

# Role of Uncoupled Endothelial Nitric Oxide Synthase in Abdominal Aortic Aneurysm Formation

## Treatment With Folic Acid

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**Abstract**—It has been shown that endothelial NO synthase (eNOS) uncoupling occurs in hypertension and atherosclerosis. However, its causal role in vascular pathogenesis has not been characterized previously. Here, we challenged eNOS preuncoupled hyperphenylalaninemia (hph)-1 mice (deficient in eNOS cofactor tetrahydrobiopterin biosynthetic enzyme GTPCHI) with angiotensin II (Ang II; 0.7 mg/kg per day, 14 days). Both wild-type and hph-1 groups developed hypertension similarly up to day 6 to 7. Thereafter,  $\approx 14\%$  of Ang II-infused (0.7 mg/kg per day) hph-1 mice ( $n=72$ ) started to die suddenly of ruptured abdominal aortic aneurysm (AAA). Among the survivors, 65% developed AAA, resulting in a total morbidity rate of 79%. In contrast, none of the Ang II-infused wild-type mice died or developed AAA. Ang II progressively deteriorated eNOS uncoupling in hph-1 mice while augmenting tetrahydrobiopterin and nitric oxide (NO) deficiencies. The abundance of the tetrahydrobiopterin salvage enzyme dihydrofolate reductase in the endothelium was decreased in hph-1 mice and further diminished by Ang II infusion. Intriguingly, restoration of dihydrofolate reductase expression by oral administration of folic acid or overexpression of dihydrofolate reductase completely prevented AAA formation in Ang II-infused hph-1 mice while attenuating progressive uncoupling of eNOS. Folic acid also attenuated vascular remodeling and inflammation characterized by medial elastin breakdown and augmented matrix metalloproteinase 2 activity and activation of matrix metalloproteinase 9, as well as macrophage infiltration. In conclusion, these data innovatively suggest a causal role of eNOS uncoupling/tetrahydrobiopterin deficiency in AAA formation. Therefore, oral folic acid administration, endothelium-targeted dihydrofolate reductase gene therapy, and perhaps other countermeasures directed against eNOS uncoupling could be used as new therapeutics for AAA. (*Hypertension*. 2012;59:158-166.)

**Key Words:** abdominal aortic aneurysm, eNOS uncoupling ■ hph-1 mice ■ tetrahydrobiopterin, angiotensin II ■ folic acid, dihydrofolate reductase

By producing nitric oxide (NO) to rapidly inactivate superoxide ( $O_2^-$ ) and other reactive oxygen species (ROS), endothelial NO synthase (eNOS) protects vascular cells from oxidative damage. Accumulating evidence has demonstrated that, when eNOS cofactor tetrahydrobiopterin ( $H_4B$ ) is deficient, eNOS becomes dysfunctional to produce  $O_2^-$  rather than NO.<sup>1-13</sup> This uncoupling of eNOS could deteriorate endothelial dysfunction, making it extremely difficult to correct. One important pathological agonist capable of transforming eNOS into the uncoupled state is angiotensin II (Ang II).<sup>1,2,5</sup> We have shown previously that Ang II uncouples eNOS via transient activation of NADPH oxidase and consequent hydrogen peroxide-dependent, endothelium-specific deficiency in  $H_4B$  salvage enzyme dihydrofolate reductase (DHFR).<sup>1,5</sup>

Ang II plays an important role in the pathogenesis of vascular diseases, such as hypertension and atherosclerosis, acting via well-characterized mechanisms, such as vasoconstriction, activation of vascular NADPH oxidase, and ROS-dependent inflammatory and hypertrophic signaling.<sup>14-18</sup> Uncoupling of eNOS, however, represents a novel mechanism whereby Ang II causes prolonged oxidative stress.<sup>1,2,5</sup> Nevertheless, it has remained unclear whether uncoupled eNOS is directly involved in the pathogenesis of vascular disease. Tetrahydrobiopterin ( $H_4B$ ) deficiency subsequent to a mutation in GTP cyclohydrolase 1 (GTPCHI) induces hyperphenylalaninemia in mice.<sup>19,20</sup> At baseline, hyperphenylalaninemia (hph)-1 mice have reduced NO bioavailability but preserved vasorelaxation because of hydrogen peroxide-dependent compensation.<sup>19</sup> After crossing with C57BL6 mice

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for >10 generations we have genotyped and characterized the *hph-1* mice using electron spin resonance for detection of  $O_2^{\cdot-}$  production. What we found was that, although in the wild-type (WT) animals *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) increased  $O_2^{\cdot-}$  production because of the loss of NO, L-NAME attenuated  $O_2^{\cdot-}$  production in *hph-1* mice, implicating uncoupling of eNOS. Therefore, *hph-1* mice can serve as an excellent model system to study contribution to vascular pathogenesis of uncoupled eNOS/H<sub>4</sub>B deficiency.

To examine whether uncoupled eNOS exaggerates pathological effects of Ang II to amplify hypertension or augment vascular remodeling, we infused WT and *hph-1* mice with Ang II for 14 days. Mean blood pressure (MBP) monitored by an intracarotid telemetry method was found increased in both groups up to day 6 to 7. Although MBP in WT mice continued to rise, it started to decline in *hph-1* mice, which was associated with sudden death (13.9%; n=72). Immediate postmortem inspection revealed rupturing abdominal aortic aneurysm (AAA). Approximately 65% of the surviving *hph-1*s developed AAA, resulting in a total morbidity rate of 79% (n=72). We further characterized AAA and revealed that progressive DHFR deficiency and uncoupling of eNOS in Ang II-infused *hph-1* underlie extensive vascular remodeling, inflammation, and AAA formation. Intriguingly, restoration of DHFR expression by oral administration of folic acid (FA), or overexpression of DHFR, completely prevented AAA formation in Ang II-infused *hph-1* mice. These treatments also blunted progressive uncoupling of eNOS, as well as vascular remodeling and inflammation characterized by matrix metalloproteinase (MMP) activation, elastin breakdown, collagen remodeling, and macrophage infiltration. Therefore these innovative findings represent first evidence that eNOS uncoupling/H<sub>4</sub>B deficiency plays a causal role in AAA formation and that oral FA administration, DHFR-targeted therapy, and perhaps other countermeasures directed against eNOS uncoupling could serve as novel and powerful therapeutic regimes for AAA, the severe and prevalent human disease for which no pharmacological treatment is currently available.

