of the protocol, maximal dilation was obtained as detailed earlier. Brachial BP (Cardiocap; GE Healthcare) was measured every 5 minutes. A Food and Drug Administration Investigational New Drug (no 105 572) was obtained for the in vivo utilization of all pharmacological agents.

**Data and Statistical Analysis**

Subject characteristics, CSE and 3-MPST expression, and \( \text{H}_2\text{S} \) production were compared using unpaired Student’s \( t \) tests. Functional data were collected at 40 Hz (Windaq; DataQ Instruments, Akron, OH). Cutaneous vascular conductance was calculated as red blood cell flux (perfusion units) divided by mean arterial pressure. Cutaneous vascular conductance was averaged during 5 minutes of baseline and vascular conductance was calculated as red blood cell flux (perfusion units) divided by mean arterial pressure. Cutaneous vascular conductance was averaged during 5 minutes of baseline and

**Results**

Subject characteristics are presented in Table. There were no statistically significant differences between groups in age,
anthropometric characteristics, or blood biochemistry. By design, resting screening BP and 24-hour BP were significantly elevated in HTN adults (all $P<0.01$). BP did not change significantly during the course of the experiment in either group (NTN, $P=0.35$; HTN, $P=0.54$).

Activity of H$_2$S-Producing Enzymes Is Reduced in HTN Adults (In Vitro)
In HTN adults, CSE and 3-MPST expression in cutaneous tissue homogenates were markedly reduced (Figure 1A and 1B). In addition, both CSE- and 3-MPST-mediated H$_2$S production were attenuated in HTN adults (Figure 1C and 1D).

Endogenous H$_2$S-Mediated Vasodilation Is Functionally Absent in HTN Adults
Maximal SNP-induced cutaneous vasodilation was reduced in HTN adults (Figure 2; $P=0.039$). As expected, ACh-induced endothelium-dependent vasodilation was attenuated in HTN adults (Figure 2). ACh-induced vasodilation at each pharmacological treatment site is presented in Figure 3. NOS inhibition attenuated ACh-induced vasodilation in both subject groups (Figure 3); however, there was no group difference in the vasodilatory response in the presence of NOS inhibition (NTN, 0.88±0.09 versus HTN, 0.89±0.07 flux/mm Hg; $P=0.92$), reflecting hypertension-induced reductions in vascular NO bioavailability. AOAA attenuated ACh-induced vasodilation in NTN adults (Figure 3B), but had no effect in HTN adults (Figure 3E), suggesting a functional lack of endogenous H$_2$S-mediated vasodilation. Combined pharmacological inhibition of both NOS and H$_2$S-producing enzymes attenuated ACh-induced vasodilation in HTN adults; however, the attenuation in cutaneous vasodilation was not statistically different from that during L-NAME treatment alone ($P>0.05$). In HTN adults, combined treatment with L-NAME+AOAA had minimal effect on vasodilatory responsiveness to exogenous ACh, such that there was no difference between the combined site and the control site.

Vascular Responsiveness to Exogenous Na$_2$S Is Preserved in HTN Adults
Maximal cutaneous vascular conductance was not different between groups or treatment sites ($P>0.05$ for all). There was no

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Expression of cystathionine γ-lyase (CSE; A) and 3-mercaptopruvate sulfurtransferase (3-MPST; B), as well as CSE-mediated (C) and 3-MPST-mediated H$_2$S production, in normotensive (NTN; filled bars) and hypertensive adults (HTN; open bars). A representative blot is shown in the first panel. *$P<0.01$ vs NTN.

