

# Hydrogen Sulfide Attenuates sFlt1-Induced Hypertension and Renal Damage by Upregulating Vascular Endothelial Growth Factor

Kim M. Holwerda,\* Suzanne D. Burke,<sup>†</sup> Marijke M. Faas,<sup>‡</sup> Zsuzsanna Zsengeller,<sup>§</sup> Isaac E. Stillman,<sup>§</sup> Peter M. Kang,<sup>†</sup> Harry van Goor,\* Amy McCurley,<sup>||</sup> Iris Z. Jaffe,<sup>||</sup> S. Ananth Karumanchi,<sup>†</sup> and A. Titia Lely<sup>||</sup>

Divisions of \*Pathology and <sup>‡</sup>Biology, Department of Pathology and Medical Biology, and <sup>†</sup>Department of Obstetrics and Gynecology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; Departments of <sup>†</sup>Medicine and <sup>§</sup>Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts; and <sup>||</sup>Molecular Cardiology Research Institute, Tufts Medical Center, Boston, Massachusetts

## ABSTRACT

Soluble fms-like tyrosine kinase 1 (sFlt1), a circulating antiangiogenic protein, is elevated in kidney diseases and contributes to the development of preeclampsia. Hydrogen sulfide is a vasorelaxant and proangiogenic gas with therapeutic potential in several diseases. Therefore, we evaluated the potential therapeutic effect and mechanisms of action of hydrogen sulfide in an animal model of sFlt1-induced hypertension, proteinuria, and glomerular endotheliosis created by adenovirus-mediated overexpression of sFlt1 in Sprague-Dawley rats. We injected sFlt1-overexpressing animals intraperitoneally with the hydrogen sulfide-donor sodium hydrosulfide (NaHS) (50  $\mu$ mol/kg, twice daily) or vehicle ( $n=7$  per group). Treatment with NaHS for 8 days significantly reduced sFlt1-induced hypertension, proteinuria, and glomerular endotheliosis. Measurement of plasma protein concentrations with ELISA revealed a reduction of free plasma sFlt1 and an increase of free plasma vascular endothelial growth factor (VEGF) after treatment with NaHS. Renal VEGF-A mRNA expression increased significantly with NaHS treatment. *In vitro*, NaHS was proangiogenic in an endothelial tube assay and attenuated the antiangiogenic effects of sFlt1. Stimulation of podocytes with NaHS resulted in both short-term VEGF release (120 minutes) and upregulation of VEGF-A mRNA levels (24 hours). Furthermore, pretreatment of mesenteric vessels with a VEGF receptor 2-neutralizing antibody significantly attenuated NaHS-induced vasodilation. These results suggest that hydrogen sulfide ameliorates sFlt1-induced hypertension, proteinuria, and glomerular endotheliosis in rats by increasing VEGF expression. Further studies are warranted to evaluate the role of hydrogen sulfide as a novel therapeutic agent for vascular disorders such as preeclampsia.

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Elevated soluble fms-like tyrosine kinase 1 (sFlt1, also referred to as soluble vascular endothelial growth factor receptor 1) levels are associated with several diseases, including preeclampsia, vasculitis, and CKD.<sup>1–3</sup> In preeclampsia, a consistent line of evidence has shown that increased sFlt1 is one of the major contributors to the development of hypertension and proteinuria.<sup>4–7</sup> sFlt1 is a splice variant of the vascular endothelial growth factor (VEGF) receptor lacking the transmembrane and cytoplasmic domains and acts as a powerful antagonist of VEGF, thereby inhibiting VEGF signaling

in the vasculature.<sup>8–10</sup> Increased levels of circulating sFlt1 lead to functional VEGF deficiency, causing

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**Correspondence:** Dr. A. Titia Lely, Department of Obstetrics and Gynecology, University Medical Center Groningen, University of Groningen, Hanzeplein 1, PO Box 30001, 9713 GZ Groningen, The Netherlands. Email: [a.t.lely@umcg.nl](mailto:a.t.lely@umcg.nl)

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endothelial dysfunction, decreased angiogenesis, impaired capillary repair, and consequently hypertension and proteinuria.<sup>5,11</sup> Indeed, VEGF inhibitors used as part of cancer chemotherapy are associated with significant hypertension and proteinuria.<sup>12–14</sup> Moreover, genetic models of glomerular VEGF deficiency are also associated with proteinuria and glomerular endothelial damage.<sup>13,15</sup> Thus far, no targeted interventions to reduce circulating sFlt1 are available in the clinic. Although recombinant VEGF or placental growth factor have proven to be effective in experimental models, no clinical trials have yet been conducted.<sup>16–19</sup> sFlt1 removal using dextran sulfate apheresis has recently shown to be a possible new strategy in patients with very severe preterm preeclampsia.<sup>20</sup>

Hydrogen sulfide (H<sub>2</sub>S) belongs to a family of gasotransmitters, along with nitric oxide and carbon monoxide. The endogenously produced gas is important in physiologic processes, such as regulating arterial diameter, blood flow, and leukocyte adhesion.<sup>21</sup> H<sub>2</sub>S has been shown to enhance vasorelaxation through an endothelial cell-independent mechanism by acting on ATP-sensitive potassium (K<sub>ATP</sub>) channels.<sup>22,23</sup> However, K<sub>ATP</sub> channel blockers do not completely abolish H<sub>2</sub>S-induced relaxation, and H<sub>2</sub>S stimulates vasorelaxation in an endothelial cell-dependent manner at lower doses.<sup>24–27</sup> This suggests that H<sub>2</sub>S likely stimulates vasorelaxation through additional pathways that have not yet been elucidated.

