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Combination of folic acid with nifedipine is completely effective in attenuating aortic aneurysm formation as a novel oral medication

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Elastin degradation
Adventitial hypertrophy

A B S T R A C T

Aortic aneurysms are prevalent and severe vascular diseases with high mortality from unpredicted ruptures, while the only treatment option is surgical correction of large aneurysms with considerable risk. We have shown that folic acid (FA) is highly effective in alleviating development of aneurysms although not sufficient to *completely* attenuate aneurysm formation. Here, we examined therapeutic effects on aneurysms of combining FA with Nifedipine as novel and potentially more effective oral medication. Oral administration with FA (15 mg/kg/day) significantly reduced incidence of AAA from 85.71% to 18.75% in Ang II-infused apolipoprotein E (apoE) null mice, while combination of FA with Nifedipine (1.5, 5.0 or 20 mg/kg/day) substantially and completely further reduced incidence of AAA to 12.5%, 11.76% and 0.00% respectively in a dose-dependent manner. The combinatory therapy substantially and completely further alleviated enlargement of abdominal aortas defined by ultrasound, vascular remodeling characterized by elastin degradation and adventitial hypertrophy, as well as aortic superoxide production and eNOS uncoupling activity also in a dose-dependent manner, with combination of FA with 20 mg/kg/day Nifedipine attenuating all of these features by 100% to control levels. Aortic NO and H_4B bioavailabilities were also dose-dependently further improved by combining FA with Nifedipine. These data establish entirely innovative and robust therapeutic regime of FA combined with Nifedipine for the treatment of aortic aneurysms. The comminatory therapy can serve as a first-in-class and most effective oral medication for aortic aneurysms, which can be rapidly translated into clinical practice to revolutionize management of the devastating vascular diseases of aortic aneurysms known as silent killers.

1. Introduction

Aortic aneurysms are prevalent and severe vascular diseases, with high mortality resulting from patients dying of unpredictable sudden rupture of the aneurysms. No treatment options have been available except for surgical correction of large aneurysms with considerable risk. Abdominal aortic aneurysm (AAA) is defined as an abdominal aortic dilation of over 3 cm in diameter, most commonly affecting the infrarenal segment [1]. It is associated with high risk of mortality in the event of aneurysm rupture, leading to around 200,000 deaths each year worldwide [2,3]. The incidence of AAA is up to 9% in population older than 65 [4], and a meta-analysis of 56 studies indicates that the prevalence of AAA in general population is at 4.8% [5]. While the most well-recognized risk factors for AAA include male gender and smoking, other risk factors have been implicated in AAA formation such as older age, family history, hypertension and hyperlipidemia [1,6,7]. The mechanisms of AAA formation are complex, primarily involving oxidative stress driven vascular remodeling that is characterized by matrix degradation and inflammation, resulting in expansion of abdominal aortas [8–10]. The only clinical intervention to treat aortic aneurysms is limited to surgical correction of large AAAs of over 5.5 cm in size, and the 30-day mortality rate is high for both endovascular aortic aneurysm repair (EVAR) and open surgical repair at 1.7–4.7% [11]. There have been no oral medicines available to treat all sizes of the aortic aneurysms including the smaller and growing aneurysms to prevent unpredictable sudden rupture and death [12].

Oxidative stress has been shown to play an important role in the formation of aortic aneurysms including AAA and thoracic aortic

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aneurysm (TAA) [3,9,13–23]. We have previously established a novel and critical role of uncoupled endothelial nitric oxide synthase (eNOS) in AAA formation via sustaining oxidative stress to induce matrix metalloproteinase (MMP) activation and matrix degradation [3,15–22]. We first demonstrated that eNOS uncoupling mediates AAA formation in a novel model of AAA, namely angiotensin II (Ang II) infused hph-1 mice in which 79% of the mice developed AAA within 2 weeks of Ang II infusion, with 14% died of ruptured aneurysm [15]. The Ang II infused hph-1 mice prove to be the most aggressive AAA model to date. Restoration of dihydrofolate reductase (DHFR) function with folic acid (FA) to recouple eNOS markedly attenuated AAA formation in these animals [15]. Moreover, we further demonstrated a novel role of eNOS uncoupling in the development of AAA in Ang II-infused apolipoprotein E (apoE) null mice, a well-established, classical model of AAA, while oral FA administration also effectively restored DHFR function to recouple eNOS, resulting in abrogated aneurysm formation [16]. Indeed, knockout of DHFR in mice facilitating uncoupling of eNOS leads to exaggerated formation of AAA [20]. We have further shown that activation of NADPH oxidase (NOX) isoforms lies upstream of uncoupled eNOS to drive AAA formation [19]. More recently, we have identified a novel microRNA, miRNA-192-5p, in mediating DHFR deficiency downstream of NOX activation in Ang II infused hph-1 mice to result in AAA formation [3]. Furthermore, we have shown that eNOS uncoupling also plays a critical role in driving TAA and AAA formation in Fbn1 Marfan Syndrome mice, a classical model for TAA, which can also be targeted by FA to alleviate formation of both TAA and AAA [22]. Therefore, these data further confirm a critical causal role of eNOS uncoupling in AAA and TAA formation, while also establishing a universal efficacy of FA in treating aortic aneurysms in different animal models, via restoration of eNOS coupling activity to attenuate oxidative stress and consequent vascular remodeling [3,15–22]. Nonetheless, FA alone is not sufficient to fully alleviate formation of aortic aneurysms.

Of note, hypertension is a risk factor for aortic aneurysms, and AAA and TAA patients often have co-existing hypertension [11,24]. Our earlier study investigated effects on AAA formation of both low and high doses of the anti-hypertensive drug Nifedipine, which is a calcium blocker [18]. Intriguingly, while low dose of Nifedipine has no effects on blood pressure, both low and high dose of Nifedipine was able to attenuate AAA formation, via restoration of DHFR and inhibition of NOX to attenuate eNOS uncoupling [18]. The high dose of Nifedipine can also reduce blood pressure at the same time, hence particularly valuable to treat aortic aneurysm patients with co-existing hypertension [18]. Nonetheless, either FA or Nifedipine alone is not sufficient to fully alleviate development of AAA. Therefore, our goal of the present study is to examine whether combination of FA with various doses of Nifedipine has augmented efficacies in treating AAA. We subjected Ang II-infused apoE null mice to oral administration of FA (15 mg/kg/day) and FA in combination with increasing doses of Nifedipine at 1.5, 5.0 or 20.0 mg/kg/day. Remarkably, the data indicate that combination of FA with various doses of Nifedipine substantially and completely further alleviated AAA formation in a dose-dependent manner. The combinatory therapies were robustly more effective in restoring eNOS coupling activity to improve NO bioavailability and attenuate oxidative stress, resulting in abrogated vascular remodeling characterized by elastin degradation and adventitial hypertrophy, with combination of FA with Nifedipine significantly and completely further inhibiting incidence of AAA in a dose-dependent manner from 18.75% in FA group (reduced from 85.71% in Ang II infused apoE null mice) to 12.50%, 11.76% and 0.00% respectively for groups of FA plus 1.5, 5.0 or 20 mg/kg/day Nifidipine. The enlargement of abdominal aortas defined by echocardiography was substantially and completely further attenuated by combining FA with Nifedipine in a dose-dependent manner, with the FA combined with high dose of Nifedipine at 20 mg/kg/day alleviating expansion of abdominal aortic areas by 100% to control levels. These findings are remarkable in establishing that orally administrated combinatory therapy of FA and Nifedipine can serve as a robust, first-in-class, and most effective oral medicine for the treatment of AAA. We anticipate that this entirely innovative oral medication can be rapidly translated into clinical practice to revolutinize management of the devastating silent killer of aortic aneurysms.

