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Hydrogen sulfide depletion contributes to microvascular remodeling in obesity

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Hydrogen sulfide depletion contributes to microvascular remodeling in obesity. *Am J Physiol Heart Circ Physiol* 310: H1071–H1080, 2016. First published March 18, 2016; doi:10.1152/ajpheart.00062.2016.—Structural remodeling of the microvasculature occurs during obesity. Based on observations that impaired H2S signaling is associated with cardiovascular pathologies, the current study was designed to test the hypothesis that altered H2S homeostasis is involved in driving the remodeling process in a diet-induced mouse model of obesity. The structural and passive mechanical properties of mesenteric resistance arterioles isolated from 30-wk-old lean and obese mice were assessed using pressure myography, and vessel H2S levels were quantified using the H2S indicator sulfindfluor 7-AM. Remodeling gene expression was assessed using quantitative RT-PCR, and histological staining was used to quantify vessel collagen and elastin. Obesity was found to be associated with decreased vessel H2S concentration, inward hypertrophic remodeling, altered collagen-to-elastin ratio, and reduced vessel stiffness. In addition, mRNA levels of fibronectin, collagen types I and III, matrix metalloproteinases 2 and 9, and tissue inhibitor of metalloproteinase 1 were increased and elastin was decreased by obesity. Evidence that decreased H2S was responsible for the genetic changes was provided by experiments in which H2S levels were manipulated, either by inhibition of the H2S-generating enzyme cystathionine γ-lyase with Nα-propargylglycine or by incubation with the H2S donor GYY4137. These data suggest that, during obesity, depletion of H2S is involved in orchestrating the genetic changes underpinning inward hypertrophic remodeling in the microvasculature.

**NEW & NOTEWORTHY**

The current study shows that obesity is associated with the depletion of steady-state H2S levels in the microvasculature and that this alters the expression of genes involved in structural remodeling.

Microvascular disease is a consequence of obesity that often precedes (17, 18, 24, 25) and, indeed, reliably predicts more serious cardiovascular complications such as hypertension and type 2 diabetes (35, 41). Structural remodeling of the microvasculature has been well documented in obesity and undoubtedly contributes to impaired vessel function in microvascular disease (17). It is defined as inward (narrowing of the lumen) or outward (widening of the lumen) remodeling, which may or may not be associated with thickening (hypertrophic remodeling) or thinning (hypotrophic remodeling) of the vessel wall (32, 38). Inward hypertrophic remodeling has been the most commonly described form in the microvasculature of obese humans and animal models and is characterized by altered composition of the extracellular matrix (ECM), as well as smooth muscle cell proliferation and migration (8). ECM remodeling during obesity progresses by changes in the deposition of collagen, elastin, and fibronectin (42, 45, 50, 54), most likely driven by a shift in the balance between increased synthesis and reduced breakdown by matrix metalloproteinase (MMP) activity and/or increased tissue inhibitor of metalloproteinase (TIMP) activity (45, 50). In addition, the profibrotic growth factors connective tissue growth factor-β (CTGF) and transforming growth factor-β (TGFβ), which synergize to regulate collagen and fibronectin deposition (37), are also upregulated by obesity (34).