In several animal models, endogenous activity of H<sub>2</sub>S-producing enzymes and exogenous H<sub>2</sub>S donors or precursors protect against ischemia and reperfusion injury and exhibit anti-inflammatory activity.<sup>28–31</sup> The proangiogenic effect of H<sub>2</sub>S was first described by Cai *et al.*, who showed that an exogenously administered H<sub>2</sub>S donor (sodium hydrosulfide [NaHS]) promoted proliferation, migration, and tube-like structure formation in endothelial cells *in vitro*.<sup>32</sup> *In vivo*, it was demonstrated that H<sub>2</sub>S is a proangiogenic factor in a model of hind limb ischemia.<sup>33</sup> These effects were associated with an increase in VEGF expression and activation of vascular endothelial growth factor receptor (VEGFR)2 signaling in vascular endothelial cells, suggesting that the effects of H<sub>2</sub>S may be mediated by VEGF and its receptor VEGFR2.<sup>33</sup> Bir *et al.* recently showed that H<sub>2</sub>S-stimulated ischemic vascular growth is dependent on augmented expression and activity of VEGF.<sup>34</sup>

We therefore hypothesized that by up-regulating VEGF, H<sub>2</sub>S may directly antagonize the detrimental effects of sFlt1 on the endothelium, consequently attenuating the development of hypertension and proteinuria. In this study, we demonstrate a protective effect of exogenous H<sub>2</sub>S administration in a rat model with overt hypertension and proteinuria induced by sFlt1 overexpression.

## RESULTS

### H<sub>2</sub>S Ameliorates Hypertension and Proteinuria in sFlt1-Transfected Rats

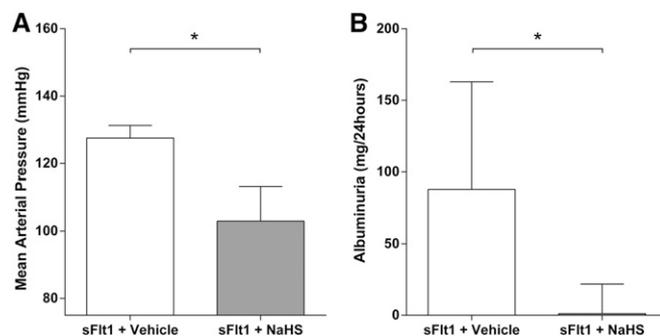
To evaluate the *in vivo* effect of exogenous H<sub>2</sub>S on BP and proteinuria, we administered 50 μmol/kg NaHS (H<sub>2</sub>S-donor) twice daily to sFlt1-overexpressing rats. BPs measured by invasive means were significantly lower in the NaHS-treated group (103 mmHg; interquartile range [IQR], 98–113 mmHg) compared with the control vehicle-treated group (128 mmHg; IQR, 124–131) (*P*<0.003; Figure 1A). These findings were also confirmed by continuous telemetry measurements during 8 days of NaHS or placebo treatment (Supplemental Figure 1). Similarly, we noted a significant and dramatic reduction in proteinuria after treatment with NaHS (Figure 1B). The sFlt1-injected rats developed a median albuminuria of 88 mg/24 h (IQR, 10–163), whereas albuminuria was almost normalized at 1 mg/24 h in the sFlt1-injected rats treated with NaHS (IQR, 0.3–22) (*P*<0.05).

### NaHS Improves Renal Glomerular Endotheliosis

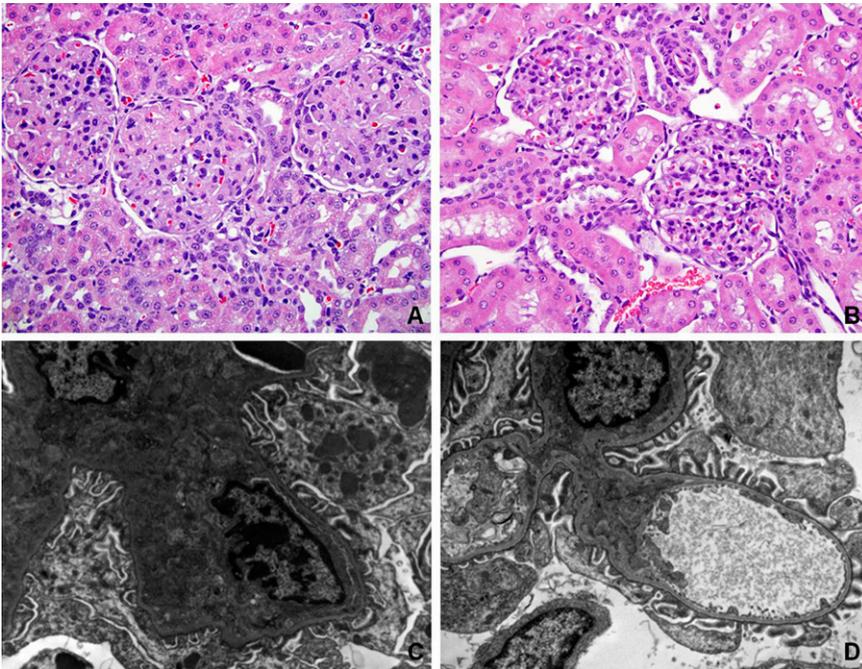
Similar to previously published data, animals overexpressing sFlt1 demonstrated marked glomerular endotheliosis with occlusion of capillary lumens (Figure 2A).<sup>5,16</sup> Interestingly, kidneys from NaHS-treated animals had more open capillaries and less glomerular damage with absence of proteinaceous deposits and distinct mesangium (Figure 2B). Electron microscopy confirmed that glomerular endotheliosis was ameliorated in NaHS-treated animals compared with vehicle-treated rats (Figure 2, C and D).