### Table. Subject Characteristics

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<th>Baseline Characteristic</th>
<th>NTN</th>
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<tr>
<td>N (M/F)</td>
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<td>14 (6/8)</td>
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<td>Age, y</td>
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<td>Height, cm</td>
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**Blood biochemistry**

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<td>Fasting glucose, mg/dL</td>
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<td>Fasting total cholesterol, mg/dL</td>
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<td>Fasting HDL, mg/dL</td>
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<td>Fasting LDL, mg/dL</td>
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<tr>
<td>Fasting triglycerides, mg/dL</td>
<td>91±11</td>
<td>127±23</td>
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</tbody>
</table>

Values are mean±SE. *$P<0.05$ vs NTN. BMI indicates body mass index; BP, blood pressure; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; HTN, hypertensive; LDL, low-density lipoprotein; and NTN, normotensive.
difference in cutaneous vascular responsiveness to exogenous Na2S between NTN and HTN adults (Figure 4; 10−1 mmol/L Na2S, 1.31±0.19 NTN versus 1.34±0.24 HTN flux/mmHg; P=0.80). NOS inhibition blunted Na2S-induced vasodilation at the highest dose in both subject groups (Figure 4; P<0.05).

Discussion

The primary novel findings of the present study are that expression and substrate-dependent activity of H2S biosynthetic enzymes are reduced and H2S-dependent ACh-induced cutaneous vasodilation is impaired in HTN adults. Contrary to our hypothesis, vascular responsiveness to exogenous H2S was preserved in HTN adults, further implicating reductions in H2S bioavailability, and not alterations in downstream vascular smooth muscle sensitivity, in contributing to hypertension-associated vascular dysfunction. Moreover, we are the first to demonstrate an interaction between the H2S and NO signaling pathways in the regulation of microvascular function in humans. Taken together, these findings suggest that reductions in both H2S- and NO-dependent mechanisms contribute to endothelial dysfunction in the microcirculation of HTN adults.

Impaired endothelium-dependent vasodilation is a well-established contributing factor to HTN pathology27 and is evident in the cutaneous microcirculation of HTN adults.23,28–30 In the present study, HTN adults exhibited endothelial dysfunction, evidenced by impaired ACh-induced vasodilation,23,31,32 but this was substantially reduced in HTN adults, confirming that reductions in vascular NO bioavailability contribute to the endothelial dysfunction characteristic of hypertension.27–30,33–35

While the roles of endothelium-derived relaxing and constricting factors, including NO and cyclooxygenase-derived metabolites, have been extensively characterized in hypertension, no studies have investigated potential alterations in H2S metabolism in human hypertension. CSE and 3-MPST are the enzymes largely responsible for endogenous endothelial-derived H2S synthesis.5,6 In young adults, these enzymes

![Figure 2.](image)

Figure 2. Cutaneous vascular conductance (CVC) in response to increasing doses of acetylcholine (ACh) in normotensive (NTN; filled symbols) and hypertensive adults (HTN; open symbols). Maximal CVC (max) was elicited by perfusion of sodium nitroprusside during local heating to 43°C at the conclusion of the ACh dose–response protocol. ACh-induced vasodilation was blunted in HTN adults. *P<0.05 vs NTN.

![Figure 3.](image)

Figure 3. Cutaneous vasodilation (cutaneous vascular conductance; CVC) in response to increasing doses of exogenous acetylcholine alone (ACh; filled circles), during nitric oxide synthase inhibition (L-NAME; open circles), during inhibition of H2S-producing enzymes (AOAA; open squares), and during combined nitric oxide and H2S inhibition (L-NAME+AOAA; open triangles) in normotensive (NTN; A–C) and hypertensive adults (HTN; D–F). Maximal CVC (max) was elicited by perfusion of sodium nitroprusside during local heating to 43°C at the conclusion of the ACh dose–response protocol. *P<0.05 vs ACh alone. AOAA indicates aminooxyacetic acid; and L-NAME, N\(^{\text{G}}\)-nitro-l-arginine methyl ester.
are present in the cutaneous microvasculature and synthetize H$_2$S, which induces vasodilation, in part, by activating intermediate calcium-dependent potassium channels. CSE and 3-MPST expression were markedly reduced in cutaneous tissue homogenates from HTN adults, consistent with previous reports. In addition, H$_2$S production was detected in each group, suggesting that the intracellular source of H$_2$S has a cytosolic and mitochondrial component. The present results demonstrate marked reductions in enzymatic H$_2$S production through both CSE and 3-MPST biosynthetic pathways in HTN adults. These in vitro amperometric H$_2$S measurements were performed in cutaneous tissue and, thus, directly relate to our functional assessments of vascular regulation. Because of the difficulty in accurately measuring H$_2$S concentration and the ambiguity of data interpretation from gross measurements of bioactive sulfide metabolites, relatively few studies have examined H$_2$S production in humans. Although low plasma H$_2$S concentration is associated with cardiovascular mortality, and plasma H$_2$S concentration appears negatively correlated with BP, to our knowledge, only one previous study has measured H$_2$S production in untreated HTN adults. Interestingly, the authors reported a greater serum H$_2$S concentration, measured via a sulfide-sensitive electrode, in HTN adults, despite lower expression of 3-MPST. We did not measure serum H$_2$S in the present study because of the aforementioned methodological difficulties and instead favored measurement of tissue-specific H$_2$S production from the substrates for CSE and 3-MPST, L-cysteine, and 3-MP, respectively. Our findings are consistent with those observed in rodent models of hypertension, in which expression and activity of H$_2$S-producing enzymes are reduced and contribute to vascular dysfunction.