The mechanisms responsible for driving remodeling during obesity have not been fully defined; however, a number of pathophysiological stimuli are known to influence the remodeling process. Indeed, many of these, including increased vessel tone (5, 31), endothelial dysfunction (10), inflammation (40), and oxidative stress (33), are hallmarks of the obese microvasculature (8). It is intriguing that the gaseous signaling molecule H2S, an important mediator in the vascular system, is known to impinge on all these pathways (61). Although a role for H2S in regulating microvascular remodeling has not been described, there are precedents for its involvement. In a rat model of hypertension, characterized by increased aortic collagen and smooth muscle hypertrophy, the impaired ability to generate endogenous H2S was treated with a H2S donor, which reduced collagen deposition and smooth muscle cell proliferation (71). Similarly, H2S donors also decreased hypoxia-induced expression levels of collagen types I and III in rat pulmonary arteries (20) and reduced levels of MMPs to help normalize the MMP-to-TIMP ratio (20, 55, 56). Knockout of the cystathionine γ-lyase (CSE) gene, which encodes for the enzyme required for the majority of H2S production in the vascular system, has been described form in the microvasculature of obese humans and animal models and is characterized by altered composition of the extracellular matrix (ECM), as well as smooth muscle cell proliferation and migration (8). ECM remodeling during obesity progresses by changes in the deposition of collagen, elastin, and fibronectin (42, 45, 50, 54), most likely driven by a shift in the balance between increased synthesis and reduced breakdown by matrix metalloproteinase (MMP) activity and/or increased tissue inhibitor of metalloproteinase (TIMP) activity (45, 50). In addition, the profibrotic growth factors connective tissue growth factor-β (CTGF) and transforming growth factor-β (TGFβ), which synergize to regulate collagen and fibronectin deposition (37), are also upregulated by obesity (34).
murine macrophages (57), it is not known if H₂S levels in the microvasculature are affected by obesity or if H₂S serves to modulate the remodeling process. The objective of the current study was to assess the role of endogenous H₂S in regulating the structural properties of the microvasculature in obesity. We hypothesized that obesity-dependent H₂S depletion acts as an important stimulus for microvasculature remodeling. This was tested using pressurized mesenteric resistance vessels isolated from lean and diet-induced obese mice by examining the level of endogenous H₂S and its role in regulating remodeling gene expression.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice (Taconic Biosciences, Germantown, NY) were housed in the biological resource facility at Rosalind Franklin University. For the duration of the study, all mice were housed with a 12:12-h light-dark cycle in 12 × 6.25-inch cages with standard enrichment and ad libitum access to food and water. Mice were fed a high-fat (diet no. D12492; 60% of caloric intake from fat) or a control (diet no. D12450B; 10% of caloric intake from fat) diet (Research Diets, New Brunswick, NJ) from 6 wk of age until they were euthanized at 28–30 wk. Euthanasia was accomplished by inhalation of a lethal dose of CO₂ followed by cervical dislocation, according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with approval of the Institutional Animal Care and Use Committee of Rosalind Franklin University of Medicine and Science. For blood pressure measurements, mice were anesthetized with 2–5% isoflurane in oxygen for induction and 0.25–4% isoflurane in oxygen for maintenance. The left common carotid artery was isolated and cannulated with an indwelling polyethylene (PE-08) catheter (0.20 mm inner diameter) filled with heparinized saline. The catheter was connected to a pressure transducer (Grass Technologies, Warwick, RI) coupled to a Powerlab 4/30 amplifier, and the mean arterial blood pressure was continuously recorded for 15 min using LabChart 7 software (ADInstruments, Colorado Springs, CO).

Cell culture. Primary aortic vascular smooth muscle cells were isolated from mouse aorta as we described previously (58). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Mediatech, Manassas, VA) in a humidified 95% air-5% CO₂ atmosphere at 37°C. All experiments were carried out between passages 3 and 10.

Pressure myography. Third-order mesenteric arteries were isolated and transferred to a myograph chamber (Living Systems Instrumentation, Burlington, VT). Each end of the vessel was mounted onto a glass cannula and pressurized under zero-flow conditions. Experiments were performed in Ca²⁺-free Hanks’ balanced salt solution containing (mM) 137.9 NaCl, 5.33 KCl, 0.44 KH₂PO₄, 0.34 Na₂HPO₄, 5.56 glucose, 4.17 NaHCO₃, 2.29 MgCl₂, 0.41 MgSO₄, 10 HEPES, and 1 EGTA, with pH adjusted to 7.4 with NaOH. Intraluminal pressure was increased stepwise from 5 to 150 mmHg, and measurements of vessel diameter and wall thickness were used to calculate the passive structural and mechanical properties as previously described (46). In experiments requiring overnight incubation with drug or vehicle control, isolated arteries were maintained in serum-free 50:50 mix of DMEM and Ham’s F-12 medium (Mediatech) in a humidified 95% air-5% CO₂ atmosphere at 37°C.