2. Materials and methods

2.1. Animals

The use of animals and experimental procedures were approved by the Institutional Animal Care and Usage Committee (IACUC) at the University of California Los Angeles (UCLA). Breeders of apoE null mice were purchased from Jackson Labs (Bar Harbor, ME, USA; Strain B6.129P2-ApoEtm1Unc/J), and bred in house. Animals were kept in ventilated cages, with free access to water and standard chow under the standard care of Division of Laboratory Animal Medicine (DLAM) staff. Male apoE null mice of 6–8 months old were used for experimentation.

2.2. Ang II infusion by osmotic minipump

The animals receiving Ang II infusion were anesthetized in an isoflurane chamber with 5% isoflurane (Piramal Healthcare), and then moved to a nose cone with sustained supply of 1.5–2% isoflurane at 2 L/min oxygen flow using a Isoflurane vaporizer (Tec 3 Isoflurane vaporizer) to maintain the anesthetic state. The back area between the shoulder blades was cleaned of hair and disinfected. Then a small incision was made at the cleaned site; the osmotic minipump (Alzet, model 2004) containing Ang II (1000 ng/kg/min, Sigma-Millipore, St. Louis, MO, USA) was subcutaneously implanted into the mice. The surgical wounds were closed with surgical staples, and then animals were allowed to recover in a heated cage.

2.3. Oral administration of folic acid (FA) and Nifedipine

For animal groups orally treated with folic acid (FA) or FA in combination with various doses of Nifedipine, standard chow was replaced with customized food tablets containing FA (15 mg/kg/day) alone, or FA in combination with Nifedipine (1.5, 5 or 20 mg/kg/day) two days prior to implantation of osmotic minipumps for Ang II infusion, and throughout the study period of 4 weeks of Ang II infusion.

2.4. Ultrasound detection of abdominal aortic size

The enlargement of abdominal aortas in various experimental groups was monitored using ultrasound as we previously published [3,15–20,22]. Animals were anesthetized with isoflurane and placed on a temperature-controlled table. 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 was placed on the gel to visualize aorta transversely (Vevo 3100. FUJIFILM VisualSonics, Inc., Toronto, Ontario, Canada). The aorta was identified using Doppler measurement for the presence of pulsatile flow. Consistent localization of abdominal aortas for image acquisition was insured by visualizing the aorta immediately superior to the branch of the left renal artery in all of the animals.

2.5. Anatomical inspection of abdominal aortas and histological analyses

At the end of the study period of 4 weeks, animals were euthanized with CO_2. The aortas were rapidly removed from the body, rinsed with ice cold Krebs/HEPES buffer, and cleaned of connective tissue and fat. The incidence of AAA was determined by ultrasound assessment of abdominal aortic expansion as described above, and by direct inspection of the abdominal aortas post-mortem, as we published previously [3,

15–22]. For histological analyses, small sections (5 mm) of the abdominal aortas of the suprarenal region were removed and fixed in 4% paraformaldehyde overnight, followed by incubation for 24 h in 10% sucrose, and then embedded in paraffin. In the case of AAA, a center section of the AAA was used for these analyses. Sections were sliced at 5 μm at UCLA Pathology Core, and subjected to hematoxylin-eosin (H&E) staining following standard protocol.

2.6. Verhoeff-Van Gieson (VVG) staining

Paraffin embedded aortic sections were deparaffinized by sequential washes in xylene (2x), descending ethanol from 100%, 90%, 75%–50%, and distilled water. Sections were stained in Verhoeff's solution for 70 min, followed by differentiation in 2% ferric chloride for 90 s. Sections were incubated with 5% sodium thiosulfate for 60 s, followed by counterstaining with Van Gieson's solution. Sections were then subjected to dehydration with 95% and 100% alcohol, and finally washed in xylene. After drying, sections were mounted with Permount mounting media (SP15-100, Thermo-Fisher Scientific, Pittsburgh, PA, USA), and images captured using a Nikon TE2000-U fluorescent microscope.

2.7. Electron spin resonance determination of total aortic superoxide production and eNOS uncoupling activity

Aortic superoxide production was determined by electron spin resonance (ESR) as we previously published [3,15,16,18–20,22,25,26]. In brief, freshly isolated aortas were homogenized in lysis buffer containing 1:100 protease inhibitor cocktail (Sigma-Millipore, St. Louis, MO, USA), centrifuged at 12,000 rpm for 15 min, and protein supernatant collected. After determination of protein concentration using a protein assay kit (Bio-Rad, Hercules, CA, USA), five μg of the protein was loaded into ice-cold and nitrogen bubbled modified Krebs/HEPES buffer (KHB, in mmol/L: NaCl, 99; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.0; $CaCl_2$, 1.9; $NaHCO_3$, 25; glucose, 11.1, NaHEPES, 20) containing diethyldithiocarbamic acid (5 mmol/L), deferoxamine (25 mmol/L), and the superoxide specific spin trap methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, 500 μmol/L, Axxora, San Diego, CA, USA). The mixture was loaded into a glass capillary (Kimble, 71900-50, Dover, OH, USA), and assayed using electron spin resonance (ESR) spectrophotometer (eScan, Bruker, Billerica, MA, USA) for total superoxide production by taking the difference in the presence or absence of PEG-SOD (polyethylene glycol-superoxide dismutase; 100 U/mL, Sigma-Millipore, St. Louis, MO, USA). To determine eNOS uncoupling activity, measurements were made with the addition of L-NAME (N(ω)-nitro-L-arginine methylester, 10 μmol/L, Cayman Chemical, Ann Arbor, MI, USA). A reduction in superoxide production with L-NAME indicates that eNOS is uncoupled producing superoxide, while an increase in superoxide production with L-NAME indicates that eNOS is coupled producing NO. The ESR settings used were: Center field, 3480; Sweep width, 9G; microwave frequency, 9.78 GHz; microwave power, 21.02 mW; modulation amplitude, 2.47 G; 512 points of resolution; receiver gain,1000.

2.8. Electron spin resonance determination of aortic nitric oxide production

Aortic nitric oxide (NO) bioavailability was determined by electron spin resonance (ESR) as we previously published [3,15,16,18–20,22,25,26]. In brief, freshly isolated aortic rings were incubated with freshly prepared NO specific spin trap $Fe^{2+}(DETC)_2$ (0.5 mmol/L) colloid in modified Krebs/HEPES buffer at 37°C for 60 min, in the presence of calcium ionophore A23187 (10 mmol/L). After incubation, the aortic pieces were snap frozen in liquid nitrogen and loaded into a finger Dewar for analysis with ESR spectrophotometer (eScan, Bruker, Billerica, MA, USA). The instrument settings used were as the followings: Center field, 3440; Sweep width, 100 G; microwave frequency, 9.796 GHz; microwave power 13.26 mW; modulation amplitude, 9.82 G; 512 points of resolution; and receiver gain 356.