## Methods

### Animals, Ang II Infusion, and Blood Pressure Measurements

The *hph-1* mice (originally in CBA background)<sup>19</sup> were backcrossed with C57BL6 mice for >10 generations and genotyped based on a protocol by Khoo et al.<sup>21</sup> Only homozygote *hph-1* mice were used for experiments. WT and *hph-1* male mice at 24 weeks of age were infused with Ang II (0.7 mg/kg per day) using subcutaneously implanted osmotic pumps (Durect Corp). During the 14-day infusion, blood pressure was monitored by telemetry method. Wireless blood pressure probes were implanted into the animals 10 days before the implantation of the osmotic pumps. The catheter of the blood pressure probe was inserted into the left carotid artery, whereas the body of the probe was inserted into the right flank. Animals were given 1 week to recover from the surgery. After this period, blood pressure was measured for 3 days to obtain a baseline. The osmotic pumps were then implanted on day 10 after surgery. Measurements were made daily from 9:00 AM to 4:00 PM at a 250-Hz sampling rate. Average blood pressure was calculated daily as the average of the entire recording period. The use of animals and experimental procedures were approved by the institutional animal care and usage committee at the University of California Los

Angeles. Electron spin resonance determination of aortic NO and superoxide production, high-performance liquid chromatography determination of aortic H<sub>4</sub>B content, and Western blot determination of endothelial DHFR expression were performed as published previously.<sup>1,2,5</sup>

### Endothelial Cell Isolation From Mouse Aortas

On harvest, whole aorta was cut open longitudinally and then digested with collagenase (0.6 mg/mL) at 37°C for 20 minutes before gentle removal of endothelial cells with a cotton applicator. The supernatant containing endothelial cells was centrifuged and lysed for Western blot analysis.

### Oral Administration of FA and DHFR Overexpression

WT and *hph-1* mice were started on continuous oral administration of FA (15 mg/kg per day) or tail vein transfection of DHFR expression vector (pcDNA3.1-DHFR)<sup>5</sup> in lipid-based reagent from Altogen Biosystems every other day for 14 days, starting 2 days before Ang II infusion. The mice were monitored twice per day during the infusion period of 2 weeks, and aortas were harvested for assessment of AAA formation, endothelial DHFR expression, and eNOS uncoupling activity by day 14. Animals that died suddenly during the infusion period of 14 days were inspected for aneurysm immediately and aortas were freshly harvested for experiments.

### Ultrasound Detection of Abdominal Aorta Size

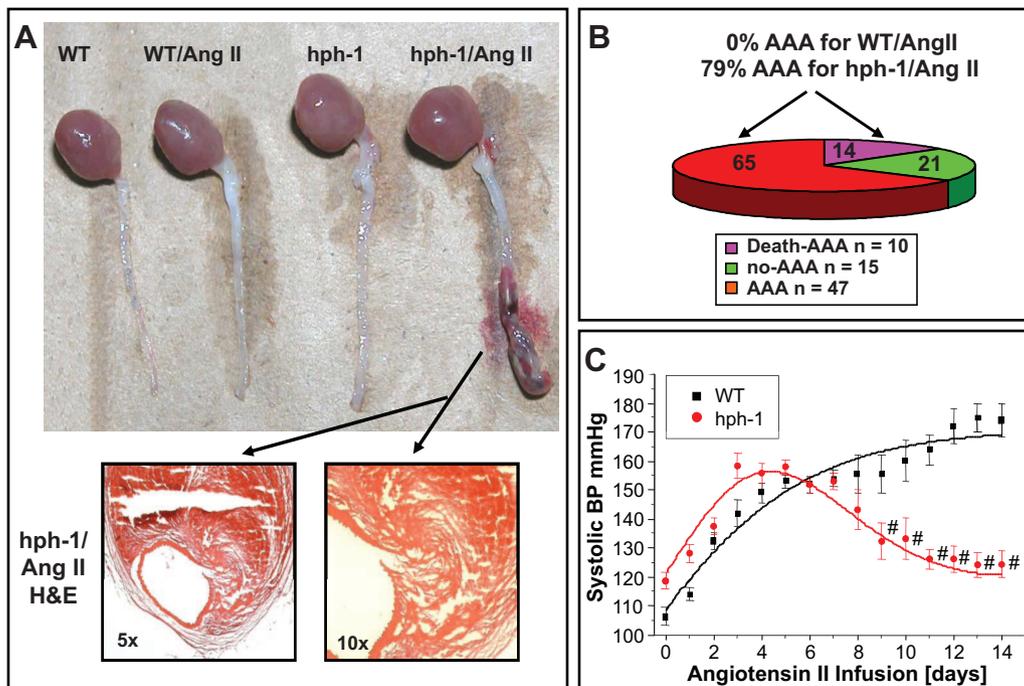
Animals were anesthetized with isoflurane and placed on a temperature-controlled table, which also measures ECG for heart rate. Isoflurane levels were adjusted throughout the experiment to maintain heart rate between 400 and 500 bpm while keeping the animal sufficiently anesthetized. Hair was removed from the abdomen using a hair removal cream, and preheated ultrasound transmission gel was applied onto the abdomen area. An ultrasound probe (Velvo 770, Visualsonics) was placed on the gel to visualize aorta transversely. The aorta was identified using Doppler measurement for the presence of pulsatile flow. Consistent localization of image acquisition was insured by visualizing the aorta immediately superior to the branch of the left renal artery in all of the animals. Images were recorded and saved onto a PC computer for offline area analysis. Hematoxylin-eosin and Verhoeff-Van Gieson stainings were conducted following standard histological protocols.