H₂S measurement. Cytoplasmic H₂S concentration was monitored using the fluorescent probe sulfofluor 7-AM (SF7-AM, Sigma-Aldrich, St. Louis, MO). For single-cell imaging, vascular smooth muscle cells cultured in flat-optical-bottomed 96-well plates were incubated with SF7-AM (1 µM) in tissue culture medium at 37°C for 30 min; then the wells were washed with normal Hanks’ balanced salt solution and mounted on the stage of an inverted wide-field fluorescence microscope (model IX71, Olympus America, Center Valley, PA). The probe was excited at 490 nm, and the emitted fluorescence was filtered at 525 nm and recorded using a charge-coupled device-based imaging system running SimplePCI software (Hamamatsu, Sewickley, PA). To image intact mesenteric arteries, vessels were loaded with 5 µM SF7-AM by incubation for 30 min at 37°C before cannulation (see above) and pressurized to 20 mmHg. The myography chamber was positioned on the stage of an inverted microscope (model IX71), and the smooth muscle layer was visualized using a water-immersion objective (×60, 1.2 numerical aperture). SF7-AM was excited using the 488-nm line of a krypton-argon laser, and emitted fluorescence was filtered at 535 nm. Confocal image slices were acquired using a VT-Infinity3 laser confocal scanner (VisiTech International, Sunderland, UK), and the mean fluorescence intensity from individually resolved myocytes in each section was quantified using HCImage software (Hamamatsu). Free sulfide was measured using reverse-phase HPLC with fluorescence detection, as described in detail previously (49). To measure H₂S production capacity in vitro, mesenteric arteries were homogenized in buffer (10 µl/mg tissue) containing 20 mM HEPES and 1 mM EDTA, with pH adjusted to 7.4 with NaOH, and protease inhibitor cocktail (Invitrogen) on ice for 10 min. After centrifugation, pyridoxyl-5-phosphate (2 mM) and l-cysteine (10 mM) were added to supernatants with or without SF7-AM (10 µM) or DL-propargylglycine (PPG, 0.4 mM) in sealed Eppendorf tubes, which were incubated for 1 h at 37°C. Samples were then plated into wells of a 96-well plate, and fluorescence intensity was measured with a POLARstar Omega plate reader (BMG Labtech, Cary, NC).

Histology. Isolated mesenteric resistance arteries were immersed in optimal cutting temperature medium (Tissue-Tek OCT, Sakura Finetek USA, Torrance, CA) and snap-frozen. Blocks were sectioned at 10 µm and stained with Picro-Sirius Red or Verhoef-Van Gieson (Electron Microscopy Sciences, Hatfield, PA) for collagen and elastin, respectively, following the manufacturers’ protocols. Bright-field (Picro-Sirius Red and Verhoef-Van Geison) and polarized-light (Picro-Sirius Red) images were acquired and processed using ImageJ (47).

Quantitative RT-PCR analysis. Isolated mesenteric arteries were first frozen in liquid nitrogen. Total RNA was extracted using TRIzol (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol, and cDNA was synthesized from 1 µg of total RNA. Real-time quantitative RT-PCR (qRT-PCR) was performed using a ViiA7 thermocycler and Sybr Green PCR Master Mix (Applied Biosystems) and validated primers. Fluorescent signals generated during PCR amplifications were normalized to an internal reference (hypoxanthine-guanine phosphoribosyltransferase), the threshold cycle (Ct) was set within the exponential phase, and the relative quantitative evaluation of target gene levels was performed using the 2−ΔΔCt method.

Analysis and statistics. In all experiments, data were pooled from multiple trials carried out on cells or arteries originating from at least three different animals and are summarized as means ± SE. Differences between means were assessed using Student’s t-test for unpaired comparisons. For multiple comparisons, one- or two-way ANOVA was employed. For all tests, the differences between means were accepted as statistically significant at the 95% level (P < 0.05).

