Induction of heme oxygenase-1 attenuates sFlt-1-induced hypertension in pregnant rats

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George EM, Arany M, Cockrell K, Storm MV, Stec DE, Granger JP. Induction of heme oxygenase-1 attenuates sFlt-1-induced hypertension in pregnant rats. Am J Physiol Regul Integr Comp Physiol 301: R1495–R1500, 2011. First published August 24, 2011; doi:10.1152/ajpregu.00325.2011.—Preeclampsia (PE) is one of the most common disorders of pregnancy. One of the initiating factors is a reduction in HO-1 induction. Finally, endothelial function was improved as measured by reductions in vascular expression of preproendothelin mRNA. In conclusion, manipulation of HO-1 induction significantly attenuates placental ischemia-induced hypertension, partially through normalization of the sFlt-1-to-VEGF ratio in the placenta. The purpose of this study was to determine whether HO-1 induction would have beneficial effects independently of sFlt-1 suppression. To that end, pregnant rats were continuously infused with recombinant sFlt-1 from gestational days 14–19, and circulating sFlt-1 increased approximately twofold, similar to rats with experimentally induced placental ischemia. In response, mean arterial pressure increased 17 mmHg, which was completely normalized by HO-1 induction. Unbound circulating VEGF was decreased ~17% in response to sFlt-1 infusion but was increased ~50% in response to HO-1 induction. Finally, endothelial function was improved as measured by reductions in vascular expression of preproendothelin mRNA. In conclusion, manipulation of HO-1 presents an intriguing therapeutic approach to the treatment of PE.

placental ischemia; preeclampsia

ONE OF THE MOST COMMON DISORDERS OF pregnancy is preeclampsia, which contributes to up to 15% of preterm births and remains a leading cause of maternal and fetal morbidity worldwide (19, 29, 35). Hallmarks of the disorder include hypertension, proteinuria, and vascular dysfunction after week 20 of pregnancy (9, 25). Despite intensive research, the exact etiology of the disease is unknown. However, there are strong indications that one of the initiating factors is a reduction in placental perfusion. This is believed to stem from the failure of fatally derived trophoblasts to adequately invade and remodel the maternal spiral arteries from high-resistance, low-capacity vessels to high-capacity, low-resistance vessels (15).

Recent research has highlighted the importance of this hypoxia and ischemia in the placenta as a central factor in the release of soluble factors that lie at the root of the widespread maternal vascular dysfunction typical of the symptomatic phase of the disease. One factor in particular, which has recently been the focus of intensive research, is the alternately spliced, soluble VEGF receptor fms-like tyrosine kinase-1 (sFlt-1), which acts as a direct VEGF antagonist, binding it and making it unavailable for normal signaling, in turn causing maternal endothelial dysfunction (32). It is now well documented that elevated circulating levels of sFlt-1 are strongly correlated with preeclampsia, appearing even before the clinical symptoms of the disorder (13, 18, 24, 28). Human trials and experimental models of preeclampsia have both demonstrated marked elevations of sFlt-1 produced directly by the placenta in response to placental insufficiency (8, 17–18). Finally, we and others have demonstrated that in pregnant animals, introduction of exogenous of sFlt-1 induces a condition mimicking preeclampsia (18, 21) The hypertension associated with this model was shown to be heavily dependent on activation of the endothelin type-A (ET-A) receptor, as administration of an ET-A receptor antagonist abolished the associated hypertension.

Heme oxygenase-1 (HO-1) has recently been demonstrated to be a promising therapeutic agent in several experimental forms of hypertension, including renovascular hypertension, pulmonary hypertension, angiotensin II-dependent hypertension, and the spontaneously hypertensive rat model (2–3, 10, 27, 34). The normal function of HO-1 is in the heme salvage pathway in which the prooxidant-free heme is converted to bilirubin, an antioxidant molecule, and carbon monoxide (CO), an anti-inflammatory agent and potent vasodilator (22–23, 33). These byproducts are hypothesized to be important pathways in the attenuation of hypertension (3). Of particular importance to preeclampsia, in vitro studies have demonstrated that either HO-1 induction, or administration of CO directly, is capable of significantly downregulating sFlt-1 from placental villous explants in response to exogenously applied VEGF (4).