### Macrophage Stainings

Formalin-fixed and paraffin-embedded tissue were sectioned at 5 μm. Paraffin was removed by washing with xylene and then rehydrated with descending concentrations of ethanol. Antigen retrieval was performed by immersing the sections in an antigen retrieval buffer (10 μmol/L of citric acid, 0.01% Tween 20) at 98.5°C for 20 minutes. Sections were washed in PBS plus 0.1% triton (PBS-T) and then blocked with 2% normal goat serum in PBS-T at room temperature for 3 hours. Sections were then incubated with primary antibody (Mac-3, BD Pharmingen, 2%, in PBS-T) overnight at 4°C. After washing with PBS-T for 1 hour, sections were incubated with secondary antibody (Alexa fluor 488, 2% in PBS-T) for 2 hours at room temperature in the dark. After washing with PBS-T for 1 hour, sections were dehydrated with ascending grades of ethanol and then xylene. Sections were mounted with Permount medium and pictures taken with a confocal microscope (Leica, SPI inverted). MMP activity assays were performed as published previously.<sup>22</sup>

### Statistical Analysis

Comparisons among different treatment groups were performed by ANOVA. When differences were indicated, the Dunnett post hoc test was used. Statistical significance was set for *P*<0.05. All of the grouped data shown in the figures were presented as mean±SEM.



**Figure 1.** Angiotensin II (Ang II) induces abdominal aortic aneurysm (AAA) formation in hyperphenylalaninemia (hph)-1 mice. Wild-type (WT) and hph-1 mice were infused with Ang II (0.7 mg/kg per day) for 14 days. **A**, Representative appearance of abdominal aortas in the different experimental groups of WT, WT/Ang II, hph-1, and hph-1/Ang II by day 14. Only Ang II-infused hph-1 mice developed AAA, with visible evidence of hemorrhage. Hematoxylin-eosin staining (arrows) of the AAA segment revealed thrombus formation. **B**, AAA morbidity and mortality rates (no-AAA vs nonlethal AAA vs lethal AAA) in Ang II-infused hph-1 mice at 21%, 65%, and 14%, respectively. **C**, Changes in mean blood pressure (MBP) in Ang II-infused hph-1 mice. The MBP was monitored by an intracarotid telemetry method (Data Sciences International) continuously for 14 days.

## Results

### Ang II Infusion of hph-1 Mice Induces AAA Formation

Twenty-four-week-old male WT ( $n=72$ ) and hph-1 mice ( $n=72$ ) were infused with Ang II (0.7 mg/kg per day) for 14 days and twice per day. Approximately 14% of Ang II-infused hph-1 mice died suddenly of ruptured AAA within 14 days (Figure 1B). Among the survivors, 65% developed AAA, resulting in a total morbidity rate of 79% (Figure 1A-1B). In contrast, none of the Ang II-infused WT mice or untreated hph-1 mice died or developed AAA (Figure 1A). The MBP was monitored by an intracarotid telemetry method and was found modestly higher in hph-1 mice as compared with WT mice at baseline (ie, day 0 Ang II infusion; Figure 1C). During Ang II infusion, MBP was increased in both genotypes up to day 6 (WT:  $101\pm 3$ – $140\pm 2$  mm Hg; hph-1:  $112\pm 3$ – $140\pm 2$  mm Hg; Figure 1C). Thereafter, MBP declined in hph-1 mice, whereas it continued to rise in WT mice (Figure 1C).

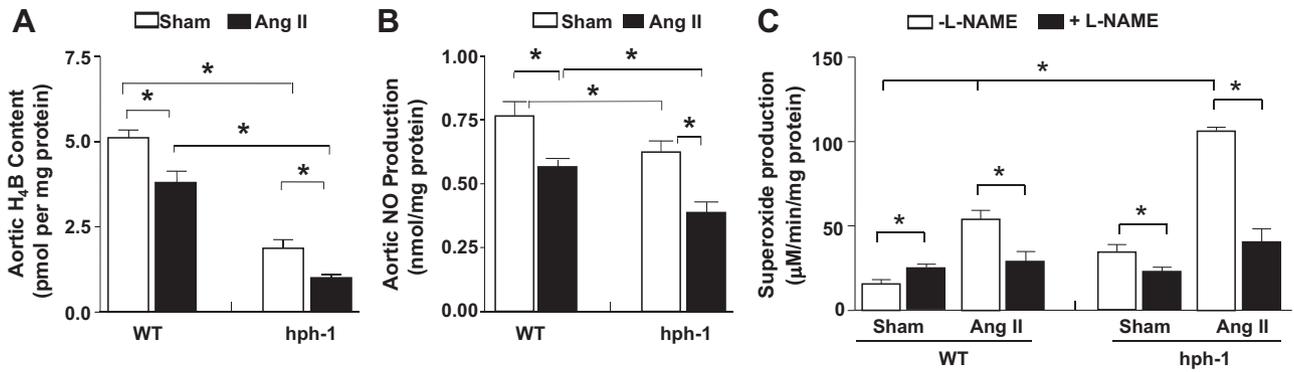
### Ang II Infusion Augments Deficiency of H<sub>4</sub>B and NO<sup>•</sup> and Aggravates eNOS Uncoupling, in hph-1 Mice

Freshly isolated aortas from Ang II-infused WT and hph-1 mice were subjected to high-performance liquid chromatography determination of H<sub>4</sub>B content and electron spin resonance determination of NO<sup>•</sup> and O<sub>2</sub><sup>•-</sup> production.<sup>1,2</sup> As compared with WT mice, the hph-1 mice exhibited reduced aortic H<sub>4</sub>B bioavailability (Figure 2A), which was further

diminished by Ang II infusion ( $1.9\pm 0.2$ – $1.0\pm 0.1$  pmol/mg of protein; Figure 2A). Ang II infusion also significantly reduced aortic H<sub>4</sub>B bioavailability in WT mice ( $5.1\pm 0.2$ – $3.8\pm 0.4$  pmol/mg of protein). Of note, aortic NO<sup>•</sup> production mirrored these changes in Ang II-infused WT and hph-1 mice (Figure 2B). Aortic O<sub>2</sub><sup>•-</sup> production was determined in the presence or absence of L-NAME. Basal O<sub>2</sub><sup>•-</sup> production was twice as high in hph-1 mice as compared with WT mice (Figure 2C, compare sham hph-1 versus sham WT). Although L-NAME increased O<sub>2</sub><sup>•-</sup> production as a result of inhibiting coupled eNOS in untreated WT mice (Figure 2C), L-NAME decreased O<sub>2</sub><sup>•-</sup> production in Ang II-infused WT mice, consistent with uncoupling of eNOS in response to Ang II, as we reported previously<sup>1,5</sup> (Figure 2C). In hph-1 mice, L-NAME decreased O<sub>2</sub><sup>•-</sup> production in untreated mice, consistent with uncoupled eNOS at baseline, in the absence of Ang II infusion (Figure 2C). Importantly, Ang II infusion further uncoupled eNOS in hph-1 mice, as demonstrated by the substantially higher production of O<sub>2</sub><sup>•-</sup> that was completely inhibited by L-NAME (Figure 2C).