RESULTS

High-fat feeding causes increased body weight, blood glucose, and blood pressure in C57BL/6 mice. At 6 wk of age, mice were placed on a high-fat (60% of caloric intake from fat) or a control (10% of caloric intake from fat) diet. After 22–24 wk, body weight of lean animals fed the control diet was 36 ± 2 (SE) g, while animals fed the high-fat diet weighed 55 ± 1 g (n = 11; P < 0.001, by Student’s t-test). In addition, mean
arterial pressure was 84 ± 3 and 123 ± 3 (SE) mmHg in lean and obese mice, respectively (n = 4; P < 0.001, by Student’s t-test), and nonfasting blood glucose was 121 ± 7 and 155 ± 7 (SE) mg/dl in lean and obese mice, respectively (n = 11; P = 0.008, by Student’s t-test). These phenotypes are typical and widely reported for this strain on similar diets (15).

**Obesity causes inward hypertrophic remodeling and reduced vessel stiffness in mesenteric arterioles.** Pressure myography was used to assess the structural and passive mechanical properties of mesenteric arterioles isolated from lean and obese mice. Luminal diameter (Fig. 1A) and wall thickness (Fig. 1B) were measured across a range of physiological intraluminal pressures and used to calculate the medial cross-sectional area (CSA) and wall-to-lumen ratio (Fig. 1, C and D). Characteristic of inward hypertrophic remodeling (38), lumens were narrower, walls were thicker, and medial CSA was larger in vessels from obese mice, resulting in increased wall-to-lumen ratio compared with lean animals. Comparison of the stress-strain relationships and incremental elastic modulus (E\textsubscript{inc}) was used to assess mesenteric arteriolar stiffness in vessels isolated from lean and obese mice. Reduced vessel stiffness in arterioles from obese mice compared with those from lean mice was indicated qualitatively by a rightward shift in the stress-strain curve for obese mice (Fig. 2A), as well as a statistically significant decrease in E\textsubscript{inc} across the range of physiological pressures (Fig. 2B).

Vascular stiffness is highly dependent on the composition of the ECM, which is made up largely of collagen and elastin (66). The collagen and elastin content of mesenteric arterioles from lean and obese mice was quantified by staining fixed sections with Picro-Sirius Red and Verhoeff-Van Gieson to label collagen and elastin, respectively (Fig. 2, C and D). Obesity was associated with increased collagen and decreased elastin content, resulting in an increased collagen-to-elastin ratio compared with lean controls (Fig. 2, E–G). In addition, growth and remodeling indexes were 35% and 62%, respectively, in vessels from the obese mice.

**Obesity alters the expression profile of ECM-regulating genes in mesenteric arterioles.** In addition to collagen and elastin, fibronectin (51) and MMPs and TIMPs (59), as well as CTGF and TGF\(\beta\) (22, 34), have been implicated in the remodeling process. The expression profile of these genes in samples of mesenteric arterioles was examined using qRT-PCR. Consistent with the Picro-Sirius Red and Verhoeff-Van Gieson labeling, collagen type I and III expression was increased by obesity and elastin was decreased (Fig. 3A). In addition, compared with samples prepared from lean mice, fibronectin, CTGF, TGF\(\beta\), TIMP1, MMP2, and MMP9 expression levels were increased in obesity (Fig. 3, B and C).

**Obesity decreases steady-state \(H_2S\) concentration in mesenteric arterioles.** The fluorescent \(H_2S\) indicator SF7-AM was used to assess steady-state \(H_2S\) concentration in the mesenteric arterioles. Vessels from lean and obese mice loaded with SF7-AM were pressurized to 20 mmHg, and sections through the smooth muscle layer were captured (Fig. 4A). \(H_2S\) levels were decreased in arterioles from obese mice compared with lean controls (Fig. 4B). In a second set of experiments, the free sulfide in lysates of mesenteric arterioles was assessed by HPLC (49). Free sulfide was lower in samples prepared from the obese mice than in samples prepared from lean mice, consistent with the SF7-AM measurements (Fig. 4D). \(H_2S\) production in the vasculature is heavily dependent on the enzyme CSE (69). The protein levels of CSE in mesenteric arterioles assessed by Western blotting, however, were similar among lean and obese mice (Fig. 4D).