H$_2$S regulates vascular tone via its direct effects on vascular smooth muscle cells, as well as by extensive interactions with other endothelium-dependent signaling pathways. H$_2$S and NO are mutually dependent, and multiple potential points of functional interaction exist, both upstream in the endothelium and downstream in the vascular smooth muscle. In this regard, NaHS-induced dilation is blunted in the presence of L-NAME in blood vessels isolated from both rodents and humans, as well as in vessels harvested from eNOS$^{-/-}$ mice, suggesting that the vasodilatory action of H$_2$S requires the presence of endogenously produced NO. In the current study, there was a modest, but significant, attenuation in Na$_2$S-induced vasodilation during NOS inhibition in NTN adults. Given that the vasodilatory effect of H$_2$S was only modestly blunted by NOS inhibition, H$_2$S-induced vasodilation likely involves parallel signaling pathways, such as KATP and KCa.

Figure 4. Cutaneous vasodilation (cutaneous vascular conductance, CVC) in response to increasing doses of the exogenous H$_2$S donor sodium sulfide (Na$_2$S) alone (ACh; filled circles) and during nitric oxide synthase inhibition (L-NAME; open circles) in normotensive (NTN; A) and hypertensive adults (HTN; B). Maximal CVC (max) was elicited by perfusion of sodium nitroprusside during local heating to 43°C at the conclusion of the Na$_2$S dose–response protocol. $^*P<0.05$ vs Na$_2$S alone. L-NAME indicates N$^\bullet$-nitro-l-arginine methyl ester.

Contrary to our hypothesis, we found that perfusion of an exogenous H$_2$S donor elicited significant vasodilation in HTN adults that was not different from that observed in NTN adults, suggesting that vascular responsiveness to exogenous H$_2$S is maintained in human hypertension. In addition, we noted an attenuation in the vasodilatory response to the highest dose of Na$_2$S during concurrent NOS inhibition in HTN adults, the magnitude of which was not different from that in NTN adults, providing evidence that the NO contribution to exogenous H$_2$S-mediated vasodilation remains intact in HTN adults. In hypertensive CSE$^{-/-}$ mice, relaxation in response to H$_2$S was augmented compared with that in wild-type mice, a response indicative of supersensitivity associated with the diminished endogenous synthesis of H$_2$S. Thus, it seems that vascular sensitivity to exogenous H$_2$S, as well as its synergistic interaction with NO, is preserved in hypertension-induced vascular dysfunction. Because bioavailability of both H$_2$S and NO is reduced in hypertension, exogenous delivery of a H$_2$S donor may be beneficial for improving vascular function through both signaling pathways, a potentially important clinical implication given the development of novel pharmacological agents with slow-release H$_2$S moieties for the treatment of hypertension. This area of research warrants future investigation.