### FA Restores Endothelial DHFR Expression and H<sub>4</sub>B Bioavailability and Prevents eNOS Uncoupling in Ang II-Infused hph-1 Mice

WT and hph-1 mice were started on oral FA (15 mg/kg per day) 2 days before Ang II infusion (0.7 mg/kg per day) and treated throughout the study period of 14 days. Both endothelial cells (digested off aortas, see Methods section) and endothelial cell-denuded aortas were analyzed for DHFR



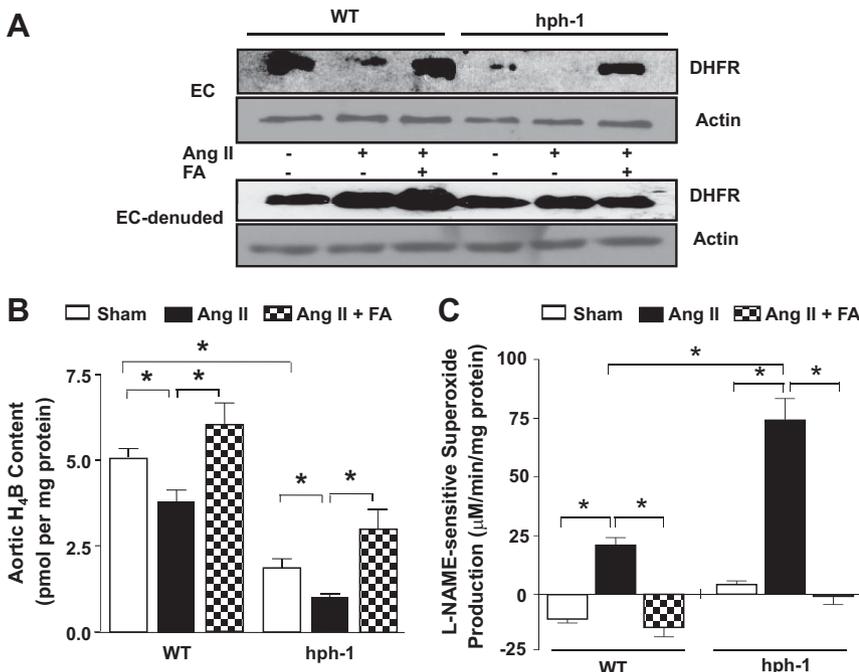
**Figure 2.** Angiotensin II (Ang II) infusion augments deficiency of tetrahydrobiopterin (H<sub>4</sub>B) and NO<sup>•</sup> and aggravates endothelial NO synthase (eNOS) uncoupling in hyperphenylalaninemia (hph)-1 mice. Wild-type and hph-1 mice were infused with Ang II (0.7 mg/kg per day) for 14 days, after which aortas were harvested for the following: (A) aortic H<sub>4</sub>B content; (B) aortic NO<sup>•</sup> production; and (C) aortic superoxide (O<sub>2</sub><sup>•-</sup>) production in the presence or absence of L-N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME). \*P<0.05.

expression. As shown, hph-1 mice exhibited reduced endothelial DHFR expression, which was further reduced by Ang II infusion. FA treatment restored endothelial DHFR expression in hph-1 mice to near WT levels (Figure 3A). The H<sub>4</sub>B deficiencies induced by Ang II in both WT and hph-1 mice were likewise prevented by FA treatment, resulting in H<sub>4</sub>B levels that were even higher than baseline in both groups (Figure 3B). Moreover, FA prevented eNOS uncoupling in both Ang II-infused WT and hph-1 mice, as evidenced by complete attenuation of L-NAME-sensitive O<sub>2</sub><sup>•-</sup> production (Figure 3C).

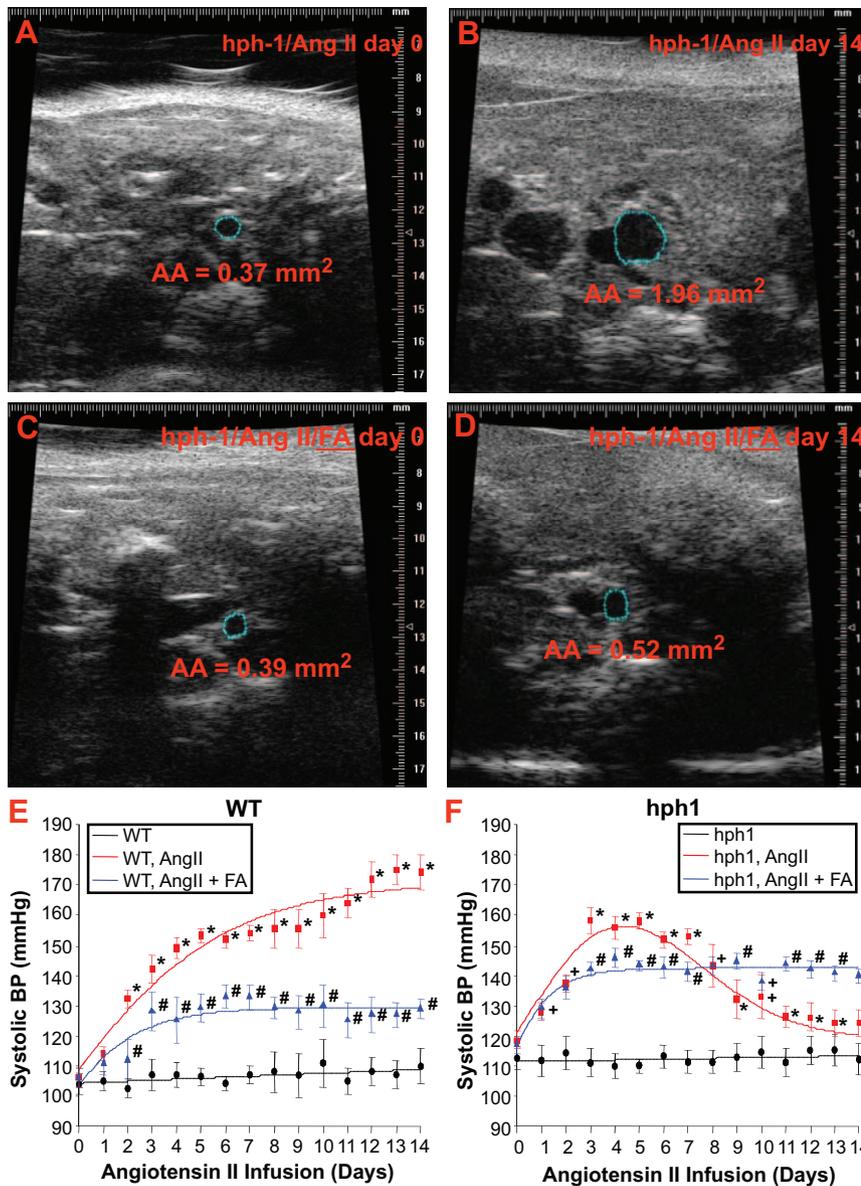
**FA Treatment Prevents AAA Formation and Normalizes Blood Pressure in Ang II-Infused hph-1 Mice**