### Proangiogenic Effects of Hydrogen Sulfide *In Vitro*

sFlt1 is able to induce an antiangiogenic state *in vitro*.<sup>5,6</sup> Figure 3 shows that incubation of human umbilical vascular endothelial cells (HUVECs) with 1 μg sFlt1 resulted in a



**Figure 1.** Effect of NaHS on hypertension and proteinuria in rats overexpressing sFlt1. (A) Mean arterial pressure is measured via carotid catheterization under anesthesia after 8 days of treatment with either vehicle or 50 μM/kg NaHS. BP is significantly lower in the NaHS-injected group compared with vehicle. (B) After 7 days of treatment with vehicle or NaHS, rats are placed in metabolic cages to collect 24-hour urine and total albumin is determined. NaHS-treated rats show significant lower albuminuria than vehicle-treated rats. Data are presented as the median (IQR). *n*=7 per group. \**P*<0.05.



**Figure 2.** NaHS reverses glomerular endotheliosis in rats overexpressing sFlt1. (A) Histopathologic analysis of renal tissue from one representative vehicle-treated rat overexpressing sFlt1 shows marked glomerular endotheliosis with occlusion of capillary lumens. (B) Histopathologic analysis of renal tissue from one representative NaHS-treated rat overexpressing sFlt1 (50  $\mu$ M/kg) shows open capillaries with absence of proteinaceous deposits and mesangium that is now distinct (stained with hematoxylin and eosin). Electron microscopy is performed for the same rats as shown in A and B. (C) Representative electron micrographs of glomeruli from a vehicle-treated rat overexpressing sFlt1 confirm glomerular endotheliosis. (D) Representative electron micrographs of glomeruli from a NaHS-treated rat overexpressing sFlt1 show ameliorated glomerular endotheliosis. Original magnification,  $\times 40$  in B;  $\times 10,000$  in D.

significantly reduced capacity to form tube-like structures (52.6% reduction with respect to the control; IQR, 80.2–25.0) ( $P < 0.05$ ). Treatment of sFlt1-incubated HUVECs with 600  $\mu$ M NaHS enhanced capillary-like structure formation by 27.0% (IQR, 81.3–15.7) compared with sFlt1 ( $P < 0.05$ ).

#### Proangiogenic Effects of Hydrogen Sulfide *In Vivo*

We first confirmed that the sFlt1 ELISA kit used in our studies measured only unbound mouse sFlt1 by generating a standard curve for sFlt1 protein in the presence of recombinant rat VEGF (Figure 4A). Using this ELISA system, we measured free sFlt1 in the plasma of the NaHS- and vehicle-treated rats (Figure 4B). Before treatment, similar concentrations of free sFlt1 in the plasma of both groups were shown (2097 ng/ml [IQR, 1297–3746] versus 2745 ng/ml [IQR, 2383–3430]) ( $P = \text{NS}$ ). After treatment, free sFlt1 concentrations significantly decreased in the NaHS-treated group compared with the levels before the start of treatment. On the other hand, free sFlt1 concentrations in the vehicle-treated group did not differ from those before treatment. Whereas the free sFlt1 in the plasma of the NaHS-treated rats decreased to 39 ng/ml (IQR, 10–1168), plasma sFlt1 in the vehicle-treated rats remained high at 1547

ng/ml (IQR, 996–3370) ( $P < 0.05$ ; Figure 3B). Compared with the pretreatment levels of free plasma sFlt1, levels of the NaHS-treated rats showed a reduction of 91% (IQR, 45–99), whereas there was a small reduction of 35% (IQR, 0–56) over time in the vehicle-treated rats ( $P < 0.05$ ). These findings were accompanied by increased circulating free VEGF levels in animals treated with NaHS (2.6 pg/ml; IQR, 0.2–19) compared with the vehicle group (0.0 pg/ml; IQR, 0.0–0.1) ( $P < 0.05$ ; Figure 4C, right panel). Free plasma VEGF levels after treatment with NaHS negatively correlated to the free plasma sFlt1 measured after treatment with NaHS ( $r = -0.79$ ;  $P < 0.05$ ). Finally, we also noted upregulation of the VEGF-A mRNA level in the kidneys after treatment with NaHS. Relative renal VEGF-A mRNA expression in vehicle-treated rats was  $6.1 \cdot 2^{-\Delta\text{CT}}$  (IQR, 4.7–6.8), whereas the NaHS-treated group showed significantly higher VEGF-A mRNA levels of  $6.9 \cdot 2^{-\Delta\text{CT}}$  (IQR, 6.2–7.5) (Figure 4C, left panel) ( $P < 0.05$ ).