Despite preserved vascular sensitivity to exogenous H$_2$S, reductions in the endogenous production and function of H$_2$S clearly contribute to the pathogenesis of hypertension-associated vascular dysfunction. Moreover, long-term systemic treatment with a H$_2$S donor improves endothelial function in hypertensive rodents, in part via increased NO bioavailability, suggesting that the loss of redundancy and synergism between the NO and H$_2$S signaling pathways contributes to vascular dysfunction in hypertension. In this regard, endogenous H$_2$S seems to be required for the full vasodilatory actions of NO. siRNA-mediated silencing of CSE attenuates, but does not completely block, dilation in response to a NO donor or to the endothelium-dependent agonist ACh, and methacholine-induced vasodilation is attenuated in CSE$^{-/-}$ mice.

In the current study, inhibition of CSE with AOAA blunted ACh-induced endothelium-dependent vasodilation in NTN adults, providing the first direct evidence in humans that H$_2$S mediates a portion of the vasodilatory response to ACh. Mechanistically, it has been suggested that ACh, via endothelial calcium mobilization, may stimulate H$_2$S release from
endothelial cells, resulting in prolonged phosphodiesterase type 5 inhibition and subsequent relaxation. It is plausible to suggest that the reductions in endogenous H\(_2\)S production in HTN adults may contribute to altered regulation of vascular responsiveness to ACh in hypertension. Consistent with this hypothesis, inhibition of CSE did not affect ACh-induced vasodilation in HTN adults, indicating that the H\(_2\)S-mediated component of cholinergic vasodilation is functionally absent in human hypertension. Interestingly, concurrent antagonism of NOS and CSE did not further attenuate ACh-induced vasodilation in either NTN or HTN adults compared with either pharmacological agent alone, a finding consistent with the notion that other parallel signaling pathways are compensatory in response to cholinergic stimulation. Collectively, the results of the present study suggest that alterations in the production and function of endogenous H\(_2\)S likely contribute to the endothelial dysfunction in human hypertension.

Additional studies are necessary to determine the potential for alterations in H\(_2\)S-dependent mechanisms to contribute to vascular dysfunction in HTN adults with additional major cardiovascular risk factors. In the present study, every attempt was made to match NTN and HTN groups for all clinical characteristics aside from resting BP. Although not statistically different, and still within normal limits, HTN adults tended to be older and have a greater body mass index. Because aging and overweight/obesity are independently associated with impairments in endothelial function, it is possible that these non-statistically significant trends in cardiovascular risk may have contributed to the observed functional differences.

**Limitations**

Because of the complexity of H\(_2\)S metabolism, it is methodologically challenging to determine the specific bioactive H\(_2\)S-derived intermediate mediating the observed vasodilatory responses. Given these technical limitations, we instead measured substrate-dependent H\(_2\)S production as a surrogate for the activity of the H\(_2\)S biosynthetic enzymes CSE and 3-MPST. We acknowledge the inability of the amperometric probe to determine the specific H\(_2\)S-derivative species mediating the observed biological responses. Nevertheless, because reductions in both H\(_2\)S biosynthetic enzyme expression and substrate-mediated H\(_2\)S production are evident in HTN adults, the corresponding signal for eliciting a physiological response is, therefore, also reduced, regardless of the specific biochemical intermediate pathway, ultimately resulting in a blunted contribution to vasodilation.

We also recognize the potential limitation of the use of AOAA to inhibit endogenous production of H\(_2\)S; however, AOAA is currently the only CSE inhibitor available for use in humans. Importantly, AOAA has recently been demonstrated to be a more specific inhibitor of CSE than cystathionine-\(\beta\)-synthase (neuronal enzymatic source of H\(_2\)S) in the vasculature. In addition, the inhibition of CSE by AOAA was verified by measurement of H\(_2\)S production using the CSE-specific substrate L-cysteine (Figure S1A in the online-only Data Supplement). Moreover, we were not able to delineate the relative contribution of 3-MPST inhibition to the observed responses. Although AOAA may indirectly inhibit 3-MPST via cysteine aminotransferase, it is not specific for 3-MPST, and thus, we cannot determine the direct extent to which H\(_2\)S production from 3-MPST contributes to vascular dysfunction in HTN adults. Additional studies are necessary once more specific inhibitors for the enzymatic sources of H\(_2\)S are available to fully elucidate the contribution of H\(_2\)S synthesized from MPST to vascular control in humans.