Recently, we have demonstrated that induction of HO-1 in the reduced uterine perfusion pressure (RUPP) model of placental ischemia is capable of significantly attenuating the hypertension and increase in vascular endothelin associated with the model. Furthermore, it was shown that HO-1 induction normalized the sFlt-1/VEGF balance in the placenta, increased bioavailable circulating VEGF, and decreased placental superoxide production (7). Here, by utilizing a previously established rat model of gestational hypertension, which infuses exogenous sFlt-1, and effectively clamps high levels of circulating sFlt-1, we have attempted to further elucidate the relative contributions of these pathological pathways by testing the hypothesis that HO-1 induction can attenuate pregnancy-
induced hypertension (PIH) independently of sFlt-1 suppression.

MATERIALS AND METHODS

Animals. Timed pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) were received on gestational day 11. All protocols were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee, and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Rats were maintained on a 12:12-h light-dark cycle, at 23°C constant temperature and were provided food and water ad libitum.

sFlt-1 administration and cobalt (III) protoporphyrin IX chloride treatment. On gestational day 14, animals in the sFlt-1 experimental groups were implanted with miniosmotic pumps containing recombinantly expressed sFlt-1 (R&D Systems, Minneapolis, MN). Lyophilized recombinant sFlt-1 was reconstituted in PBS and administered in miniosmotic pumps (model 2001; Alzet) at a final dose of 7.4 μg·kg\(^{-1}\)·day\(^{-1}\) until terminal harvest on gestational day 19. Briefly, animals were anesthetized by controlled 3% isoflurane (Webster), and a midline lower abdominal incision was made. Pumps were introduced into the intraperitoneal space, and the incision was closed by nonabsorbable continuous sutures. For HO-1 induction, cobalt (III) protoporphyrin IX chloride (CoPP; Frontier Scientific, Logan, UT) was injected at a dose of 5 mg/kg ip on day 14 of gestation, as described previously (11, 26).

Measurement of mean arterial pressure. On gestational day 18, rats were anesthetized as above and implanted with indwelling carotid catheters of V-3 tubing (SCI) that were tunneled under the skin and externalized at the back of the neck. The following day, rats were placed in individual restraining cages and acclimatized. Mean arterial pressure was measured in the conscious rats for 1 h via Cobe III pressure transducers (CDX Sema). Each experimental group had 10–11 rats.

Tissue harvest. Rats were anesthetized as above. The uterus was exteriorized at the back of the neck. The following day, rats were anesthetized as above and implanted with indwelling carotid catheters of V-3 tubing (SCI) that were tunneled under the skin and externalized at the back of the neck. The following day, rats were placed in individual restraining cages and acclimatized. Mean arterial pressure was measured in the conscious rats for 1 h via Cobe III pressure transducers (CDX Sema). Each experimental group had 10–11 rats.

Measurement of sFlt-1 and VEGF. Placental tissue was prepared from representative placenta from individual rats. Briefly, the frozen tissue was mechanically ground by mortar and pestle in liquid nitrogen. Tissue fragments were resuspended in radiimunoassay buffer with protease inhibitor cocktail, PMSF, and sodium orthovanadate (Santa Cruz Biotechnology, Santa Cruz, CA). Homogenization was performed in glass tissue dounces and the solution was cleared by centrifugation at 12,000 g for 20 min. The resulting protein concentration was measured by the bicinchoninic acid method (Pierce Biotechnology). VEGF and sFlt-1 were both measured by sandwich ELISA (R&D Systems) in duplicate according to manufacturer’s protocols. Measurements of free plasma VEGF were performed with the same kit. Each group had six rats.

Western blot analysis. For Western blot analysis, 50 μg of protein was subjected to SDS-PAGE on 7.5% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA). Membranes were blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE) for 2 h at room temperature. Primary incubation was undertaken overnight at 4°C with a rabbit anti-HO-1 polyclonal antibody (StressGen, Vancouver, CA) at 1:2,000 and a mouse anti-β-actin antibody (Gentest) at 1:5,000. Secondary antibody incubation was done with Alexa Fluor 680 goat anti-rabbit (Molecular Probes) and IRDye 800 goat anti-mouse IgG (Rockland) for 1 h at room temperature. Fluorescence was detected on an Odyssey infrared imager (LI-COR) for simultaneous detection of both species. Blot analysis was performed with the supplied Odyssey software, and HO-1 was normalized to β-actin, with less than three rats in each group.