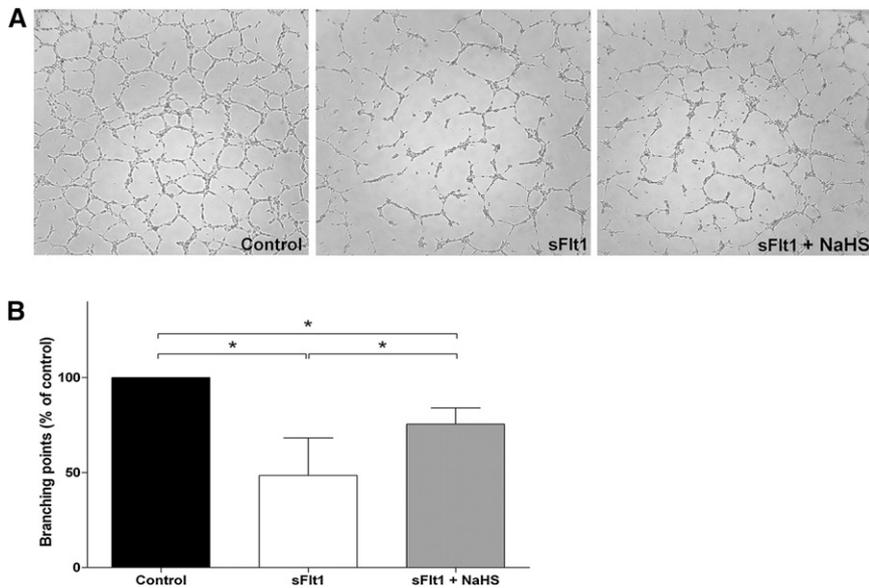
#### NaHS Causes VEGF Release and Upregulation of VEGF mRNA by Podocytes *In Vitro*

To study the short- and late-term effects of NaHS on VEGF protein and mRNA *in vitro*, podocytes were stimulated with NaHS.

Both VEGF protein release (short term) and production of VEGF mRNA (late term) were evaluated. As shown in Figure 5A, NaHS treatment resulted in a significant increase of VEGF protein release by podocytes (210 pg/ml [IQR, 149–265] for 1000  $\mu$ M NaHS compared with 97 pg/ml [IQR, 116–130] for PBS) ( $P < 0.05$  after 120 minutes). After 60 minutes, an increase was observed at both 100 and 1000  $\mu$ M NaHS, but this effect was borderline significant ( $P = 0.07$ ). VEGF mRNA was upregulated after 24-hour stimulation with 100  $\mu$ M NaHS ( $0.84 \cdot 2^{-\Delta\text{CT}}$ ; IQR, 0.64–1.60) compared with PBS ( $0.46 \cdot 2^{-\Delta\text{CT}}$ ; IQR, 0.36–0.69) ( $P < 0.05$ ; Figure 5B). After 60 and 120 minutes, no effects of NaHS on VEGF mRNA levels were observed.

#### NaHS-Mediated Vasodilation Is Dependent on VEGF Signaling

To explore whether the vascular effect of NaHS is dependent on VEGF signaling, we evaluated the vasodilatory properties of NaHS in the presence of VEGFR2-neutralizing antibody in mesenteric microvessels from mice. After precontraction of the vessels with phenylephrine, VEGF induces vasorelaxation in a dose-dependent manner (Figure 6A). This response is significantly inhibited in the presence of a VEGFR2



**Figure 3.** NaHS is proangiogenic *in vitro*. (A) An endothelial tube assay is performed using recombinant sFlt1 or NaHS (600  $\mu$ M) to treat the cells. A representative experiment is shown for the following conditions: control, recombinant sFlt1, and recombinant sFlt1 in combination with NaHS. (B) Quantification of the endothelial tube assay. The tubes are quantified by counting branching points and are normalized to the control condition (100%). Statistical analysis is performed using the Kruskal–Wallis test. After administration of recombinant sFlt1 to the endothelial cells, significantly decreased angiogenesis is shown. NaHS is able to restore this decrease, but angiogenesis in the sFlt1 plus NaHS condition is still significantly lower compared with control. Experiments were repeated five times. Data are presented as the median (IQR). \* $P < 0.05$ .

neutralizing antibody. Figure 6B shows that NaHS-dependent vasodilation is significantly attenuated in vessels pretreated with the same concentration of VEGFR2-neutralizing antibody.

## DISCUSSION

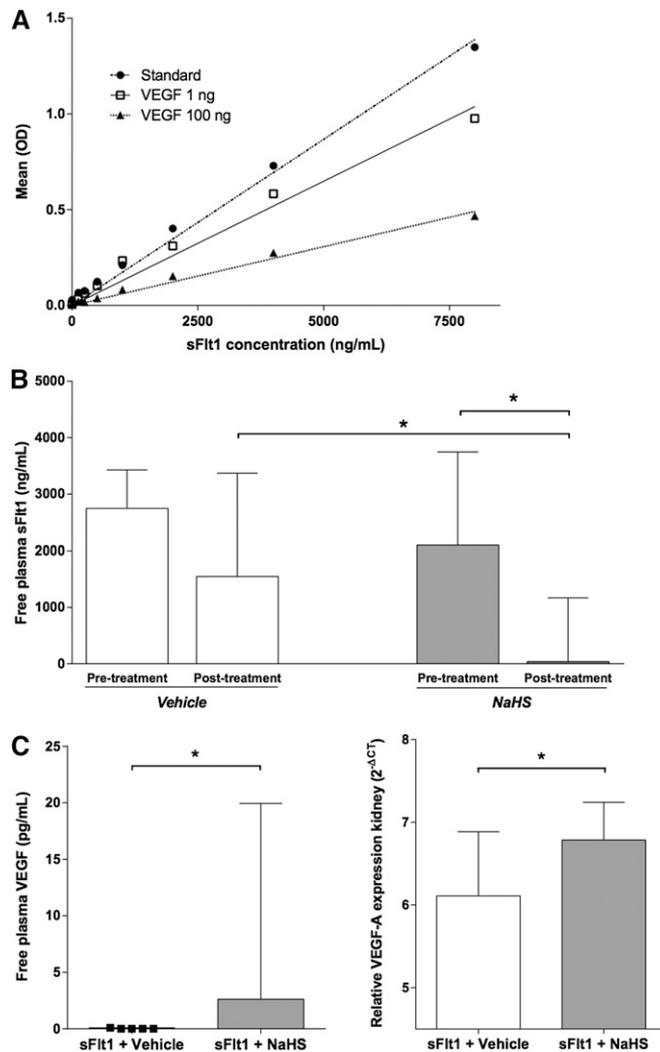
A consistent line of evidence has shown that excess sFlt1 antagonizes VEGF and consequently contributes to endothelial dysfunction.<sup>3,5,6,35,36</sup> H<sub>2</sub>S is known for its proangiogenic, antihypertensive, and organ protective properties.<sup>28,37,38</sup> In this study, we investigated the effects of exogenously administered H<sub>2</sub>S in a rat model for severe endothelial dysfunction, induced by overexpression of sFlt1. As previously described, elevation of plasma sFlt1 by adenoviral overexpression resulted in hypertension, proteinuria, and glomerular endotheliosis. We noted that administration of the H<sub>2</sub>S-donor NaHS markedly attenuated hypertension and proteinuria induced by sFlt1 and that this effect was associated with upregulation of tissue and systemic VEGF by NaHS.