2.9. HPLC determination of aortic H_4B bioavailability

For determination of aortic H_4B levels as we previously published [3,15–20,25,26], freshly isolated aortas were lysed in H_4B lysis buffer (0.1 mol/L phosphoric acid, 1 mmol/L EDTA, 10 mmol/L DLDithiothreitol), centrifuged at 12,000 rpm for 10 min, and then supernatants subjected to differential oxidation in acidic (0.2 mol/L trichloroacetic acid with 2.5% I_2 and 10% KI) and alkalytic (0.1 mol/L NaOH with 0.9% I_2 and 1.5% KI) solutions. After centrifugation, 10 μl of the supernatant was injected into a HPLC system equipped with a fluorescent detector (Schimadzu America Inc, Carlsbad, CA, USA). Excitation and emission wavelengths of 350 nm and 450 nm were used to measure H_4B levels.

2.10. Statistical analyses

All grouped data are presented as Mean ± SEM. Statistical analyses were carried out with the Prism software. Comparisons between multiple groups were done using one-way ANOVA followed by the Newman-Keuls post-hoc test. Statistical significance was set at $p < 0.05$. Comparisons of the AAA incidence rates were done using a contingency table, also with a statistical threshold of 0.05.

3. Results

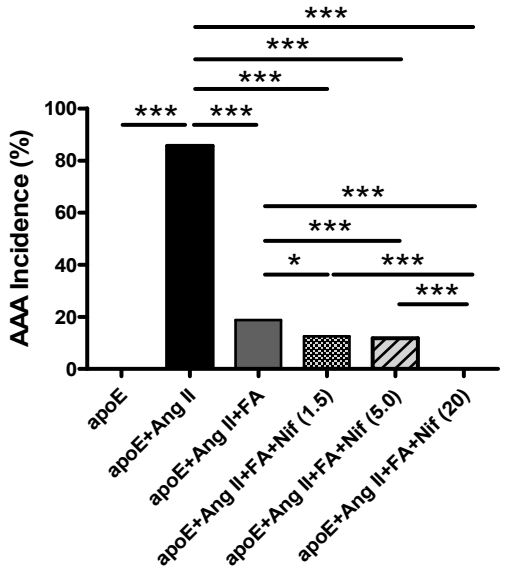
3.1. Combination of FA with Nifedipine substantially and completely further attenuated incidence of AAA in a dose-dependent manner

We examined incidence of AAA in Ang II infused apoE null mice subjected to oral administration of FA alone, or combinatory therapy of FA with various doses of Nifedipine (1.5, 5 or 20 mg/kg/day). As shown in Fig. 1A-B, after 4 weeks of Ang II infusion, 85.71% (30 out of 35) of the Ang II infused apoE null mice developed AAA. This is in comparable range to our previous findings [16]. Oral FA administration significantly reduced incidence of AAA to 18.75% ($p < 0.001$, 6 out of 32), which is consistent to our previous observation [16]. Intriguingly, compared to FA alone group, combination of FA with various doses of Nifedipine (1.5, 5 or 20 mg/kg/day) substantially and completely further reduced incidence of AAA to 12.50% ($p < 0.05$, 3 out of 24), 11.76% ($p < 0.001$, 2 out of 17) and 0.00% ($p < 0.001$, 0 out of 18) respectively in a dose-dependent manner, indicating that combinatory therapy of FA and Nifedipine is robustly more effective in treating aortic aneurysms.

3.2. Combination of FA with Nifedipine substantially and completely further attenuated enlargement of abdominal aortas in a dose-dependent manner

To examine enlargement of abdominal aortas in Ang II infused apoE null mice that were treated with FA alone, or FA in combination with 1.5, 5 or 20 mg/kg/day Nifedipine, sizes of abdominal aortas were monitored weekly using ultrasound. Fig. 2A shows representative ultrasound images of abdominal aortas from all experimental groups across the 4 weeks, while Fig. 2B shows grouped data. Of note, abdominal aortas of Ang II infused apoE null mice were markedly enlarged comparing to untreated apoE null mice, which was significantly attenuated by oral administration of FA (Fig. 2A-B, ##$p < 0.01$ or ###$p < 0.001$ vs. apoE + Ang II of the same week). Remarkably, combination of FA with 1.5, 5 or 20 mg/kg/day Nifedipine substantially or completely further attenuated expansion of abdominal aortas in a dose-dependent manner, with the FA plus 20 mg/kg/day Nifedipine group showing reduction of aortic enlargement to baseline levels (Fig. 2A-B, @@$p < 0.01$ or @@@$p < 0.001$ for 1.5 mg/kg/day Nifedipine group; &&$p < 0.01$ or &&&$p < 0.001$ for 5.0 mg/kg/day Nifedipine group; $$$p < 0.001$ for 20 mg/kg/day Nifedipine group). Of note,

A



B

Groups	No AAA	AAA	Percentage (AAA)
apoE	34	0	0.00%
apoE+Ang II	5	30	85.71%
apoE+Ang II+FA	26	6	18.75%
apoE+Ang II+FA (1.5)	21	3	12.50%
apoE+Ang II+FA+Nif (5.0)	15	2	11.76%
apoE+Ang II+FA+Nif (20)	18	0	0.00%

Fig. 1. Combination of FA with Nifedipine substantially and completely further attenuated incidence of aortic aneurysm in a dose-dependent manner. ApoE null mice of 6–8 months old were infused with angiotensin II (Ang II, 1000 ng/kg/min) for 4 weeks in the presence of regular chew, customized chew containing folic acid (FA, 15 mg/kg/day), or customized chew containing FA and various doses of Nifedipine (1.5, 5.0, and 20 mg/kg/day). (A) Incidence of abdominal aortic aneurysm (AAA) in cross different groups. The data indicate that with regular chow, the AAA incidence in Ang II infused apoE mice is 85.71%, while it was attenuated by oral administration of FA to 18.75%. Combination of FA with 1.5, 5 or 20 mg/kg/day Nifedipine substantially further attenuated AAA incidence rate to 12.50%, 11.76% and 0.00% respectively. *p < 0.05, ***p < 0.001, n = 17–35. (B) Actual numbers of animals examined across different groups and numbers of animals developed AAA: apoE, n = 34/0; apoE + Ang II, n = 35/30; apoE + Ang II + FA, n = 32/6; apoE + Ang II + FA + Nif (1.5), n = 24/3; apoE + Ang II + FA + Nif (5.0), n = 17/2; , apoE + Ang II + FA + Nif (20.0), n = 18/0.

at some time points, Nifedipine 5.0 or 20 mg/kg/day group was significantly more effective than Nifedipine 1.5 mg/kg/day group (￥p < 0.05; %p < 0.05 or %%% p < 0.001).

3.3. Combination of FA and Nifedipine substantially and completely further attenuated vascular remodeling in a dose-dependent manner

Formation of aortic aneurysms is accompanied by extensive vascular remodeling featured by matrix degradation to allow expansion of aortas. To examine the extent of the vascular remodeling that occurred during AAA in the presence of various treatments, freshly isolated aortas were embedded in paraffin, sectioned and stained with hematoxylin-eosin (H&E). As shown in Fig. 3, oral administration of FA markedly attenuated vascular remodeling featured by elastic degradation and adventitial hypertrophy in Ang II infused apoE null mice. Combination of FA with 1.5, 5 or 20 mg/kg/day Nifedipine substantially and completely further attenuated vascular remodeling in a dose-dependent manner. The black arrows in the figures indicate elastin degradation in Ang II infused apoE mice and restoration of which in different treatment groups of FA alone or FA in combination with increasing doses of Nifedipine.