HO-1 activity assays. Determination of HO-1 activity and tissue levels was performed as described previously (31). Briefly, for HO-1 activity, tissue lysates were prepared and measured as above. Reactions were performed in 1.2 ml volume consisting of: 2 mM glucose-6-phosphate, 0.2 units glucose-6-phosphatedehydrogenase, 0.8 mM NADP, 20 μM hemin, and 0.5 mg of protein from lysates. Incubations were allowed to proceed for 1 h at 37°C. Resulting bilirubin was chloroform extracted and concentration was determined by change in optical density at 464–530 nm, utilizing an extinction coefficient of 40 mM/cm. Activity was expressed as picomole bilirubin formed per hour per milligram of protein. All values were normalized to normal pregnant control levels and expressed as percent of control. A minimum of five samples were analyzed in each group.

Detection of proproendothelin by quantitative RT-PCR. Preproendothelin (preproET) mRNA levels were determined by quantitative RT-PCR. Aortas from harvested rats were quick frozen immediately in liquid nitrogen. For RNA isolation, the samples were crushed in liquid nitrogen, and total RNA was isolated with the RNeasy Mini Kit (Qiagen) per the manufacturer’s instructions. cDNA was produced from 750 ng total RNA by Superscript II in concert with oligo(dT) primers (Invitrogen). Measurements were made from n = 5 in each experimental group. Custom primers (Invitrogen) were used for both ET-1 and β-actin measurements. Endothelin-1 primers used were forward (5′-GAGCGGTCTGCCCAGTA-3′) and reverse (5′-TGTACTGGATCTTGGCCTGG-3′). β-Actin primers used were forward (5′-ATCAAAGATTTGCTCTCTCTGA-3′) and reverse (5′-GAGCCACCATGCCACAGAG-3′). Real-time amplification was carried out with SYBR-Green Supermix (Bio-Rad) with a Bio-Rad iCycler undergoing 40 rounds of amplification. Relative fold change was determined by the 2ΔΔCt method, with six rats in all experimental groups.

Statistical analysis. Data are means ± SE. Comparisons between groups were performed by unpaired Student’s t-test with a significance threshold value of P < 0.05. All statistical comparisons and graphs were generated with OriginPro 8 (Microcal).

RESULTS

CoPP induces HO-1 expression in the liver and placenta of sFlt-1-infused rats. To experimentally induce the expression of HO-1, CoPP was administered intraperitoneally on gestational day 14. Western blot analysis of liver tissue lysates indicated that there was no significant increase in liver HO-1 in response to sFlt-1 infusion. Administration of CoPP caused an approximately twofold increase in liver HO-1, which failed to reach statistical significance. When CoPP was coadministered with sFlt-1 infusion, however, there was a dramatic ~5.5-fold increase in HO-1 expression in the liver compared with sFlt-1-infused animals (Fig. 1, A–B).

In the placenta, there was a statistically insignificant 1.6-fold increase in HO-1 in response to sFlt-1 infusion, similar to levels previously reported in rats undergoing placental ischemia. Also in agreement with previous findings, there was no noticeable increase in placental HO-1 levels in control animals in response to CoPP administration. When animals were coadministered both sFlt-1 and CoPP, there was a statistically significant ~1.8-fold increase in HO-1 expression in the placenta compared with CoPP alone (Fig. 1, C–D).

As it is well documented that the activity of HO-1 does not necessarily correlate with the level of protein expression, we also determined the activity of HO-1 in the placentas of all experimental groups. As can be seen in Fig. 2, despite increased expression of HO-1 in response to sFlt-1, HO-1 activ-
ity in the placenta is unchanged. Likewise, administration of CoPP alone had no discernable effect on HO-1 expression, but there was a trend, which did not reach significance (P = 0.08), for increased HO-1 activity. Coadministration of sFlt-1 and CoPP, however, demonstrated a robust, statistically significant increase in liver HO-1 when compared with sFlt-1 infusion alone (P < 0.05) (n = 3). NP, normal pregnant.