**Perspectives**

Endothelial dysfunction is thought to be the primary causative event in the development of atherosclerosis, occurs before angiographic evidence of disease, and predicts future cardiovascular events and mortality. The pathogenesis of vascular dysfunction associated with hypertension is a complex, multifaceted process that is thought to manifest to a proportional extent in multiple tissue beds. In this regard, the progression of endothelial dysfunction in the cutaneous microvasculature mirrors that in the coronary and renal circulations, making it an accessible and representative model to examine vascular function. Using this methodology, we are the first to demonstrate that reductions in the enzymatic production and function of endogenous H\(_2\)S contribute to microvascular endothelial dysfunction in HTN adults. Additional studies are warranted to examine the systemic effects of H\(_2\)S in targeted pharmacotherapy for HTN vascular pathology. Stage I clinical trials indicate that antihypertensive medications with H\(_2\)S-releasing moieties demonstrate superior clinical benefit; however, future investigations as to whether this is because of the independent vasodilatory effect of H\(_2\)S, via its synergistic interaction with the NO signaling pathway or through alterations in the enzymatic production of other endothelium-dependent signaling molecules within the vasculature (eg, prostanooids) are necessary.

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**Disclosures**

None.

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### What Is New?

- These findings show that the hydrogen sulfide (H2S)-dependent contribution to vasodilation is functionally absent in hypertensive adults, likely because of a reduction in the endogenous production of H2S within the vasculature.
- Vascular responsiveness to exogenous H2S is preserved in hypertensive adults.
- We additionally demonstrate an interaction between H2S and nitric oxide signaling pathways in the regulation of vascular function in humans.

### What Is Relevant?

- Given that rodent models of hypertension demonstrate reductions in the endogenous production and function of H2S clearly contribute to the pathogenesis of hypertension-associated vascular dysfunction in rodent models, translating these findings to human hypertension may provide novel insight into the mechanisms of endothelial dysfunction.
- These findings are clinically relevant, and additional studies are warranted to examine the systemic effects of H2S in targeted pharmacotherapy for hypertensive vascular pathology.

### Summary

Reductions in both H2S- and nitric oxide–dependent mechanisms contribute to microvascular endothelial dysfunction in hypertensive adults.
Impaired Hydrogen Sulfide–Mediated Vasodilation Contributes to Microvascular Endothelial Dysfunction in Hypertensive Adults

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Impaired hydrogen sulfide-mediated vasodilation contributes to microvascular endothelial
dysfunction in hypertensive adults

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METHODS

Subjects: Hypertension was defined in accordance with JNC7 criteria\textsuperscript{1} and confirmed on at least two separate occasions by a trained investigator using guidelines set forth by the American Heart Association\textsuperscript{2}. To confirm the diagnosis of hypertension, ambulatory BP monitoring (Ambulo 2400; Mortara Instrument Inc, Milwaukee, WI, USA) was additionally used because of its greater accuracy for describing the BP profile in the daily routine and avoiding “white coat” hypertension\textsuperscript{3}. The average blood pressure values obtained in 24-hr ambulatory monitoring are typically lower than those obtained by office measurements due to nocturnal dipping; thus, the consensus values for the diagnosis of hypertension using 24-hr ambulatory measures are systolic BP >130 mmHg or diastolic blood pressure >80 mmHg\textsuperscript{3,4}. These diagnostic thresholds for ambulatory BP monitoring yielded 10 yr cardiovascular risks similar to those using diagnostic values obtained on office measurement\textsuperscript{5}. Ambulatory measures were obtained every 30 min while awake and every 60 min while asleep.

No subjects had any evidence of overt cardiovascular disease, aside from hypertension, or any evidence or diagnosis of associated comorbidities, including renal, pulmonary, neurological, or dermatological disease. Although elevated BP is often accompanied by the presence of additional cardiovascular risk factors\textsuperscript{6}, in order to more specifically isolate hypertension-related alterations in H\textsubscript{2}S-dependent vasodilation, only adults with high blood pressure in the absence of any additional cardiovascular risk factors were included in the present study. Women taking, or who had recently taken, hormone replacement therapy were excluded. Protocols were conducted on separate visits; before each visit, participants abstained from eating (4 hr), caffeinated and alcoholic beverages (12 hr), and strenuous physical activity (24 hr). All protocols were performed in a thermoneutral laboratory (22°C).