3.4. Combination of FA with Nifedipine substantially and completely further attenuated elastin degradation in a dose-dependent manner

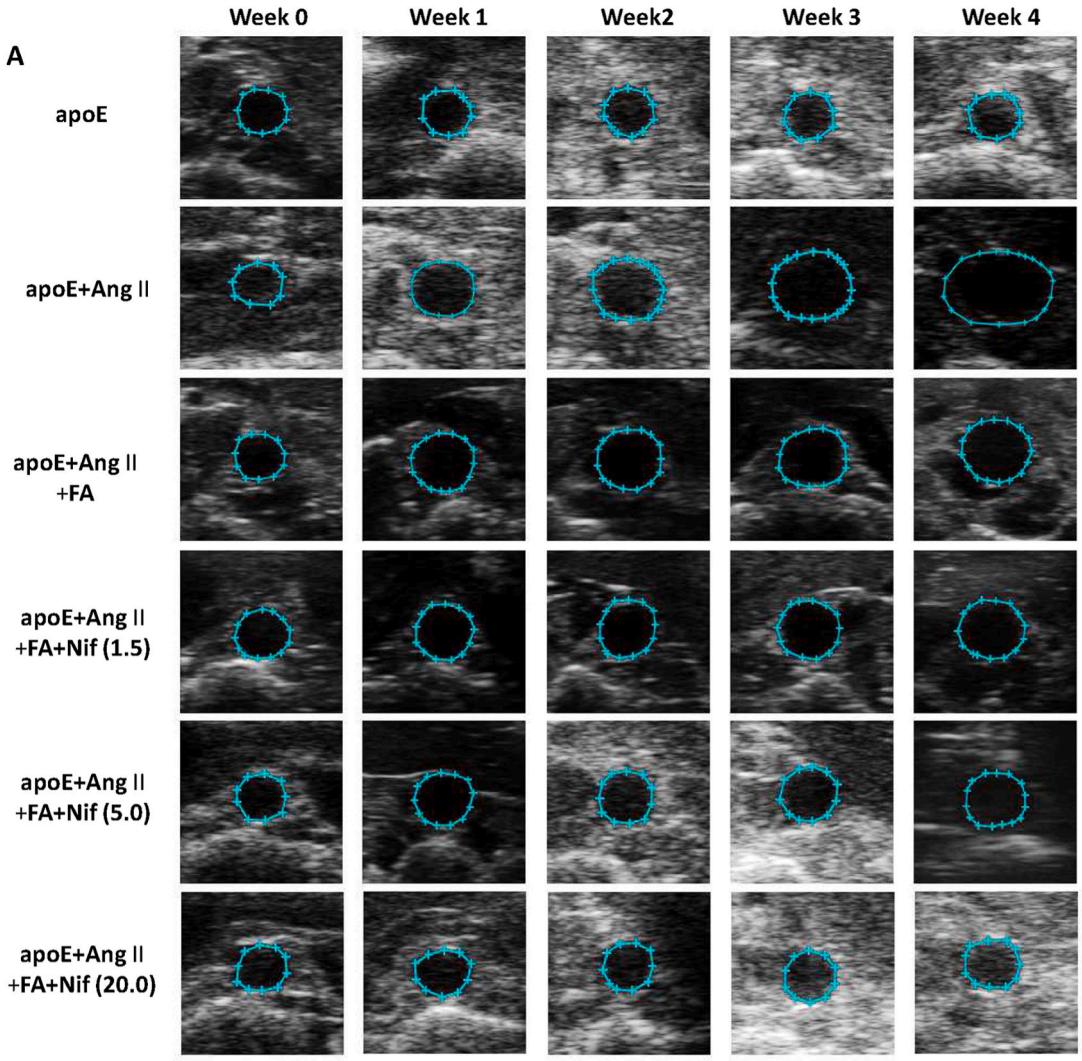
To assess medial elastin degradation, we performed VVG staining using aortic sections from all experimental groups. As shown in Fig. 4, elastin degradation featured by flattening and breakdown was obvious in Ang II infused apoE null mice, which was significantly attenuated by oral administration of FA. Importantly, combination of FA with various doses of Nifedipine (1.5, 5 or 20 mg/kg/day) substantially and completely further alleviated elastin degradation in a dose-dependent manner. The black arrows in the figures indicate elastin flattening and breakdown in Ang II infused apoE mice and restoration of which in different treatment groups of FA alone or FA in combination with increasing doses of Nifedipine.

3.5. Combination of FA with Nifedipine substantially and completely further attenuated total superoxide production and eNOS uncoupling activity in a dose-dependent manner

We have previously established a novel mediator role of eNOS uncoupling in AAA formation in both novel and classical models of Ang II infused hph-1 and apoE null mice [15–19]. In the present study, we examined changes in aortic total superoxide production and eNOS uncoupling activity from all treatment groups. Freshly prepared aortic lysates were subjected to electron spin resonance (ESR) determination of superoxide production with and without L-NAME, an inhibitor of NOS. If eNOS is functional and coupled, its inhibition by L-NAME will increase the measured superoxide, as eNOS is producing NO to scavenge superoxide. However, if eNOS is dysfunctional, uncoupled and producing superoxide, its inhibition with L-NAME will lead to a decrease in measured superoxide.

As is shown in Fig. 5A, there was a significant increase in total superoxide production in aortas isolated from Ang II infused apoE null mice, which was markedly abrogated by oral FA administration. This is similar to our previous findings, representing the molecular mechanism underlying protective effects of FA on AAA formation [16]. Intriguingly, combination of FA with various doses of Nifedipine (1.5, 5 or 20 mg/kg/day) substantially and completely further attenuated total superoxide production in a dose-dependent manner.

As shown in Fig. 5B, there was a marked increase in eNOS uncoupling activity in Ang II infused apoE null mice, which is consistent to our