C–D: in the placenta, sFlt-1 infusion increased the tissue levels of HO-1, although this did not reach significance. CoPP administration alone had no effect. Coadministration of CoPP and sFlt-1, however, resulted in a significant increase in the placental levels of HO-1 compared with sFlt-1 infusion alone (P < 0.05) (n = 4).

Effect of HO-1 induction in sFlt-1-induced hypertension. To induce sFlt-1-induced hypertension, recombinant sFlt-1 was administered intraperitoneally via osmotic minipump beginning on gestational day 14. In response to sFlt-1 infusion, mean arterial pressure was increased significantly by 17 mmHg (Fig. 3) on gestational day 19 (103 ± 1 vs. 120 ± 2 mmHg, P < 0.05). Administration of CoPP alone had no significant effect on arterial pressure (105 ± 3 mmHg). However, when CoPP was administered to sFlt-1-infused animals, the associated hypertension was significantly decreased (103 ± 2 mmHg) to levels seen in control animals. No effect was seen on either litter size or pup weight in response to any experimental treatment (data not shown). This data indicates that HO-1 induction abolishes sFlt-1-induced PIH.

HO-1 induction alters angiogenic balance in sFlt-1-infused pregnant rats. We have previously reported that HO-1 induction has significant effects on both sFlt-1 and VEGF in the placenta of RUPP-treated rats. In response to sFlt-1 infusion, the levels of both of these proteins are significantly elevated in the placentas of the treated animal compared with control rats (Fig. 4, A–B). Administration of CoPP has no significant effect on the expression of either protein in either normal pregnant or sFlt-1-infused animals, indicating that HO-1 induction is not significantly altering the production of angiogenic factors in the placenta during sFlt-1 infusion. However, interesting changes were noted in the level of circulating free, bioavailable VEGF in the maternal circulation. In response to sFlt-1 infusion, there was a trend for decreased free VEGF in the circulation (1,030 ± 88 vs. 867 ± 51 pg/ml, P = 0.14), although this failed to reach statistical significance (Fig. 4C). Again, CoPP administration had no discernable effect on free-circulating VEGF (1,007 ± 50 pg/ml). However, in response to CoPP in sFlt-1-infused rats there is a significant ~27% increase in free VEGF in the maternal circulation (1,309 ± 154 pg/ml), which indicates that HO-1 induction increases circulating VEGF in the maternal circulation independently of sFlt-1 suppression.

HO-1 induction decreases vascular sFlt-1-induced preproET-1 production. To determine the effect of sFlt-1 infusion on vascular production of endothelin-1, RNA was isolated from the thoracic aorta. mRNA message levels of the precursor

Fig. 1. Western blot analysis of heme oxygenase-1 (HO-1) production in the liver and placenta of fms-like tyrosine kinase-1 (sFlt-1)-infused rats and those treated with cobalt (II) protoporphyrin IX chloride (CoPP). A–B: in response to sFlt-1 infusion, there was no significant change in HO-1 levels in liver tissue. Treatment with CoPP produced a small nonsignificant increase in liver HO-1. Coadministration of sFlt-1 and CoPP, however, demonstrated a robust, statistically significant increase in liver HO-1 when compared with sFlt-1 infusion alone (P < 0.05) (n = 3). NP, normal pregnant. C–D: in the placenta, sFlt-1 infusion increased the tissue levels of HO-1, although this did not reach significance. CoPP administration alone had no effect. Coadministration of CoPP and sFlt-1, however, resulted in a significant increase in the placental levels of HO-1 compared with sFlt-1 infusion alone (P < 0.05) (n = 4).
protein preproET were measured by quantitative real-time PCR and message levels normalized to β-actin expression. As shown in Fig. 5, in response to sFlt-1 infusion, there was a significant and robust 2.1-fold increase in the production of preproET message (1 ± 0.2 vs. 2.1 ± 0.3 fold change, P < 0.05). Administration of CoPP alone to control animals had no significant effect on preproET expression (1.2 ± 0.2 fold change). However, in sFlt-1-infused animals undergoing HO-1 induction, there was a significant decrease in preproET expression compared with sFlt-1-infused animals (2.1 ± 0.3 vs. 0.5 ± 0.2 fold change, P < 0.05) to levels half of that seen in normal pregnant animals. This data indicates that sFlt-1 infusion significantly increases vascular preproET expression and that HO-1 induction significantly attenuates this effect.