![Fig. 1. Mesenteric resistance vessels undergo inward hypertrophic remodeling in obesity. Passive structural properties of resistance vessels were measured using pressure myography.](http://ajpheart.physiology.org/)}
H$_2$S levels alter the expression profile of ECM-regulating genes in mesenteric arterioles. The CSE inhibitor PPG was used to further assess the role of H$_2$S in regulating remodeling gene expression. To validate the use of PPG, cultured aortic smooth muscle cells isolated from lean mice were treated with vehicle control or PPG for 4 h, and L-cysteine (the substrate for CSE) or vehicle control was added to cultures for 1 h before loading with SF7-AM. As expected, H$_2$S levels were depleted in the presence of PPG; moreover, additional H$_2$S production evoked by L-cysteine was effectively blocked by PPG (Fig. 5, A and B), consistent with its role as an inhibitor of CSE. To assess the effect of H$_2$S depletion on remodeling gene expression, mesenteric arterioles from lean and obese mice were isolated and placed in tissue culture overnight with or without PPG. Treatment with PPG increased expression of fibronectin, collagen types I and III, and elastin (Fig. 5C), as well as CTGF and TGFβ (Fig. 5D) and MMP2 and MMP9 (Fig. 5E), in the vessels. With the exception of elastin, the pattern of gene expression changes induced by H$_2$S depletion by PPG was qualitatively similar to that observed during obesity.

Exogenous H$_2$S reverses the effect of obesity on expression of a subset of ECM-regulating genes in mesenteric arterioles. There is evidence that H$_2$S can regulate gene expression in several pathways (48, 70), including ECM remodeling (20, 55, 56). We next examined the relationship between obesity-dependent H$_2$S depletion and changes in remodeling gene expression by experimentally increasing H$_2$S levels in mesenteric arterioles. Isolated vessels were placed in tissue culture overnight with or without the H$_2$S donor GYY4137 (43), and the expression levels of remodeling genes were determined on the following day by qRT-PCR. Incubation with GYY4137 significantly reduced the expression of fibronectin, collagen types I and III, CTGF, and MMP9 in vessels from the obese mice (Fig. 6A), previously shown to be upregulated by obesity (Fig. 3). Furthermore, the expression levels of these specific genes were not significantly different from those measured in lean controls (Fig. 6B). In contrast, TIMP1, MMP2, and MMP12 remained elevated in the presence of GYY4137, suggesting that H$_2$S exerts influence over a subset of remodeling genes.
DISCUSSION

The C57BL/6 mouse model of diet-induced obesity has been extensively characterized and exhibits phenotypes that mirror those of human metabolic syndrome (16), including visceral obesity, hyperglycemia, hyperinsulinemia, and hypertension (3, 26, 65). This model was employed in the current study to assess the effects of obesity on the passive structural and mechanical properties of mesenteric resistance arterioles. Obesity was found to be associated with an increased medial CSA, wall thickness, and wall-to-lumen ratio that, together with a decreased luminal diameter (Fig. 1), define the remodeling in these vessels as inward hypertrophic remodeling (8, 38, 46). Smooth muscle hypertrophy and hyperplasia typically account for increased medial thickening (21). The contribution of smooth muscle to hypertrophy in the current study is suggested by the calculated values of the growth and remodeling indexes (19). This remains speculative, however, in the absence of smooth muscle cell size and number measurements. Interestingly, we report smooth muscle cell H2S depletion during obesity (Fig. 4), and in light of previous work demonstrating that H2S depletion promotes smooth muscle cell proliferation (20, 68, 71), it would not be surprising if smooth muscle cell numbers were increased by obesity in the current study. While ours is the first study to describe remodeling in mesenteric vessels of the diet-induced obese mouse, the data are in agreement with previous studies using genetic models, which have reported hypertrophic remodeling in mesenteric vessels from New Zealand obese mice (30) and nonobese obese Zucker rats (7). These findings are also consistent with previous assessments of obesity-specific remodeling in human subcutaneous arterioles (11, 17, 18), as well as rat cerebral (12) and porcine coronary (54) vessels.