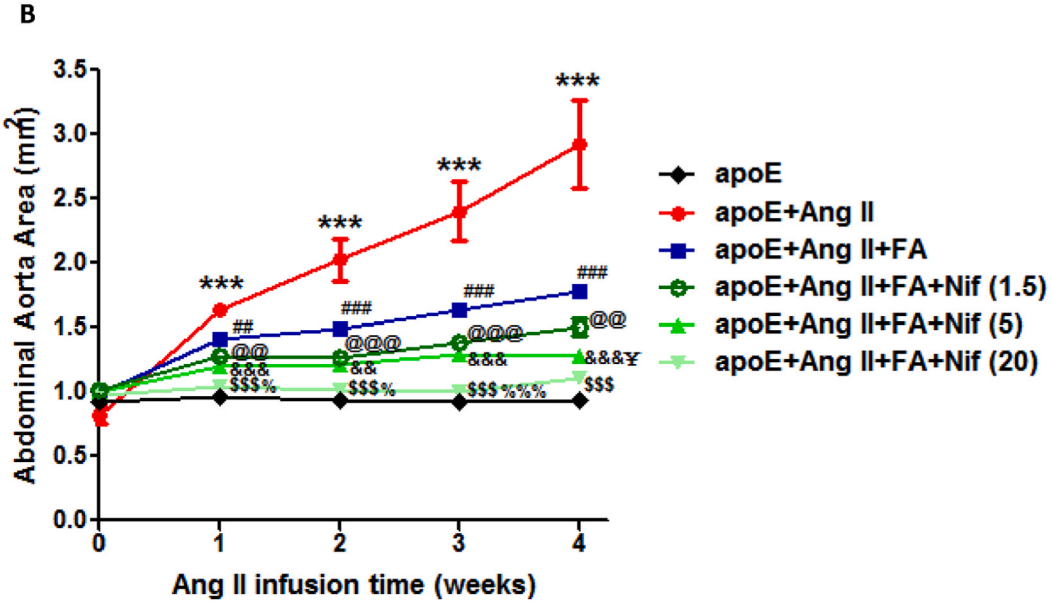


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Fig. 2. Combination of FA with Nifedipine substantially and completely further attenuated enlargement of abdominal aortas in a dose-dependent manner. ApoE null mice of 6–8 months old were infused with angiotensin II (Ang II, 1000 ng/kg/min) for 4 weeks in the presence of regular chew, customized chew containing folic acid (FA, 15 mg/kg/day), or customized chew containing FA and various doses of Nifedipine (1.5, 5.0, and 20 mg/kg/day). Enlargement of abdominal aortas was monitored weekly using ultrasound. (A) Representative weekly ultrasound images taken from different experimental groups. (B) Grouped data of ultrasound measurements of abdominal aortic areas. Infusion of Ang II into apo E null mice resulted in marked enlargement in abdominal aortas (***p < 0.001 vs. apoE of the same week). Oral administration with FA alone significantly attenuated enlargement of abdominal aortas (##p < 0.01 or ###p < 0.001 vs. apoE + Ang II of the same week). Compared to the FA alone group, Nifedipine at doses of 1.5, 5.0 and 20 mg/kg/day substantially and completely further attenuated enlargement of abdominal aortas in a dose-dependent manner, with the FA plus 20 mg/kg/day Nifedipine group showing reduction in aortic enlargement by 100% to control levels (@@p < 0.01 or @@@p < 0.001 for 1.5 mg/kg/day Nifedipine group; &&p < 0.01 or &&&p < 0.001 for 5.0 mg/kg/day Nifedipine group; $$$p < 0.001 for 20 mg/kg/day Nifedipine group). Of note, at some time points, Nifedipine 5.0 or 20 mg/kg/day group was significantly more effective than Nifedipine 1.5 mg/kg/day group (¥p < 0.05; %p < 0.05 or %%% p < 0.001). n = 4–19.

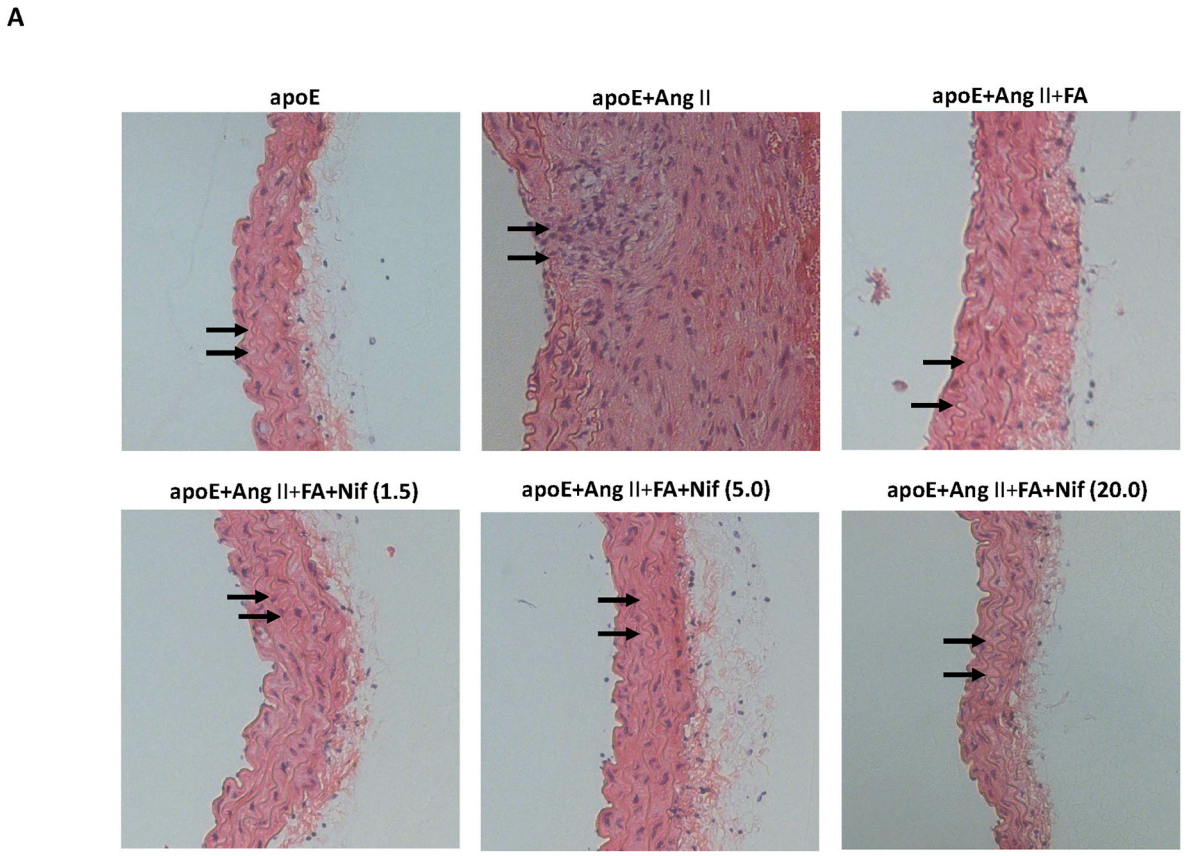

Fig. 3. Combination of FA with Nifedipine substantially and completely further attenuated vascular remodeling in a dose-dependent manner. ApoE null mice of 6–8 months old were infused with angiotensin II (Ang II, 1000 ng/kg/min) for 4 weeks in the presence of regular chew, customized chew containing folic acid (FA, 15 mg/kg/day), or customized chew containing FA and various doses of Nifedipine (1.5, 5.0, and 20 mg/kg/day). Data of H&E staining indicate that vascular remodeling characterized by elastic degradation (black arrows) and adventitial hypertrophy in Ang II infused apoE null mice was significantly attenuated by oral administration of FA, which was substantially and completely further alleviated (black arrows) by combination of FA with various doses of Nifedipine in a dose-dependent manner.

previous findings [16]. At baseline, eNOS is minimally uncoupled in apoE null mice that is well-compensated. Oral administration with FA reversed eNOS uncoupling activity in Ang II infused apoE null mice (Fig. 5B). It is important to note that combination of FA with 1.5, 5 or 20 mg/kg/day Nifedipine also completely attenuated eNOS uncoupling activity while abrogating total superoxide production more effectively in a dose-dependent manner (Fig. 5A-B). These data indicate that recoupling of eNOS and further reduction in total superoxide production underlie improved efficacies in attenuating AAA formation by combinatory therapy of FA and Nifedipine.