To the best of our knowledge, this is the first study providing evidence that H<sub>2</sub>S is able to lower free plasma sFlt1 concentrations and attenuate the detrimental vascular phenotype of

high circulating sFlt1. Although H<sub>2</sub>S could have had direct effects on sFlt1, it is more likely that reduction of free sFlt1 is due to an increase of VEGF by H<sub>2</sub>S. Indeed, we found an upregulation of free plasma VEGF after treatment with H<sub>2</sub>S. These free plasma levels are negatively correlated with free plasma sFlt1 levels, suggesting that the balance can indeed be shifted from antiangiogenic to proangiogenic by H<sub>2</sub>S. In addition to increased plasma VEGF, we also found increased VEGF mRNA expression in the kidney. We are the first to show that VEGF release is increased by H<sub>2</sub>S in podocyte cell culture after 2 hours. The mechanism of upregulation requires further studies, because we found increased release of VEGF in the absence of an increased mRNA expression after 2 hours of incubation with H<sub>2</sub>S. Possible mechanisms include a role for matrix metalloproteinases, which are known to modulate VEGF release from the inside of the cell. Besides increased plasma VEGF, we found increased VEGF mRNA expression in the kidney. This may also be a direct effect of H<sub>2</sub>S, because we found increased VEGF mRNA expression in our culture model after 24 hours of H<sub>2</sub>S incubation compared with vehicle incubation. Our VEGF mRNA data are in agreement with

previous experiments showing that H<sub>2</sub>S is able to induce VEGF expression; *e.g.*, Bir *et al.* showed that this is mediated via hypoxia-inducible factor 1- $\alpha$ .<sup>33,34</sup>

In addition, we confirmed the proangiogenic effect of H<sub>2</sub>S *in vitro*. Maynard *et al.* revealed that sFlt1 inhibits capillary formation of endothelial cells in an *in vitro* angiogenesis assay.<sup>5</sup> We showed that administration of H<sub>2</sub>S to sFlt1-stimulated endothelial cells prevented the disrupted tube formation. These data support previous observations that H<sub>2</sub>S has proangiogenic effects.<sup>32</sup> When administered twice daily, H<sub>2</sub>S induced a significant reduction in BP in the adv-sFlt1 infected model. Importantly, in the majority of the rats, mean arterial pressure was determined at the end of the experiment under anesthesia. To exclude the BP-lowering effects of anesthesia, we also confirmed the antihypertensive phenotype of hydrogen sulfide using telemetry, which has been shown to be more reliable. Moreover, BP-lowering effects of H<sub>2</sub>S have also been previously reported in various other models of both experimental and spontaneous hypertension in rats.<sup>38–40</sup> It is known that H<sub>2</sub>S-induced vasorelaxation is mainly mediated by the opening of K<sub>ATP</sub> channels in vascular smooth muscle cells.<sup>22</sup> However, in this study, the antihypertensive effect of H<sub>2</sub>S is accompanied by an increase in plasma VEGF, which itself is a vasodilator.<sup>41</sup> Therefore, we propose that H<sub>2</sub>S provides a



**Figure 4.** Effect of NaHS on sFlt1 and VEGF expression in rats. (A) A standard curve for recombinant VEGF protein is generated in the absence (standard) or in the presence of 1 or 100 ng VEGF using a murine ELISA for measurement of sFlt1 levels as described in Concise Methods. (B) Free plasma sFlt1 (ng/ml) before and after 8 days of treatment with either vehicle or NaHS. NaHS-treated rats (50  $\mu$ M/kg) show a significant reduction in free plasma sFlt1 after treatment, whereas vehicle-treated rats do not. The free plasma sFlt1 levels in the NaHS-treated rats are significantly lower compared with the vehicle group. (C) Free plasma VEGF (pg/ml) after 8 days of treatment with NaHS (left). After treatment with NaHS, VEGF levels are significantly higher compared with vehicle treatment. Renal VEGF mRNA levels are shown with respect to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT; right). Renal VEGF mRNA expression is significantly higher in NaHS-treated rats compared with the vehicle group.  $n=7$  per group. Data are presented as the median (IQR). \* $P<0.05$ .