Quantification of endogenous expression and activity of H\textsubscript{2}S biosynthetic enzymes

Samples were homogenized in 1xPBS buffer containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) and centrifuged at 12,000g for 20 min to recover proteins. Protein concentration was determined using the BioRad Protein assay reagent. For western blot analysis, 1 μg of total protein were resolved by SDS-PAGE and electrotransferred to nitrocellulose membrane (Hybond-ECL; Amersham Life Sciences, Little Chalfont, UK). Antibodies used were rabbit polyclonal anti-CSE antibody (dilution 1:1000; Protein Tech, Chicago, IL, USA), rabbit polyclonal anti-3-MPST antibody (dilution 1:1000; Atlas Antibodies, Stockholm, Sweden) and mouse monoclonal anti-GAPDH antibody (dilution 1:5000; Novus Biologicals, Littleton, CO, USA). Rat liver homogenate was used as a positive control for CSE expression and rat carotid artery homogenate was used as a positive control for 3-MPST. Densitometry analysis was performed using ImageJ software (NIH, Bethesda, MD, USA). Protein expression data were normalized relative to GAPDH. For analysis, the average CSE and 3-MPST/GAPDH ratio in NTN samples was set to 100% and all samples were compared against this normalized value.

For CSE-mediated H\textsubscript{2}S production, 25 μl of homogenates were incubated with 2 mmol∙L\textsuperscript{-1} pyridoxal-5’-phosphate (PLP; Sigma-Aldrich Corp.) and 50 mol∙L\textsuperscript{-1} L-cysteine (Sigma-Aldrich Corp.) for 50 min at 37°C in 2 ml gas-tight vials (Fisher Scientific, Hampton, NH, USA). For MPST-mediated H\textsubscript{2}S production, homogenates were incubated with 1 mmol∙L\textsuperscript{-1} dithiothreitol (DTT; New England BioLabs, Ipswich, MA, USA) and 5 mmol∙L\textsuperscript{-1} MPST substrate 3-MP (Sigma-Aldrich Corp.), as above. Next, 200 μl of 10xPBS, adjusted to pH 6.0, was added to the
reaction to promote release of H₂S and then to stop the reaction. The reaction was incubated at 37°C for an additional 10 min. The head-space gas was then sampled to detect H₂S production. Experiments were performed in triplicate.

Assessment of the efficacy of AOAA as an inhibitor of H₂S production

In these experiments, we utilized mouse liver homogenates to examine the kinetics of H₂S production with increasing doses of L-cysteine in the presence or absence of AOAA (Fig. S1A; 5 μmol∙L⁻¹ = 5x IC₅₀), as previously described⁸⁻⁹. AOAA significantly blunted H₂S production ($V_{max}$: 1,777±109.4 (-) AOAA vs. 693.6±32.65 (+) AOAA nmol∙L⁻¹∙h⁻¹; $K_m$: 21.38±4.3 (-) AOAA vs. 71.04±7.7 (+) AOAA mM L-cysteine; P<0.05 for both). In cutaneous tissue homogenates from healthy adults, 5 μmol∙L⁻¹ AOAA effectively inhibited >70% of L-cysteine-dependent H₂S production (Fig. S1B). To overcome diffusion and bioavailability limitations that are encountered with intradermal microdialysis fibers, we elected to utilize 0.5 mmol∙L⁻¹ AOAA in in vivo experiments.
REFERENCES


Figure S1. *In vitro* amperometric measurement of tissue H$_2$S production, characterized in mouse liver homogenates (Panel A) and human cutaneous tissue homogenates (Panel B), in the presence (filled symbols) or absence (open symbols) of aminoxyacetic acid (AOAA). *P*<0.05 vs. (-) AOAA.