3.6. Combination of FA with Nifedipine substantially further improved NO bioavailability in a dose-dependent manner

Since FA plus Nifedipine recoupled eNOS to diminish superoxide production as described above, we next examined aortic NO bioavailability from all experimental groups using electron spin resonance (ESR). The data indicate that there was a significant decrease in NO bioavailability in aortas isolated from Ang II infused apoE null mice, which was significantly restored by oral FA administration (Fig. 6). Intriguingly, combination of FA with various doses of Nifedipine (1.5, 5 or 20 mg/kg/day) substantially further improved aortic NO bioavailability in a dose-dependent manner, going along with restoration of eNOS function to mediate protection against formation of AAA.

3.7. Combination of FA with Nifedipine substantially further improved aortic H_4B bioavailability in a dose-dependent manner

As an essential cofactor of eNOS, H_4B deficiency is indicative of eNOS uncoupling [21,27]. Herein, we determined aortic H_4B

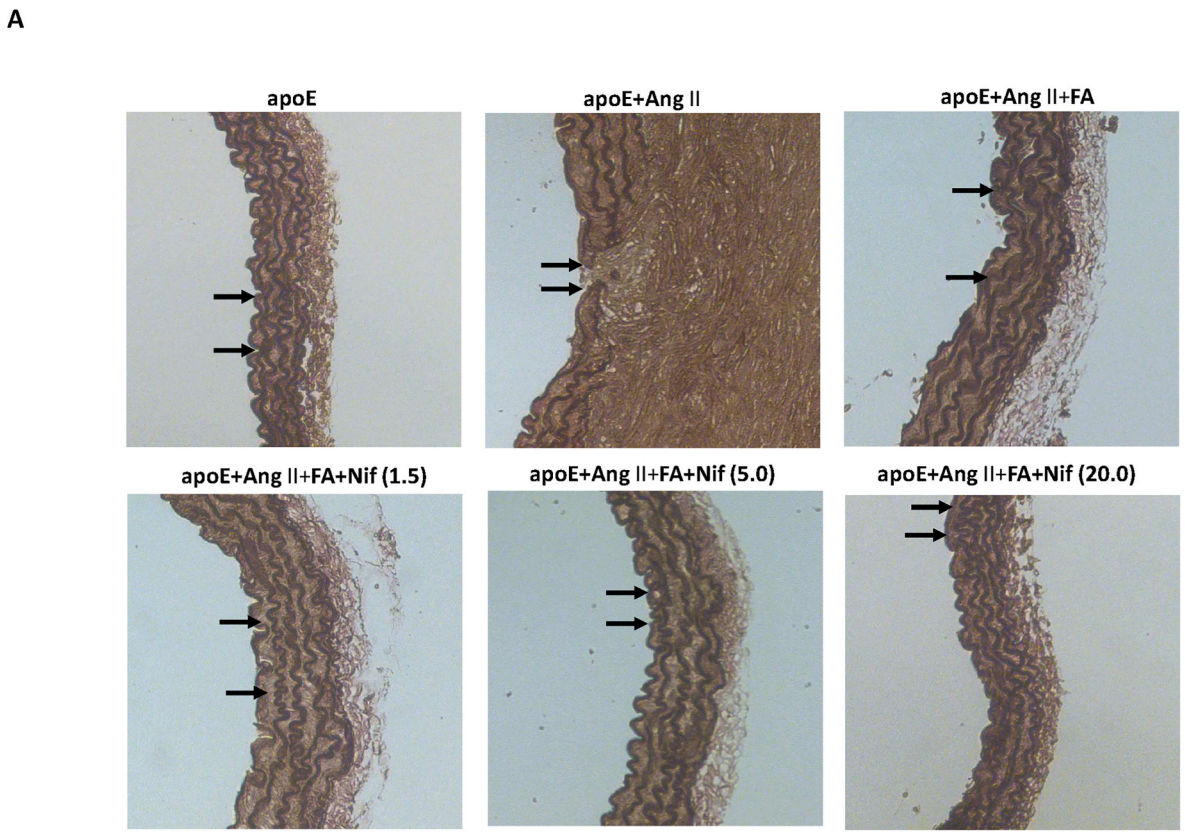

Fig. 4. Combination of FA with Nifedipine substantially and completely further attenuated elastin degradation in a dose-dependent manner. ApoE null mice of 6–8 months old were infused with angiotensin II (Ang II, 1000 ng/kg/min) for 4 weeks in the presence of regular chew, customized chew containing folic acid (FA, 15 mg/kg/day), or customized chew containing FA and various doses of Nifedipine (1.5, 5.0, and 20 mg/kg/day). Data of VVG staining indicate that medial elastin degradation featured by elastin flattening and breakdown (black arrows) in Ang II infused apoE null mice was significantly attenuated by oral administration of FA, which was substantially and completely further alleviated by combination of FA with various doses of Nifedipine in a dose-dependent manner (black arrows).

bioavailability from all experimental groups using HPLC. As shown in Fig. 7, Ang II induced H_4B deficiency in apoE null mice was abrogated by oral FA administration, while combination of FA with various doses of Nifedipine (1.5, 5 or 20 mg/kg/day) substantially further improved aortic H_4B bioavailability in a dose-dependent manner. Taken together with data describe above, our findings in the present study strongly indicate that combination of FA with 1.5, 5 or 20 mg/kg/day Nifedipine proves to be a robust, first-in-class, and most effective therapeutic regime for AAA, via attenuation of eNOS uncoupling activity to abrogate oxidative stress and consequent vascular remodeling.

4. Discussion

In the present study, we demonstrated for the first time that combinatory therapy of FA and Nifedipine, two compounds approved by FDA for different indications of nutrient supplementation to pregnant women and blood pressure lowering respectively, substantially and completely attenuates formation of aortic aneurysm in a classical model of Ang II-infused apoE null mice, with the addition of Nifedipine showing a remarkable dose-dependent effect in augmenting the efficacy of FA in treating aortic aneurysm. AAA incidence determined by ultrasound definition of aortic expansion and post-mortem inspection was synergistically, substantially and completely further abrogated by combination of FA with 1.5, 5 or 20 mg/kg/day Nifedipine in a dose-dependent manner. Combinatory therapy of FA and Nifedipine substantially and completely further alleviated vascular remodeling featured by elastin degradation and adventitial hypertrophy in a dose-dependent manner. These protective effects on AAA incidence and related pathophysiological changes are attributed to augmented effects of combinatory therapy on restoration of eNOS function and abrogation of oxidative stress. Combination of FA with various doses of Nifedipine substantially and completely further attenuated superoxide production in a dose-dependent manner while completely recoupling eNOS to restore NO bioavailability also in a dose-dependent manner. The aortic bioavailability of eNOS cofactor H_4B was indeed further improved by the combinatory therapy, going along with the outcome of eNOS recoupling. These data strongly demonstrate that combinatory therapy of FA and Nifedipine can serve as a novel oral medication that is most effective in the treatment of aortic aneurysms.