vasorelaxant effect by not only opening  $K_{ATP}$  channels but also through interacting with the VEGF signaling pathway. Indeed, we revealed that  $H_2S$  vasodilation is dependent on VEGF signaling because blockade of VEGFR2 attenuates  $H_2S$ -mediated vasodilation. Interestingly, a recent publication shows that VEGFR2 functions as a  $H_2S$ -targeting receptor in promoting endothelial cell migration.<sup>42</sup> Therefore, it is possible that  $H_2S$

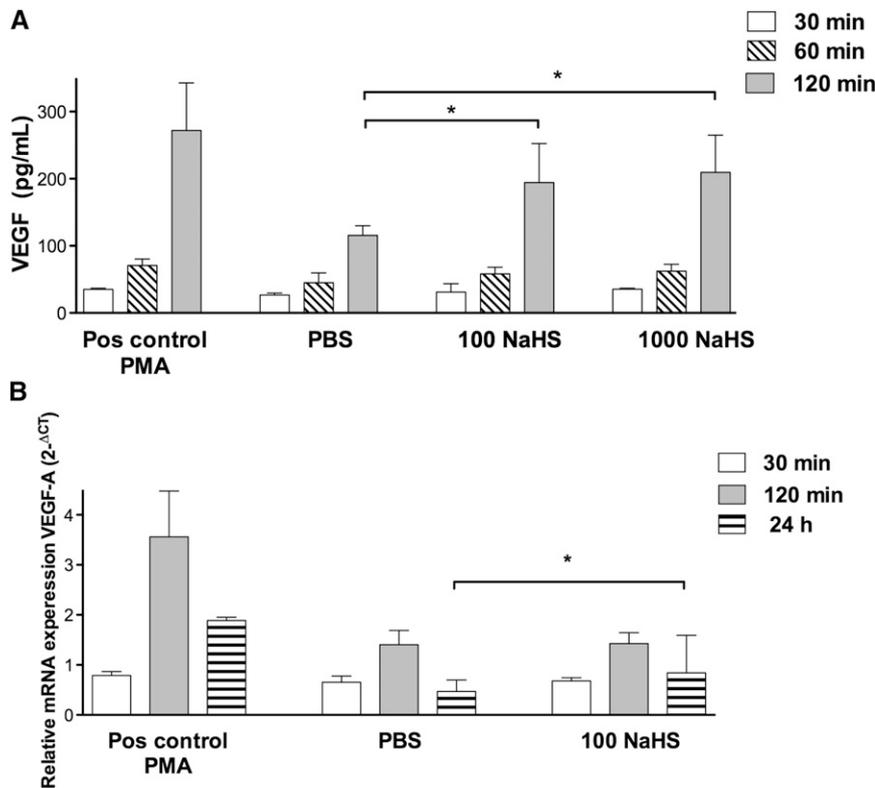
ameliorates hypertension in the sFlt1 over-expression model partly by upregulation of VEGF and/or direct action on VEGFR2.

Additional mechanisms by which  $H_2S$  induced vasorelaxation should also be considered in our study. Vasodilation by interaction of  $H_2S$  with the vasoactive agent nitric oxide (NO) has been reported.<sup>34,41,43</sup> However, we did not find that  $H_2S$  treatment caused significant changes in NO metabolite levels in plasma and urine (Supplemental Figure 2). This suggests that NO was not a major player in the vasodilatory effects of  $H_2S$  in our model. However, it is unknown whether the activity of the NO synthases was altered. Furthermore, Lu *et al.* showed that  $H_2S$  is able to lower BP by inhibiting plasma renin activity. Whether this is the case in the present model remains to be elucidated. We also do not know whether  $H_2S$  will downregulate endothelin synthesis, which was recently shown to be a key downstream signaling pathway that mediates sFlt1-induced vascular disease.<sup>44</sup> Finally,  $H_2S$  is known for its antioxidative and ischemia/hypoxia protective features.<sup>45</sup> Because massive sFlt1 production is thought to be secondary to hypoxia, we suggest that cytoprotection could be another protective effect of  $H_2S$  in the sFlt1-induced phenotype.<sup>46</sup>

This study revealed a significant reduction in sFlt1-induced proteinuria and kidney damage by administration of exogenous  $H_2S$ . The decrease of hypertension in the present model may have induced the reduction in proteinuria, because hypertension itself may induce kidney damage that in turn results in proteinuria. On the other hand, higher VEGF induced by NaHS could be directly protective for the kidney. This is in line with data of Eremina *et al.*, who showed that mice with reduced production of VEGF by podocytes exhibit massive proteinuria and glomerular endotheliosis,<sup>13</sup> suggesting the presence of a direct effect of sFlt1 on the development of proteinuria. We revealed an increased VEGF-

A mRNA expression in kidneys during  $H_2S$  treatment, implying a renal BP-independent protective effect on the kidney by NaHS.