Fig. 2. Placental HO-1 activity increases in response to CoPP administration. Continuous sFlt-1 infusion failed to increase placental HO-1 activity compared with normal pregnant controls. Administration of CoPP alone lead to an increase in HO-1 activity compared with normal pregnant controls, although this failed to reach significance (P = 0.13). With infusion of sFlt-1 and administration of CoPP, however, there was an ~2-fold statistically significant increase in HO-1 activity (P < 0.05). NP, n = 5; sFlt-1, n = 5; NP+CoPP, n = 8; sFlt-1+CoPP, n = 5.

Fig. 3. Mean arterial pressure (MAP) in response to sFlt-1 infusion and HO-1 induction. In response to sFlt-1 infusion, there was an ~17 mmHg increase (P < 0.05) in MAP measured via indwelling carotid catheters. Administration of CoPP had no significant effect on MAP; however, induction of HO-1 in sFlt-1-infused rats completely normalized the hypertension seen in the sFlt-1-infused rats compared with sFlt-1-infused control rats (P < 0.05). NP, n = 10; sFlt-1, n = 11; NP+CoPP, n = 10; sFlt-1+CoPP, n = 11.

Fig. 4. Effect of sFlt-1 infusion and HO-1 induction on angiogenic factors. A–B: in response to sFlt-1 infusion, levels of both sFlt-1 and VEGF are increased in the placenta of the rat compared with normal pregnant controls (P < 0.05). Induction of HO-1 had no significant effect on either protein, remaining at levels similar to those seen in sFlt-1-infused rats alone. C: sFlt-1 infusion decreased the amount of free VEGF found in the maternal circulation, although this failed to meet significance (P = 0.14). In response to HO-1 induction there was a significant increase in the circulating free VEGF compared with sFlt-1 infusion alone (P < 0.05). For each group, n = 6.

Fig. 5. Placental VEGF levels in response to sFlt-1 infusion and HO-1 induction. A: sFlt-1 infusion increased VEGF levels in the placenta of the rat compared with normal pregnant controls (P < 0.05). B: Induction of HO-1 had no significant effect on VEGF levels, remaining at levels similar to those seen in sFlt-1-infused rats alone. C: sFlt-1 infusion decreased the amount of free VEGF found in the maternal circulation, although this failed to meet significance (P = 0.14). In response to HO-1 induction there was a significant increase in the circulating free VEGF compared with sFlt-1 infusion alone (P < 0.05). For each group, n = 6.
Effects in the attenuation of PIH by HO-1, in this study, we observed that placental levels of sFlt-1 are elevated to levels typically seen in the RUPP animals, leading to a trend for decreased bioavailability of circulating VEGF. As a result, we observed an approximately 17 mmHg increase in arterial pressure, which is in line with previous studies (21). Surprisingly, induction of HO-1 by CoPP administration resulted in a complete normalization of this hypertensive response. This would seem to indicate that the attenuation of PIH by HO-1 is independent of its ability to negatively regulate sFlt-1.

Interestingly, when circulating levels of free VEGF were examined in these sFlt-1/CoPP-treated animals there was a significant increase noted. One possible route through which this might occur is through HO-1’s ability to induce expression of VEGF, which has been reported in both endothelial cells and vascular smooth muscle cells (5–6). This effect has been further attributed in the literature to CO, which has been shown to have similar effects as the protein itself, and has recently been traced to the p38-dependent activation of Sp1 (6, 12, 14, 16). It is possible, therefore, that one of the main mechanisms by which HO-1 induction is attenuating hypertension in both the RUPP and sFlt-1 infusion models is through induction of VEGF from the maternal vascular smooth muscle and endothelium. This would explain the increase in systemic VEGF bioavailability that is occurring independently of sFlt-1 regulatory activity.