We also report for the first time that mesenteric vessel stiffness was decreased by obesity, as indicated by a rightward shift in the stress-strain relationship and decreased elastic modulus (Fig. 2, A and B), similar to that reported for human subcutaneous (17) and porcine coronary (54) vessels. The underlying cause of the decreased stiffness, however, could not be determined from the current dataset. Indeed, we observed an increased collagen-to-elastin ratio (Fig. 2, E–G), which has often been interpreted as a cause of increased, rather than decreased, vessel stiffness. The relationship between collagen, elastin, and stiffness, however, is likely to be more complex and to depend on the relative abundance and organization of collagen subtypes, as well as smooth muscle density and cytoskeletal remodeling (2, 32). The pattern of microvascular remodeling is also in contrast to that observed in larger conduit vessels. Here, obesity has been associated with increased vessel stiffness (6, 52) and linked to the development of hypertension (62). While microvascular remodeling is not a prerequisite for hypertension (22), it does shape the nature of remodeling, as evidenced by the finding that microvascular stiffness was decreased in hypertensive, but not normotensive, obese humans (17, 18). Indeed, this is consistent with the correlation between hypertension and decreased stiffness reported here.

A primary objective of the current study was to identify mechanisms responsible for driving the genetic changes involved in ECM regulation during obesity. To achieve this, we quantified the obesity-dependent changes in expression of a subset of genes involved in determining the composition and organization of the ECM (Fig. 3). We hypothesized that changes in H2S act as a major stimulus for genetic changes driven by obesity. In support of this, we discovered that endogenous steady-state levels of H2S were depleted by obesity (Fig. 4). Two distinct approaches were used to quantify H2S levels. The first employed the H2S-sensitive fluorescent probe SF7-AM (Fig. 4, A and B). The advantage of this technique is the high sensitivity and selectivity for H2S over other sulfur and reactive species (28). Our observation that H2S was depleted during obesity was supported by a second approach (Fig. 4E) that measured free sulfide using reverse-phase HPLC with fluorescence detection, developed by Christopher Kevil’s group at Louisiana State University Health Sciences Center (49).

Depletion of H2S is likely a result of decreased production or increased consumption. Physiologically, H2S can be generated through a number of enzymatic and nonenzymatic pathways, but it is generally well accepted that its synthesis in the peripheral vasculature is heavily dependent on CSE (39, 61,
Levels of CSE protein were determined in mesenteric vessels from lean and obese mice (Fig. 4D) but were found to be similar, suggesting that the ability to produce H2S was unaffected by obesity. This was confirmed experimentally by assay of CSE-dependent H2S production capacity in arterioles isolated from lean and obese animals (Fig. 4F). If we assume that other potential sources of H2S generation are not impaired by obesity, our data point to increased consumption as a possible cause of H2S depletion in mesenteric vessels. While the mechanism for H2S depletion in the current context remains elusive, it is worth noting that H2S depletion has been linked with inflammation by our group (57) as well as others (13). Furthermore, inflammation is associated with increased oxidative stress, and increased interactions between reactive oxygen species and H2S deplete H2S and account for decreased circulating levels in diabetic patients and animal models (23, 53). Based on these precedents, we speculate that increased consumption driven by inflammation and oxidative stress could represent the mechanism responsible for H2S depletion in mesenteric vessels.