In addition to an antiangiogenic state due to high sFlt1 in preeclampsia, there are also alterations in the  $H_2S$  pathway in this disease.<sup>47,48</sup> Preeclampsia is associated with elevated levels of homocysteine, a key protein in the trans-sulfurization



**Figure 5.** Stimulation of human podocytes with NaHS causes VEGF release and upregulation of VEGF mRNA. (A) Human podocytes treated with PMA (positive control, 50 ng/ml), PBS (control), 100  $\mu$ M NaHS, or 1000  $\mu$ M NaHS. VEGF (pg/ml) release is increased after 120 minutes of stimulation in the 100 and 1000  $\mu$ M NaHS-treated groups compared with the PBS-treated group. (B) After 24 hours of stimulation with 100  $\mu$ M NaHS, an increase in VEGF-A mRNA is present compared with PBS (control). Data are presented as the median (IQR). \* $P < 0.05$ .  $n = 5$  per group. Pos, positive control; PMA, phorbol 12-myristate 13-acetate.

pathway and a precursor of H<sub>2</sub>S.<sup>49,50</sup> The enzymes cystathionine- $\beta$ -synthase (CBS) and cystathionine- $\gamma$ -lyase (CSE) are involved in catalyzing homocysteine and cysteine, respectively, as well as in producing H<sub>2</sub>S. Genetic deficiency of CBS leads to homocysteinemia, which is associated with endothelial dysfunction and hypertension,<sup>51</sup> whereas CSE-deficient mice suffer from hypertension.<sup>52</sup> Interestingly, the expression of CBS and CSE in women with preeclampsia is decreased in the placenta.<sup>47,48</sup> The administration of H<sub>2</sub>S had the same effect on sFlt1 levels in pregnant rats, compared with nonpregnant rats (data not shown). Importantly, pups from NaHS-treated mothers showed no apparent dysmorphic characteristics and there were no adverse effects of NaHS on placental and pup weight (Supplemental Figure 3). In view of these results, we propose that H<sub>2</sub>S could be a possible treatment for preeclampsia. Future studies should further explore the protective effects of NaHS in pregnant rats with overexpression of sFlt1.

In summary, we have demonstrated that administration of exogenous H<sub>2</sub>S ameliorates sFlt1-induced vascular disease by promoting VEGF upregulation. These findings may have important implications in developing novel therapeutics to treat

preeclampsia or to reverse VEGF inhibitor side effects in cancer patients. Additional studies using H<sub>2</sub>S in pregnant models of preeclampsia and growth restriction are warranted to evaluate safety and biologic efficacy before proceeding with human trials.

## CONCISE METHODS

### Animal Model and NaHS Treatment

All animal protocols were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. Sprague-Dawley female rats (200–250 g) were intravenously injected into the tail vein with  $1 \times 10^{10}$  PFU/kg of adenovirus expressing sFlt1. The recombinant adenovirus expressing murine sFlt(1–3) was previously described,<sup>53</sup> and was amplified at a commercial facility (Vector Biolabs, Philadelphia, PA). Within 72 hours, plasma mouse sFlt1 levels were confirmed using a mouse sFlt1 ELISA kit (R&D Systems, Inc., Minneapolis, MN) and animals were stratified to the treatment or control groups. Both groups were treated twice daily with intraperitoneal injections for 8 days with either 50  $\mu$ M/kg NaHS (Sigma-Aldrich, St. Louis, MO) ( $n = 7$ ) or PBS vehicle ( $n = 7$ ). Twenty-four hours before termination, the rats were housed in metabolic cages. Terminal BPs were measured *via* carotid catheterization under anesthesia immediately before harvesting blood and kidneys, as previously described.<sup>5</sup>

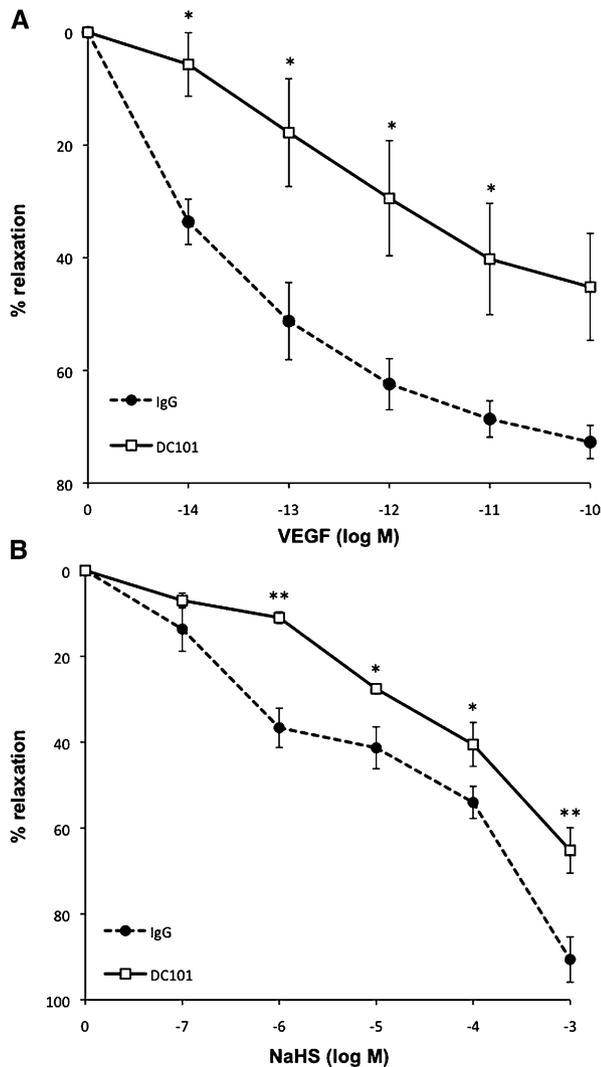
In a similar design, NaHS or vehicle was administered to pregnant sFlt1-infected rats. After sFlt1 infection, rats were stratified to treatment and control groups and intraperitoneally injected twice daily for 8 days with either 50  $\mu$ M/kg NaHS or PBS. Pups and placentae were analyzed to assess NaHS toxicity to pregnancy.