Oxidative stress has been implicated in the pathogenesis of AAA [3,9,14–22]. Specifically, we have previously established a novel and critical role of uncoupled eNOS in mediating formation of AAA by driving oxidative stress and consequent vascular remodeling [3,15–22]. We initially demonstrated that in Ang II infused hph-1 mice, a newly established, robust model of AAA, uncoupling of eNOS mediates AAA formation that can be attenuated by FA restoration of endothelial dihydrofolate reductase (DHFR) function/H_4B bioavailability to recouple eNOS [15]. We further showed that in a classical AAA model of Ang II-infused apoE null mice, eNOS uncoupling also mediates sustained oxidative stress and AAA formation that can be alleviated by oral FA administration [16]. In both Ang II-infused hph-1 and apoE null mice, oral FA administration is significantly effective in reducing incidence of AAA, preventing enlargement of abdominal aorta, and alleviating maladaptive vascular remodeling [16]. This is mediated by FA recoupling of eNOS to attenuate superoxide production while restoring NO bioavailability, resulting in abrogated oxidative stress by shutting down the enzymatic system of uncoupled eNOS [16]. In addition, we have recently shown that eNOS uncoupling plays a similar causal role in TAA

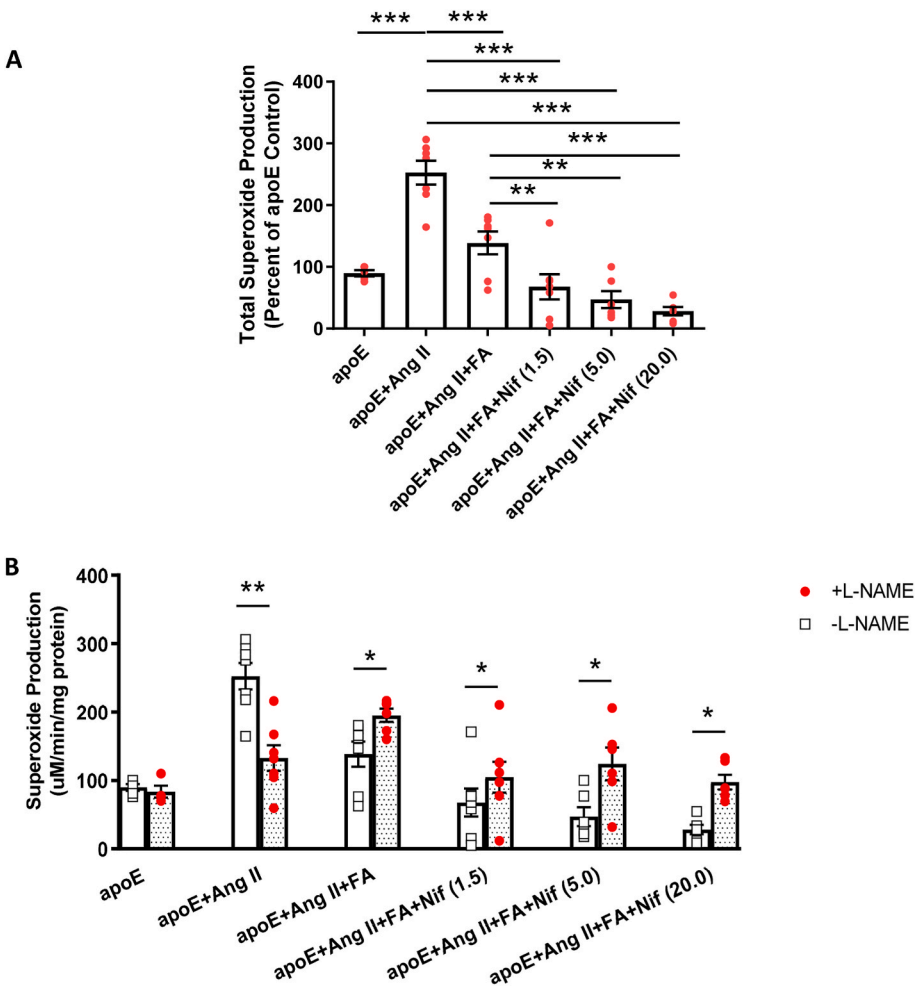

Fig. 5. Combination of FA and Nifedipine substantially and completely further attenuated total superoxide production and eNOS uncoupling in a dose-dependent manner. ApoE null mice of 6–8 months old were infused with angiotensin II (Ang II, 1000 ng/kg/min) for 4 weeks in the presence of regular chew, or oral administration of folic acid (FA, 15 mg/kg/day) in combination with various doses of Nifedipine (1.5, 5.0, and 20 mg/kg/day). (A) Total superoxide production determined by electron spin resonance (ESR) as we previously published. The data indicate that there was a significant increase in total superoxide production in aortas isolated from Ang II infused apoE null mice, which was markedly abrogated by oral FA administration. Combination of FA with 1.5, 5 and 20 mg/kg/day of Nifedipine substantially and completely further attenuated total superoxide production in a dose-dependent manner. $^{**}p < 0.01$, $^{***}p < 0.001$, n = 5–7. (B) Aortic eNOS uncoupling activity determined by ESR as we previously published. The data indicate that eNOS uncoupling activity, reflected by L-NAME-inhibitable superoxide production, was completely attenuated by oral administration of FA alone or in combination with various dosing of Nifedipine. $^{*}p < 0.05$, $^{**}p < 0.01$, n = 5–7. A reduction in superoxide production with L-NAME indicates that eNOS is uncoupled producing superoxide, while an increase in superoxide production with L-NAME indicates that eNOS is coupled producing NO. Open squares indicate conditions without L-NAME while red dots indicate conditions with L-NAME.

formation in a classical model of Fbn1 Marfan Syndrome mice, in which recoupling of eNOS with FA diet also attenuated formation of TAA and AAA [22]. Data from the present study further confirm these findings to establish a critical role of eNOS uncoupling in formation of aortic aneurysms including AAA and TAA, targeting of which by FA proves to be a highly effective treatment option. Nonetheless, FA alone cannot completely attenuate aneurysm formation. We therefore propose that combinatory therapy of FA with anti-hypertensive drug of Nifedipine, shown in our previous studies to be partially effective in alleviating AAA formation as well [18], may prove to have synergistic and augmented effects in treating AAA.

Our hypothesis of adding Nifedipine to FA administration is based on our earlier notion that Nifedipine is significantly effective in attenuating AAA formation in Ang II infused hph-1 mice [18]. Whereas hypertension is not a decisive risk factor for AAA formation, it may facilitate disease development. In a previous study, we treated Ang II infused hph-1 mice with both low and high dose of Nifedipine (5 and 20 mg/kg/day), which significantly abrogated AAA formation and related pathophysiological and molecular changes [18]. We have further shown that these effects are mediated by improved NO bioavailability and decreased superoxide production consequent to recoupling of eNOS [18], while the role of eNOS uncoupling in mediating aneurysm formation is a common mechanism for both AAA and TAA. Whereas low dose of Nifedipine had no effects on blood pressure, high dose of Nifedipine effectively lowered blood pressure in Ang II infused hph-1 mice, making it particularly beneficial to treat AAA and TAA patients with co-existing hypertension [11,18,24]. Furthermore, we demonstrated that both doses of Nifedipine are inhibitory of NADPH oxidase activity, which lies upstream of uncoupled eNOS [18]. This mechanism is different from what is driven by FA, which primarily recouples eNOS by restoring DHFR function [3,15–17,19,20,22].