The functional consequences of H2S depletion in microvasculature are likely to be many. Although our focus has been on remodeling and the regulation of gene expression, H2S is known to regulate contractility, either directly through its role as a vasodilator (39, 69) or by cross talk with nitric oxide (64). Although the role of H2S in mediating vessel contractility was not rigorously investigated in the current study, we did show that H2S-dependent vasorelaxation induced by L-cysteine was attenuated in obesity (Fig. 4C), suggesting that reduced availability of H2S may be involved in mediating the increased contractility reported in the microvasculature in obesity (3, 7, 17, 18, 30). Such a model would fit well with the concept that increased contractility is a major determinant of remodeling (5), suggesting a scheme in which H2S depletion influences remodeling through its effect on contractility. Alternatively, H2S depletion could be driving remodeling through effects on

Fig. 4. Steady-state H2S levels are reduced in mesenteric resistance vessels during obesity. A: representative confocal sections through the smooth muscle layer of pressurized arterioles isolated from lean and obese mice in the presence and absence of L-cysteine (0.5 mM) and labeled with the H2S indicator sulfidefluor 7-AM (SF7-AM). B: SF7-AM quantification. au, Arbitrary units. Values are means ± SE from multiple fields and arterioles isolated from 3 lean and 3 obese mice. Differences were compared by 1-way ANOVA. C: relaxation in response to cumulative addition of L-cysteine in vessels preconstricted with phenylephrine (10 μM). Values are means ± SE of 7 vessels from 3 lean animals and 6 vessels from 3 obese animals. Differences were compared by 2-way ANOVA. D: Western blot for cystathionine γ-lyase (CSE) protein in mesenteric arterioles. Tissue lysates were prepared from 3 individual lean (lanes 1–3) and 3 obese (lanes 4–6) mice. E: HPLC quantification of total sulfide. Values are means ± SE of arterioles isolated from 4 lean and 4 obese mice. *Statistical significance (by Student’s t-test). F: H2S production capacity in mesenteric vessel lysates incubated with L-cysteine (10 mM) and treated with or without DL-propargylglycine (PPG, 0.4 mM). Values are means ± SE. Differences were compared by 1-way ANOVA.
inflammation or oxidative stress (23, 53, 60), which have also been shown to facilitate vascular remodeling (4, 10, 27). Regardless of the stimulus, the progression of microvascular remodeling undoubtedly depends on a change of expression profile of the genes involved in ECM regulation. Our focus therefore was to define the genetic changes induced specifically by H2S depletion in obesity.

Obesity induced the upregulation of fibronectin, collagen types I and III, CTGF, TGFβ, TIMP1, MMP2, and MMP9 and the downregulation of elastin (Fig. 3). A role for H2S in mediating these changes was then established using two complementary approaches. 1) H2S was experimentally depleted in vessels from lean animals by treatment with the CSE inhibitor PPG, which has been widely used to reduce cellular H2S with few off-target effects (61) and validated by us using SF7-AM measurements (Fig. 5, A and B). 2) H2S levels were rescued in vessels from obese animals by treatment with the H2S donor GYY4137 (43).

Similar to the effect of obesity (Fig. 3A), PPG-dependent H2S depletion in vessels from lean mice resulted in elevated fibronectin and collagen types I and III (Fig. 5C). In vessels from the obese mice, GYY4137 treatment effectively reduced these genes (Fig. 6A) to levels not significantly different from those in lean controls (Fig. 6B). These data are consistent with previously reported effects of the H2S donors on collagen types I and III in vasculature (20, 71) and heart (36). The influence of H2S on elastin expression, however, was not as easily interpreted. A decreased expression was associated with obesity (Fig. 3A); however, expression levels were increased by H2S depletion in normal vessels (Fig. 5C), and GYY4137 was without effect in vessels from obese mice (Fig. 6B). These data would suggest that while elastin expression can be regulated by H2S, it may not likely to exert much influence in the obese microvasculature.