One indicator that would appear to bear this out is the change in preproET levels seen in the maternal vasculature in response to both sFlt-1 infusion and HO-1 induction. In response to sFlt-1 treatment, the maternal vascular production of ET-1 was significantly increased approximately twofold. This increase was not only blocked by HO-1 induction but was reduced to levels approximately half of those seen in normal pregnant animals. This appears to be in direct correlation with the levels of free VEGF that were found in the maternal circulation, suggesting that VEGF, by promotion of endothelial function, might be playing a role in the suppression of ET-1. Alternatively, there is evidence that CO directly inhibits stimulated ET-1 production from both endothelial cells and from vascular smooth muscle cells (20, 30). It is possible then that HO-1 induction is suppressing sFlt-1 induced increases in vascular ET-1 through two distinct mechanisms, VEGF induction and ET-1 suppression, independently of its ability to suppress sFlt-1. As we have previously demonstrated that sFlt-1-induced hypertension is heavily dependent on the activation of the ET-A receptor (21), reductions in ET-1 seen here are suggestive of one mechanism by which HO-1 is reducing blood pressure in this model.

In summary, an effective intervention in the treatment of preeclampsia remains elusive. HO-1 is a promising avenue for the treatment of preeclampsia, as it appears to affect multiple pathways in the pathological manifestation of the disorder. Continuing work examining the utility of the protein or its specific metabolic byproducts in the treatment of preeclampsia should prove instructive and may prove useful in the development of novel therapies for the treatment of preeclampsia.

**DISCUSSION**

Preeclampsia remains one of the leading causes of fetal and maternal morbidity and effective pharmacological interventions remain elusive. One potential target for intervention that has been suggested for the treatment of preeclampsia is the protein HO-1, an important component of the heme salvage pathway (1). As a normal consequence of its function in the cell, HO-1 produces two bioactive byproducts, the vasodilator CO and the antioxidant bilirubin (23, 33). HO-1 has been actively investigated for the treatment of hypertension and has proven to effectively attenuate the symptoms associated with a number of forms of experimental hypertension (2–3, 27, 34).

Of particular interest for the treatment of preeclampsia, strong in vitro evidence has indicated that HO-1, and CO specifically, is an important regulator of two important pathological factors in the development of the disorder. First, it has been shown that HO-1 and CO can negatively regulate stimulated sFlt-1 release from human placental explants (4). In addition, HO-1-derived CO from smooth muscle cells has been shown to downregulate expression of preproET in an in vitro system (20). Recently, we have examined the ability of HO-1 induction to attenuate the pathology associated with RUPP-induced hypertension, a useful rat model for the symptomatic phase of preeclampsia (7). In this model, HO-1 induction did attenuate the hypertension associated with RUPP treatment by approximately 50%. Several factors were implicated with this decrease in pressure. Angiogenic balance of sFlt-1 and VEGF produced by the placenta was significantly altered, resulting in significantly higher levels of free-circulating VEGF in the maternal circulation. Placental superoxide, an important factor in the development of RUPP hypertension and human preeclampsia, was significantly reduced by HO-1 induction.

To further determine the relative contributions of these effects in the attenuation of PIH by HO-1, in this study, we have utilized the sFlt-1 infusion model of PIH. The rationale for this choice is that sFlt-1 levels would be clamped at artificially high levels, independently of placenta sFlt-1 production. Indeed, as observed in Fig. 3, placental levels of sFlt-1 are elevated to levels typically seen in the RUPP animals, leading to a trend for decreased bioavailability of circulating VEGF. As a result, we observed an approximately 17 mmHg increase in arterial pressure, which is in line with previous studies (21). Surprisingly, induction of HO-1 by CoPP administration resulted in a complete normalization of this hypertensive response. This would seem to indicate that the attenuation of PIH by HO-1 is independent of its ability to negatively regulate sFlt-1.

Interestingly, when circulating levels of free VEGF were examined in these sFlt-1/CoPP-treated animals there was a significant increase noted. One possible route through which this might occur is through HO-1’s ability to induce expression of VEGF, which has been reported in both endothelial cells and vascular smooth muscle cells (5–6). This effect has been further attributed in the literature to CO, which has been shown to have similar effects as the protein itself, and has recently been traced to the p38-dependent activation of Sp1 (6, 12, 14, 16). It is possible, therefore, that one of the main mechanisms by which HO-1 induction is attenuating hypertension in both the RUPP and sFlt-1 infusion models is through induction of VEGF from the maternal vascular smooth muscle and endothelium. This would explain the increase in systemic VEGF bioavailability that is occurring independently of sFlt-1 regulatory activity.

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