Therefore, in the present study we subjected Ang II-infused apoE null mice to combinatory therapy of FA and various doses of Nifedipine, to examine potential synergistic and augmented effects of the treatment on attenuating aortic aneurysm formation. Intriguingly, FA combined with 1.5, 5 or 20 mg/kg/day Nifedipine substantially and completely further reduced incidence of AAA to 12.50%, 11.76% and 0.00% respectively in a dose-dependent manner, which represent substantially improved efficacies comparing to FA alone (18.75%). The combinatory therapy substantially and completely further abrogated expansion of abdominal aortas in a dose-dependent manner compared to the FA alone group, with FA plus 20 mg/kg/day of Nifedipine attenuating sizes of the abdominal aortas by 100% to control levels, again confirming a substantial further improvement in the efficacy of treating AAA. Moreover, vascular remodeling featured by elastin degradation and adventitial hypertrophy was also substantially and completely further alleviated in FA plus Nifedipine groups in a dose-dependent manner.

Of note, combinatory therapy of FA and various doses of Nifedipine substantially and completely further improved efficacy of FA in attenuating total superoxide production and eNOS uncoupling activity in a dose-dependent manner. Though the treatment with FA more directly targets the coupling state of eNOS [3,15–17,19,20,22], Nifedipine by original approval targets a seemingly unrelated pathway of calcium channel in the cell. Our previous study indicates that Nifedipine has an inhibitory effect on NADPH oxidase that is upstream of uncoupled eNOS, besides its upregulating effects on DHFR [18]. This differential

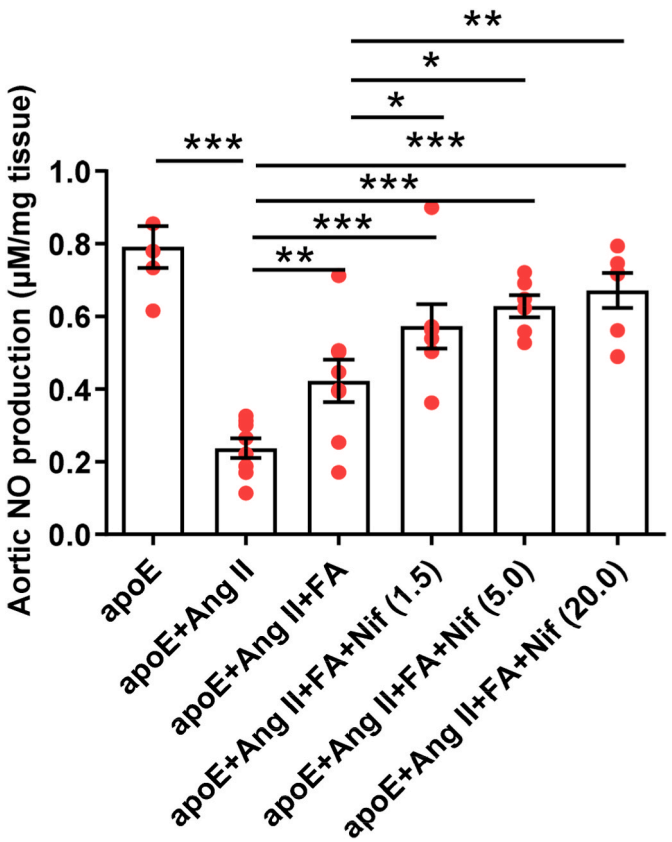

Fig. 6. Combination of FA and Nifedipine substantially further improved NO bioavailability in a dose-dependent manner. ApoE null mice were infused with Ang II, fed regular chow or chow mixed with FA (15 mg/kg/day) or chow mixed with FA plus different concentration of Nifedipine (1.5, 5 and 20 mg/kg/day). ApoE null mice of 6–8 months old were infused with angiotensin II (Ang II, 1000 ng/kg/min) for 4 weeks in the presence of regular chew, or oral administration of folic acid (FA, 15 mg/kg/day) in combination with various doses of Nifedipine (1.5, 5.0, and 20 mg/kg/day). Aortic NO bioavailability was determined by electron spin resonance (ESR) as we previously published. The data indicate that there was a significant decrease in NO bioavailability in aortas isolated from Ang II infused apoE null mice, which was significantly restored by oral FA administration. Combination of FA with 1.5, 5 and 20 mg/kg/day of Nifedipine substantially and dose-dependently further restored NO bioavailability. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 6–8$.

mechanism might explain the synergistic effects between FA and Nifedipine in attenuating aortic aneurysm formation via enhanced efficacies in restoration of eNOS function/NO bioavailability to abrogate oxidative stress and consequent vascular remodeling. Indeed, H_4B deficiency as an indicator of eNOS uncoupling activity, was also markedly further alleviated by combination of FA with various doses of Nifedipine in a dose-dependent manner.

In conclusion, our data for the first time strongly demonstrate that combination of FA with various doses of Nifedipine substantially and completely further attenuates formation of aortic aneurysms and related pathophysiological and molecular changes in a dose-dependent manner. Oral administration of FA and Nifedipine proves to be a robust, first-in-class, and most effective oral medicine for the treatment of aortic aneurysms including AAA and TAA which are driven by the common pathway of eNOS uncoupling, with composition containing high dose of Nifedipine particularly suitable for treatment of AAA patients with co-existing hypertension. These findings are of great translational potential for immediate implementation of entirely innovative oral medication to robustly treat the prevalent and devasting human disease of aortic aneurysms.

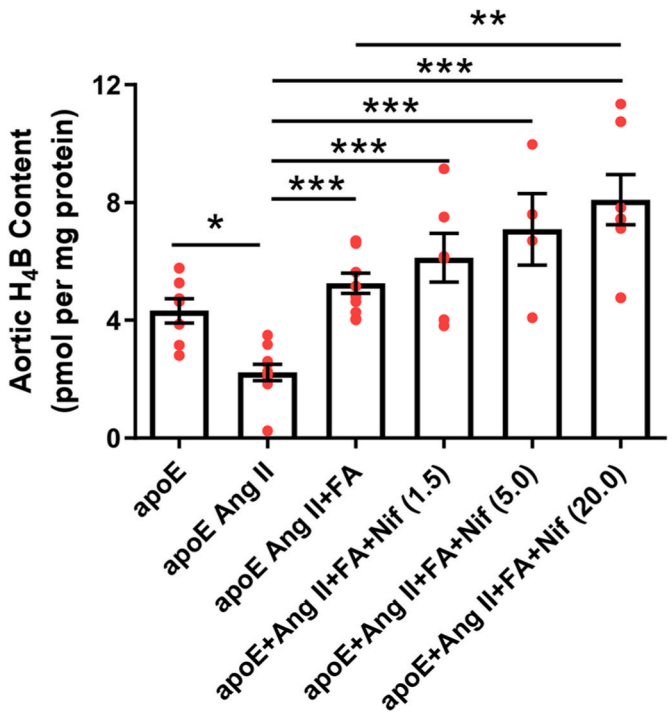

Fig. 7. Combination of FA and Nifedipine substantially further improved aortic H_4B bioavailability in a dose-dependent manner. ApoE null mice were infused with Ang II, fed regular chow or chow mixed with FA (15 mg/kg/day) or chow mixed with FA plus different concentration of Nifedipine (1.5, 5 and 20 mg/kg/day). Aortic H_4B bioavailability was determined by HPLC as we previously published. The data indicate that there was a significant decrease in aortic H_4B levels in Ang II infused apoE null mice, which was markedly restored by oral FA administration. Combination of FA with 1.5, 5 and 20 mg/kg/day of Nifedipine substantially and dose-dependently further restored H_4B bioavailability. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 4–10$.

Data availability

Data will be made available on request.

Acknowledgement

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