CTGF and TGFβ expression can be regulated by H2S (Fig. 5D). Importantly, we provide evidence for H2S-dependent regulation of these genes during obesity (Fig. 6). Treatment with GYY4137 significantly reduced CTGF in vessels from the obese mice (Fig. 6A). Although TGFβ was not significantly inhibited by GYY4137 (Fig. 6A), it was reduced to levels comparable to those observed in lean controls (Fig. 6B). Nevertheless, our demonstration that CTGF and TGFβ were upregulated in obesity and potentially under the influence of H2S-dependent regulation is significant because of their synergistic effects on smooth muscle cell proliferation and collagen and fibronectin synthesis (1, 14, 44). Interestingly, H2S depletion has been linked to increased smooth muscle cell proliferation (69). Although speculative, our observations suggest a possible mechanism in which smooth muscle cell pro-
liferation is stimulated by H$_2$S depletion-dependent CTGF and TGF$eta$ expression.

Although members of the MMP family number many more than were assessed in the current study, our focus on MMP2, MMP9, and MMP12 was guided by their known importance in remodeling (9), as well as reported expression sensitivity to H$_2$S (20, 55, 56). We were not surprised, then, to observe robust upregulation of MMP2 and MMP9 with obesity (Fig. 3C), which was recapitulated in H$_2$S-depleted vessels from lean animals (Fig. 5E). While levels of MMP2 did trend lower in arterioles from the obese mice treated with GYY4137, MMP9 was significantly reduced to levels comparable to those of lean controls (Fig. 6, A and B). We might have expected MMP2 expression to be more sensitive to H$_2$S, consistent with previous studies (55, 67) and our own observation that CSE inhibition increased its expression (Fig. 5E). Perhaps this reflects differences in sensitivity to H$_2$S, for which it is difficult to control in experiments designed to manipulate cellular H$_2$S levels pharmacologically. MMP12 was selected for inclusion in our panel because of its role in degrading elastin; however, its expression was unaffected by obesity or H$_2$S (Figs. 3–6), although its expression was previously shown to be downregulated in carotid arteries by the H$_2$S donor NaHS (56). By virtue of their effects on MMPs, TIMPs are also important regulators of remodeling (32). Expression levels of TIMP1 and TIMP2 were assessed, and TIMP1 was found to be upregulated by obesity (Fig. 3C). While CSE inhibition alone failed to increase TIMP1, the obesity-dependent increase was normalized by treatment with GYY4137 (Fig. 6), suggesting that H$_2$S does not act directly on expression during obesity. Taken together, our approach has enabled us to assess the effect of interactions between obesity and H$_2$S depletion on the regulation of key ECM remodeling genes and to conclude that H$_2$S depletion is a key step in reshaping the genetic landscape dictating microvascular remodeling.

In conclusion, structural remodeling of the microvasculature is a predictor of cardiovascular-related comorbidities in high-risk populations such as obese individuals (41). Furthermore, hypertrophic remodeling, even in the absence of hypertension or hyperglycemia, correlates with the worst prognosis (18). The current study established that obesity-driven inward hypertrophic remodeling of the microvasculature is associated with depletion of the gaseous signaling molecule H$_2$S and that this is a critical determinant of genetic changes underpinning the remodeling process.

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### Fig. 6. Exogenous H$_2$S decreases remodeling gene expression in mesenteric vessels from obese, but not lean, mice.

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<th>obese control</th>
<th>obese + GYY4137</th>
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<tr>
<td>FN1</td>
<td>1.0 (P=0.038)</td>
<td>0.5 (P=0.006)</td>
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<tr>
<td>COL1A1</td>
<td>1.0 (P=0.004)</td>
<td>0.5 (P=0.005)</td>
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<tr>
<td>COL3A1</td>
<td>1.0 (P=0.005)</td>
<td>0.5 (P=0.008)</td>
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<td>ELN</td>
<td>1.0 (P=0.001)</td>
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<td>CTGF</td>
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<td>TGF$eta$</td>
<td>1.0 (P=0.008)</td>
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*Statistical significance (by Student’s t-test).
HYDROGEN SULFIDE AND MICROVASCULAR REMODELING

GRANTS

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DISCLAIMERS

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AUTHOR CONTRIBUTIONS

J.C., G.V.V., and C.W. developed the concept and designed the research; J.C. performed the experiments; J.C. analyzed the data; J.C. and C.W. approved the final version of the manuscript.

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