For this study, a total of 21 FA-treated Ang II-infused hph-1 mice was used, and none of these animals developed AAA. Statistical analysis using a 2x2 contingency table shows that this reduction in AAA development was significant

(P<0.0001). On days 0 and 14, abdominal aorta dimensions were monitored using ultrasound (Velvo 770 high-resolution echo system equipped with a 45 MHz transducer, Visualsonics). Ang II infusion induced dramatic expansion of abdominal aorta in hph-1 mice (0.37–1.96 mm<sup>2</sup>), which was prevented by FA treatment (0.39–0.52 mm<sup>2</sup>; Figure 4A through 4D). By contrast, in WT mice, Ang II induced a minimal increase in abdominal aorta size (0.53–0.66 mm<sup>2</sup>). In addition, FA was highly effective in attenuating Ang II-induced hypertension in WT mice (Figure 4E), and it also prevented the decline in MBP in Ang II-infused hph-1 mice (Figure 4F). These data suggest that, in WT mice infused with Ang II, uncoupling of eNOS leads to elevated MBP, most likely consequent to reduced NO<sup>•</sup> bioavailability, whereas in hph-1 mice, which exhibit a more profound degree of eNOS uncoupling, the excessive production of ROS paradoxically lowers MBP, or that the reduction in MBP is mediated by ROS-independent mechanisms.



**Figure 3.** Folic acid (FA) prevents endothelial NO synthase (eNOS) uncoupling in angiotensin II (Ang II)-infused hyperphenylalaninemia (hph)-1 mice via restoration of endothelial dihydrofolate reductase (DHFR) expression. Wild-type and hph-1 mice were started on oral administration of FA (15 mg/kg per day) 2 days before Ang II infusion (0.7 mg/kg per day) and treated throughout the study period of 14 days, after which aortas were harvested for the following: (A) endothelial and non-endothelial DHFR expression in aortic preparations; (B) aortic tetrahydrobiopterin (H<sub>4</sub>B) content; and (C) aortic eNOS uncoupling activity (indicated by N<sup>G</sup>-nitro-L-arginine methyl ester [L-NAME]-sensitive O<sub>2</sub><sup>•-</sup> production). \*P<0.01.



**Figure 4.** Folic acid (FA) prevents abdominal aortic aneurysm (AAA) formation and normalizes blood pressure in angiotensin II (Ang II)-infused hyperphenylalaninemia (hph)-1 mice. Wild-type (WT) and hph-1 mice were started on oral administration of FA (15 mg/kg per day) 2 days before Ang II infusion (0.7 mg/kg per day) and treated throughout the study period of 14 days. At days 0 and 14, abdominal ultrasound (Velvo 770 high-resolution echo system, Visualsonics) was performed to assess abdominal aorta (AA) dimensions (**A** through **D**) in Ang II-infused wild-type and hph-1 mice treated with or without FA. Aortic cross-sectional areas are depicted by blue circles, and calculated areas are listed below each image. Mean blood pressure (MBP) was assessed by telemetry during the course of the study (**E** and **F**), as described in Figure 1.

### FA Prevents Progressive Uncoupling of eNOS and Vascular Remodeling in Ang II-Infused hph-1 Mice

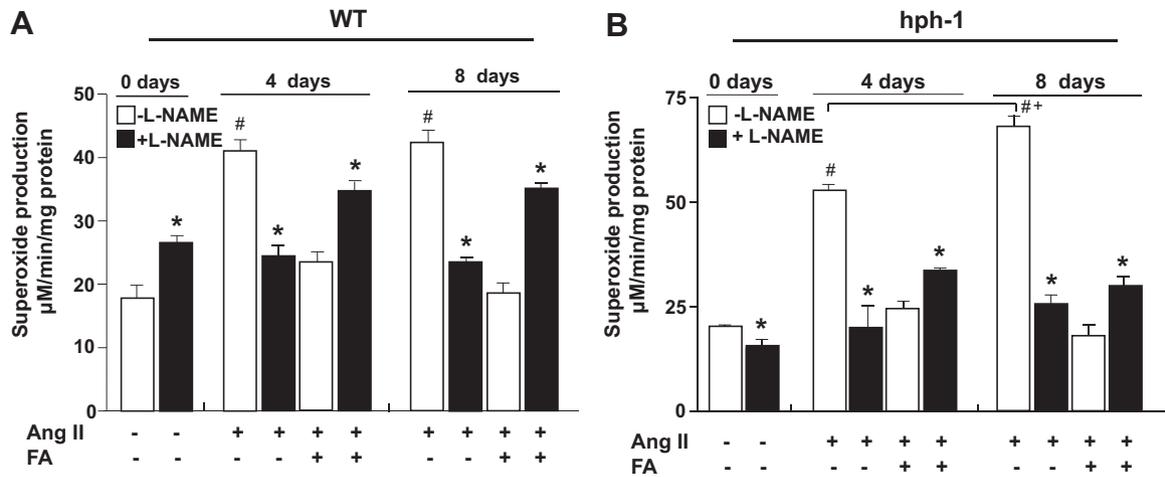
To examine the effects of FA on eNOS uncoupling at different time points during AAA development, we followed  $O_2^{\cdot -}$  production on days 4 and 8 day after initiation of Ang II infusion. Aortas were harvested and subjected to electron spin resonance detection of  $O_2^{\cdot -}$  in the presence or absence of L-NAME. In WT mice, eNOS uncoupling activity remained steady in response to Ang II for both time points examined. As is obvious in Figure 5A, L-NAME-sensitive  $O_2^{\cdot -}$  production that is reflective of eNOS uncoupling activity was similar on days 4 and 8. In contrast, Ang II induced progressive uncoupling of eNOS in hph-1 mice. As demonstrated in Figure 5B, the L-NAME-inhibitable fraction of  $O_2^{\cdot -}$  production was augmented on day 8 compared with what was observed on day 4. Importantly, FA consistently suppressed eNOS uncoupling activity in hph-1 mice at both time points.