### Histopathology and Electron Microscopy

Harvested kidneys were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. For electron microscopy, renal tissue was embedded in araldite-Epon mixture; 1- $\mu$ m sections were stained with methylene blue and assessed before ultrastructural study.

### Endothelial Tube Assay

HUVECs (20,000 cells per 200  $\mu$ l) were plated onto Matrigel (9.0 mg/ml; BD Biosciences, Bedford, MA) coated wells with or without 1  $\mu$ g recombinant sFlt (R&D Systems, Inc.) and treated with 600  $\mu$ M NaHS or PBS. After 8 hours, tube formation was assessed through an inverted phase contrast microscope at  $\times 4$  (Nikon Corporation, Tokyo, Japan) and was quantitatively analyzed (number of branching points) using ImageJ software (National Institutes of Health, Bethesda, MD). The experiment was repeated five times.



**Figure 6.** The role of VEGF signaling in NaHS-mediated vascular relaxation. (A) Treatment with the VEGFR2 neutralizing antibody DC101 (50  $\mu\text{g/ml}$ ) attenuates VEGF-mediated relaxation compared with vehicle-treated vessels (IgG antibody; 50  $\mu\text{g/ml}$ ). (B) Treatment with DC101 (50  $\mu\text{g/ml}$ ) attenuated NaHS-mediated relaxation compared with vehicle-treated vessels (IgG antibody; 50  $\mu\text{g/ml}$ ). Responses are expressed as the percentage of relaxation from precontraction and each value represents the mean  $\pm$  SEM.  $n=3-4$  mice per group. \* $P<0.05$  vehicle-treated versus DC101-treated mice; \*\* $P<0.01$  vehicle-treated versus DC101-treated mice.

#### Stimulation of Podocytes with NaHS *In Vitro*

Established lines of human glomerular visceral epithelial cells (GVECs/podocytes) were used.<sup>54</sup> Human GVECs were seeded into 12-well plates and treated with 100 or 1000  $\mu\text{M}$  NaHS or PBS. Phorbol 12-myristate 13-acetate (Sigma-Aldrich) was used as a positive control for VEGF upregulation (50 ng/ml). After 30, 60, and 120 minutes, medium was removed and VEGF concentrations were measured using ELISA (R&D Systems, Inc.). In a parallel experiment, podocytes were harvested and stored for mRNA isolation and

real-time RT-PCR after 30 minutes, and 2 and 24 hours. All experiments were repeated five times.

#### ELISA

ELISA kits were used to measure plasma mouse sFlt-1 and rat and human VEGF (R&D Systems, Inc.). Urinary albumin was determined using the Exocell Nephurat kit (Philadelphia, PA).

#### RNA Isolation and Real-Time RT-PCR

Total RNA from whole rat kidneys and human GVECs was extracted using Trizol (Invitrogen, Carlsbad, CA), and cDNA was synthesized using Superscript II RT and random hexamer primers (Invitrogen). VEGF-A mRNA was quantified with TaqMan real-time quantitative RT-PCR using the VEGF-A gene expression assays (Rn01511605\_m1 [rat] and Hs00900055\_m1 [human]; Applied Biosystems, Foster City, CA) with hypoxanthine-guanine phosphoribosyltransferase as a reference gene in each reaction. The average Ct values for the target gene VEGF-A were divided by the average housekeeping gene, generating a  $2^{-\Delta\text{CT}}$  value.

#### Mesenteric Vessel Wire Myograph Studies

Rings from second-order mesenteric resistance arteries were harvested from male wild-type C57BL/6 mice and mounted (Danish Myo Technology Aarhus, Denmark) for isometric tension recordings using PowerLab software (AD Instruments, Dunedin, New Zealand) as previously described.<sup>55</sup> Concentration-response relaxation curves were built in the presence of VEGFR2 neutralizing antibody DC101 (50  $\mu\text{g/ml}$ ) or IgG antibody (50  $\mu\text{g/ml}$ ) by precontracting vessels with phenylephrine at 10  $\mu\text{M}$  before administration of VEGF and NaHS. Data from two to four rings per mouse were averaged, with three to four mice for each wire myograph study.

#### Implantation of Telemetric Devices and Telemetric Data Acquisition

Systolic, diastolic, and mean arterial pressures were measured using TA11PA-C40 radiotransmitters (Data Sciences International, St. Paul, MN). The transmitter catheter was surgically secured in the lower abdominal aorta. After 7 days of recovery, continuous data collection was started using the Dataquest A.R.T. Acquisition System (Data Sciences International). All hemodynamic data were analyzed using 24-hour means.

#### Measurement of Plasma and Urine Nitrite/Nitrate

The stable end products of NO, nitrite and nitrate were measured in plasma and urine according to the method described by Moshage *et al.*<sup>56</sup>

#### Statistical Analyses

Results are presented as the median (IQR) or mean  $\pm$  SEM. Between-group comparisons were made using the Mann-Whitney *U* test and Wilcoxon signed rank test, and within-group differences were assessed with two-factor repeated-measures ANOVA with the Newman-Keuls post-test. Correlation coefficients were analyzed using linear regression analysis. Significant differences are reported when  $P<0.05$ .

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## DISCLOSURES

S.A.K. is a coinventor on patents related to diagnosis/therapy of preeclampsia, discloses financial interest in Aggamin, LLC, and is a consultant to Siemens Diagnostics.

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