In additional experiments, we found that FA also prevented Ang II-induced vascular remodeling in hph-1 mice. As

indicated by hematoxylin-eosin staining, FA abrogated medial degradation and adventitial inflammatory cell recruitment in Ang II-infused hph-1 mice (Figure 6A). More specifically, FA attenuated medial elastin flattening and rarefaction of elastin fibers, as shown by Verhoeff-Van Gieson (VVG) staining (Figure 6B). Infiltrating macrophages, one of the major sources of matrix degradation enzymes, including MMP9, were also dramatically upregulated with Ang II-infused mice, which was completely attenuated with FA treatment (Figure 6C). Furthermore, FA attenuated Ang II-induced augmentation of MMP2 activation and MMP9 activation in hph-1 mice, whereas it also inhibited Ang II-induced MMP2 activation in WT mice (Figure 7).

### DHFR Gene Therapy Recouples eNOS and Prevents AAA Formation in Ang II-Infused hph-1 Mice

We have shown previously that DHFR gene therapy is effective in recoupling eNOS in Ang II-infused WT mice.<sup>1</sup>



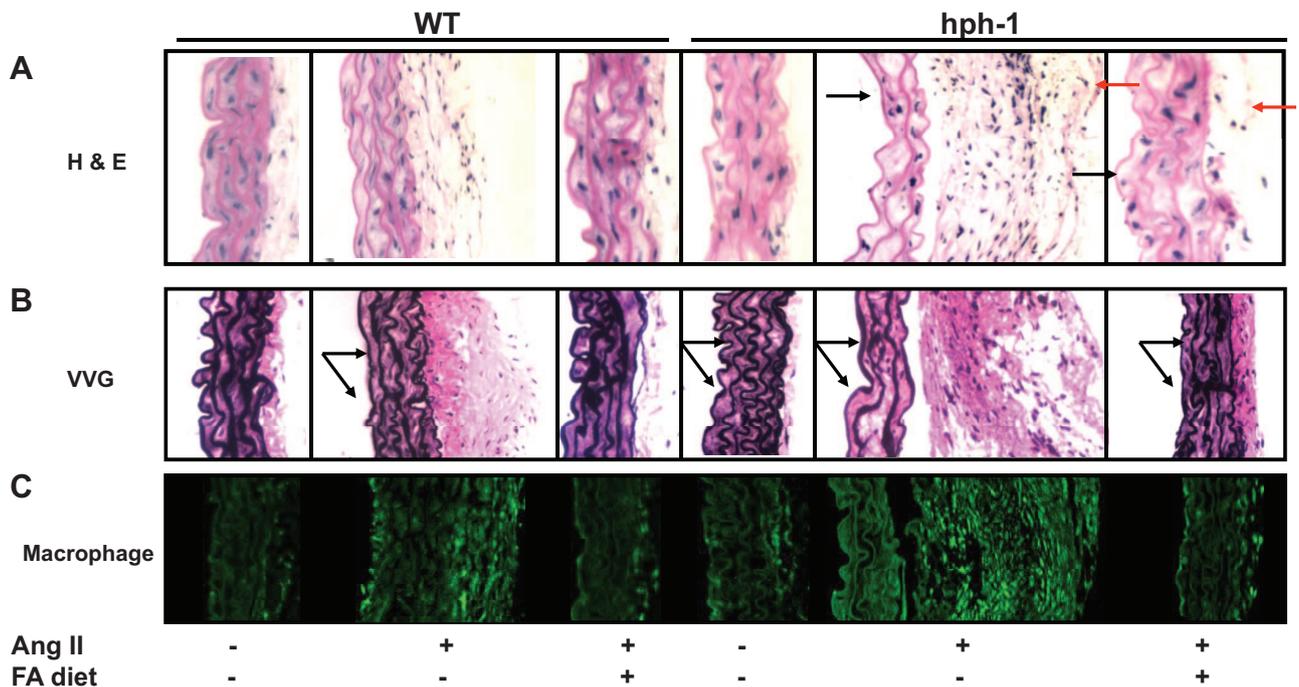
**Figure 5.** Folic acid (FA) prevents progressive uncoupling of endothelial NO synthase (eNOS) in angiotensin II (Ang II)-infused hyperphenylalaninemia (hph)-1 mice. Wild-type (WT) and hph-1 mice were started on oral administration of FA (15 mg/kg per day) 2 days before Ang II infusion (0.7 mg/kg per day) and treated throughout the study period of 14 days. Aortas were harvested on days 0, 4, or 8 for analysis of  $O_2^{\cdot -}$  production in the presence or absence of  $N^G$ -nitro-L-arginine methyl ester (L-NAME) in (A) WT and (B) hph-1 mice. \* $P < 0.05$  vs L-NAME, # $P < 0.05$  vs WT sham, + $P < 0.05$  vs 4 days.

To test whether DHFR gene therapy is enough to overcome its deficiency in Ang II-infused hph-1 mice that is crucial for AAA formation, WT or hph-1 mice were transfected with DHFR before initiation of Ang II infusion and throughout the entire infusion period. Endothelial DHFR expression was markedly enhanced after successful in vivo transfection of a DHFR-containing expression vector (Figure 8A),<sup>5</sup> and this was effective in completely attenuating augmented eNOS uncoupling in Ang II-infused hph-1 mice (L-NAME) instead of increased  $O_2^{\cdot -}$  production as in WT controls (Figure 8B).

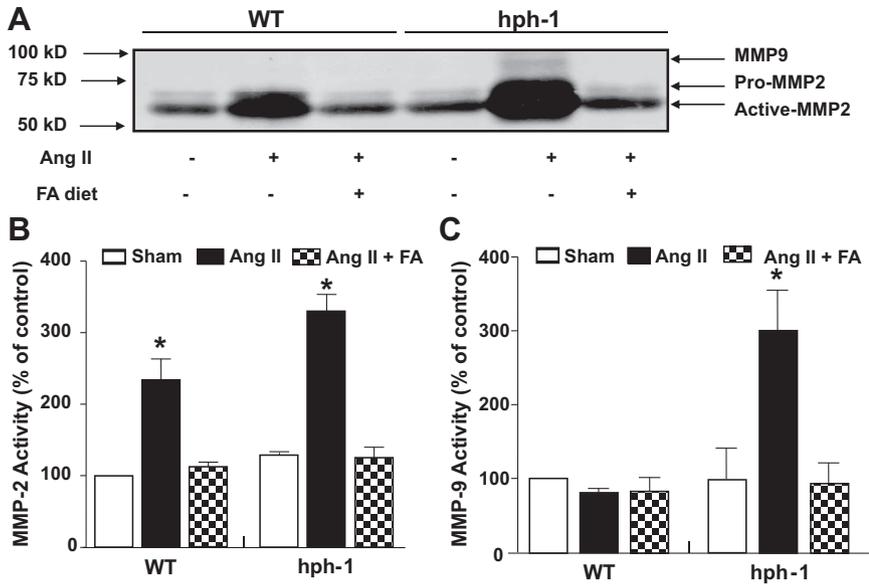
None of the Ang II-infused hph-1 mice in which DHFR was overexpressed developed AAA.

## Discussion

The most significant finding of the present study is the first demonstration of a causal role of eNOS uncoupling/ $H_4B$  deficiency and the therapeutic potential of eNOS recoupling, in AAA formation. In hph-1 mice where eNOS is uncoupled at baseline, we found that Ang II infusion induces AAA formation in conjunction with further uncoupling of eNOS.



**Figure 6.** Folic acid (FA) prevents vascular remodeling in angiotensin II (Ang II)-infused hyperphenylalaninemia (hph)-1 mice. Wild-type and hph-1 mice were treated with or without FA (15 mg/kg per day) beginning 2 days before Ang II (0.7 mg/kg per day) or vehicle infusion and treated throughout the study period of 14 days, after which aortas were harvested for (A) hematoxylin-eosin (H&E) staining (black arrows and red arrows indicating FA-induced changes in media and adventitia, respectively); (B) Verhoeff-Van Gieson (VVG) staining (black arrows showing elastin changes); and (C) macrophage staining indicating macrophage infiltration.

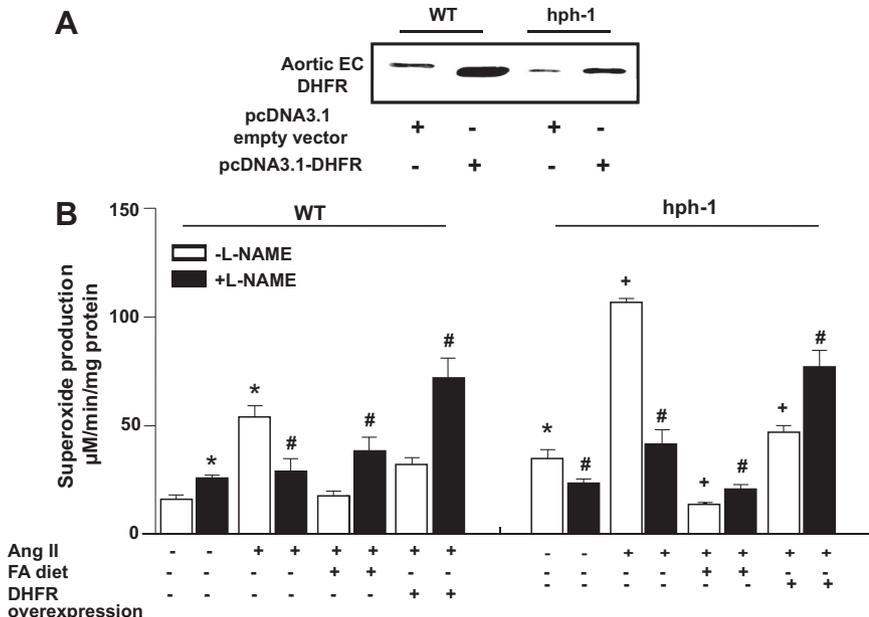


**Figure 7.** Folic acid (FA) prevents angiotensin II (Ang II)-induced matrix metalloproteinase (MMP) 2 and MMP9 activation in hyperphenylalaninemia (hph)-1 mice. Wild-type and hph-1 mice were treated with or without FA (15 mg/kg per day) beginning 2 days before Ang II (0.7 mg/kg per day) or vehicle (sham) infusion. After the 14-day study period, aortas were harvested to assess MMP activity. **A**, Representative zymogram showing MMP2 and MMP9 activities. **B**, Quantitative data of MMP2 activity. **C**, Quantitative data of MMP9 activity. \* $P < 0.05$  vs sham.

Treatment with oral FA effectively prevented both eNOS uncoupling and AAA formation in hph-1 mice. Moreover, FA attenuated Ang II-induced vascular remodeling in both hph-1 and WT mice and modulated blood pressure responses to Ang II differentially in both groups of animals. These findings suggest that eNOS uncoupling predisposes to AAA formation and that strategies directed at eNOS recoupling could be of benefit in treating this vascular disorder.

Elevated parameters of oxidative stress have been detected both systemically and locally in human AAA.<sup>23,24</sup> Moreover, increased aortic oxidative stress has been reported in conjunction with AAA induced experimentally in animal models, and countermeasures against oxidative stress have proven effective in preventing AAA formation in Ang II-infused mice, although not in humans.<sup>25-27</sup> Despite a previously established role of vascular smooth muscle, whether other cellular or enzymatic sources of oxidative stress are involved

in the pathogenesis of AAA, however, remains to be fully understood. Here, we report for the first time that uncoupled eNOS can contribute to oxidative stress leading to severe vascular remodeling and AAA formation in a murine model. The infrarenal pattern of the AAA is very similar to what is found in humans. In addition, the pathological features that we observed in mice with uncoupled eNOS resemble those observed in human AAA, including adventitial inflammation, activation of MMPs, and matrix degradation (Figures 6 and 7). Moreover, the AAA was prone to rupture leading to sudden death, as demonstrated by a mortality rate of 14% in these Ang II-infused hph-1 mice (Figure 1B). Of note, the uncoupling process makes eNOS a peroxynitrite generator, implicating that peroxynitrite, rather than other ROS, might serve as an important redox-signaling mediator for AAA formation. Deletion of eNOS in high-fat-fed apolipoprotein E null mice resulted in spontaneous AAA formation, although



**Figure 8.** Dihydrofolate reductase (DHFR) overexpression recouples endothelial NO synthase (eNOS) in angiotensin II (Ang II)-infused hyperphenylalaninemia (hph)-1 mice. Wild-type (WT) and hph-1 mice were subjected to tail vein transfusion of DHFR expression vector (pcDNA3.1-DHFR)<sup>5</sup> in lipid-based reagent from Altogen Biosystems every other day for 14 days, starting 2 days before Ang II infusion. At the end of the 14 day infusion, aortas were harvested for **(A)** endothelial DHFR expression in aortic preparations by Western blotting and **(B)** aortic superoxide production in the presence or absence of *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME). \* $P < 0.05$  vs wild-type (WT) sham, # $P < 0.05$  vs L-NAME, + $P < 0.05$  vs hph-1 sham.

the incidence rate was much lower at 25%.<sup>28,29</sup> Notably, eNOS uncoupling has also been observed in apolipoprotein E-deficient mice at baseline.<sup>7,30</sup> Mice deficient in eNOS exhibit increased oxidative stress consequent to a loss in NO<sup>•</sup> production. However, these mice do not develop eNOS uncoupling, because they lack functional eNOS protein. Taken together, these data suggest that an eNOS uncoupling-dependent NO<sup>•</sup>/ROS imbalance in the vasculature, rather than loss of NO<sup>•</sup> production, per se, is more profoundly inductive of AAA formation. Our data also suggest a novel role for endothelial cells in oxidant generation that is involved in promoting AAA formation.

Ang II infusion induced a rapid rise in blood pressure in the hph-1 mice, similar to what was observed in the WT mice. However, in hph-1 mice, blood pressure began to progressively fall after day 6 of Ang II infusion, reaching pretreatment values by day 11. In contrast, in WT mice, blood pressure continued to rise throughout the course of Ang II infusion. Treatment with FA, which has been demonstrated to recouple eNOS,<sup>1,31,32</sup> attenuated both the rise in blood pressure in WT mice and the fall in blood pressure in hph-1 mice resulted from Ang II infusion, suggesting the involvement of eNOS uncoupling in both responses. In the case of WT animals, the eNOS uncoupling induced by Ang II likely contributed to hypertension by reducing NO<sup>•</sup> bioavailability. Reduced NO<sup>•</sup> bioavailability likely also contributed to the increased blood pressure at baseline in hph-1 mice and to the initial pressor response to Ang II infusion (Figure 1C). The subsequent fall in blood pressure in Ang II-infused hph-1 mice also appears to result from eNOS uncoupling, although the exact mechanism remains to be determined. It is possible that the growing aneurysm affects hemodynamics, hence, blood pressure. Modulation of blood pressure by oxidative stress is complex and dependent on the balance between destruction of NO<sup>•</sup> and production of ROS that can have vasoconstrictor or vasodilator effects. For example, hydrogen peroxide has been shown to mediate compensatory vasodilatation in hypertensive animals.<sup>6</sup> Then scavenging of eNOS-derived hydrogen peroxide by recoupling of eNOS may increase vasocontractility and blood pressure. Considering that WT mice develop sustained hypertension but no AAA formation in response to Ang II infusion, whereas hph-1 mice develop AAA formation but only transient hypertension, our data are consistent with the previous notions that hypertension is not a decisive risk factor for AAA development, although it may facilitate the disease process.<sup>28,29,33</sup>

It was also important to note that eNOS was progressively uncoupled in Ang II-infused hph-1 mice but not in WT mice (Figure 6), which is consistent with progressive DHFR deficiency in hph-1 mice that was fully corrected by FA treatment (Figure 3A). These data indicate a critical role for endothelial DHFR deficiency in mediating eNOS uncoupling and AAA formation. DHFR is expressed in vascular cells other than endothelial cells; however, only endothelial DHFR abundance correlates with aortic H<sub>4</sub>B and NO<sup>•</sup> bioavailability (Figure 3A).<sup>1,5</sup>

Previous studies have shown that MMPs, specifically MMP-2 and -9, are major players in the development of

AAA.<sup>34–36</sup> In our study, we observed an increase in the activities of both of these enzymes in the Ang II-infused hph-1 animals (Figure 7B and 7C), which matches well with those earlier observations. Interestingly, MMP-2 activity was also increased in Ang II-infused WT animals, which did not develop AAA. This seems to suggest that MMP2 activation alone is not sufficient for AAA development. Of note, MMP-9 activity was found increased only in Ang II-infused hph-1 mice. Furthermore, macrophage staining (Figure 6C) in these animals was dramatically more abundant than in the Ang II-infused WT animals. These findings are in agreement with previous observations that the source of MMP-9 in AAA is generally macrophages.<sup>36</sup> Taken together, our data suggest that Ang II infusion causes an increase in MMP-2 production in the aortas of both WT and hph-1 animals, mostly likely from vascular smooth muscle cells<sup>34,36,37</sup> and endothelial cells.<sup>38,39</sup> However, an increase in MMP-9 from stimulated vascular cells and infiltrating macrophages is necessary for AAA to occur.

### Perspectives

These data innovatively suggest a causal role of eNOS uncoupling/H<sub>4</sub>B deficiency in AAA formation and raise the possibility that oral FA administration, DHFR gene therapy, and perhaps other countermeasures directed against eNOS uncoupling could be of benefit in treating AAA.

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### Disclosures

None.

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## Role of Uncoupled Endothelial Nitric Oxide Synthase in Abdominal Aortic Aneurysm Formation: Treatment With Folic Acid

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