AORTIC ANEURYSM

Targeting MicroRNA-192-5p, a Downstream Effector of NOXs (NADPH Oxidases), Reverses Endothelial DHFR (Dihydrofolate Reductase) Deficiency to Attenuate Abdominal Aortic Aneurysm Formation

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ABSTRACT: We have shown that endothelial-specific DHFR (dihydrofolate reductase) deficiency underlies eNOS (endothelial NO synthase) uncoupling and formation of abdominal aortic aneurysm (AAA). Here, we examined a novel role of microRNA-192-5p in mediating NOX (NADPH oxidase)-dependent DHFR deficiency and AAA formation. microRNA-192-5p is predicted to target DHFR. Intriguingly, homo sapiens−microRNA-192-5p expression was substantially upregulated in human patients with AAA. In human aortic endothelial cells exposed to hydrogen peroxide (H_2O_2), homo sapiens−microRNA-192-5p expression was significantly upregulated. This was accompanied by a marked downregulation in DHFR mRNA and protein expression, which was restored by homo sapiens−microRNA-192-5p−specific inhibitor. Of note, microRNA-192-5p expression was markedly upregulated in Ang II (angiotensin II)−infused hph-1 (hyperphenylalaninemia 1) mice, which was attenuated in hph-1−NOX1, hph-1−NOX2, hph-1−neutrophil cytosol factor 1, and hph-1−NOX4 double mutant mice where AAA incidence was also abrogated, indicating a downstream effector role of microRNA-192-5p following NOX activation. In vivo treatment with mus musculus−microRNA-192-5p inhibitor attenuated expansion of abdominal aortas in Ang II−infused hph-1 mice as defined by ultrasound and postmortem inspection. It also reversed features of vascular remodeling including matrix degradation, adventitial hypertrophy, and formation of intraluminal thrombi. These animals had restored DHFR mRNA and protein expression, attenuated superoxide production, recoupled eNOS, and preserved NO bioavailability. In conclusion, our data for the first time demonstrate a critical role of microRNA-192-5p in mediating NOX-dependent DHFR deficiency and AAA formation, inhibition of which is robustly effective in attenuating development of AAA. Since the mouse and human microRNA-192-5p sequences are identical, the microRNA-192-5p inhibitors may be readily translatable into novel therapeutics for the treatment of AAA. (*Hypertension*. 2021;78:282−293. DOI: 10.1161/HYPERTENSIONAHA.120.15070.) ● **Data Supplement**

Key Words: angiotensin II ■ aortic aneurysm, abdominal ■ dihydrofolate reductase ■ endothelial nitric oxide synthase ■ microRNA ■ NADPH oxidase ■ oxidative stress

Abdominal aortic aneurysm (AAA), characterized by a localized dilatation over 30 mm in diameter, is a severe and lethal vascular disease.[1] Patients often do not have symptoms till late-stage and die of ruptured aneurysm suddenly, which accounts for >9750 deaths per year in the United States and 200 000 deaths worldwide.[2] Open repair and endovascular aneurysm repair are the only therapeutic options for aneurysms over 5.5 cm

Novelty and Significance

What Is New?

- First identification of an intermediate role of microRNA-192-5p in DHFR (dihydrofolate reductase) deficiency and abdominal aortic aneurysm (AAA) formation downstream of NOX (NADPH oxidase) activation.
- First identification that targeting microRNA-192-5p to restore DHFR and eNOS (endothelial NO synthase) coupling activity is highly effective in attenuating AAA formation.

What Is Relevant?

- Our data reveal a novel mechanism underlying oxidative stress–induced AAA formation.
- Our data establish a novel therapeutic approach for the treatment and prevention of AAA.

Summary

These new findings indicate that specifically targeting microRNA-192-5p may serve as a novel therapeutic option for the treatment and prevention of AAA.

Nonstandard Abbreviations and Acronyms

AAA	abdominal aortic aneurysm
Ang II	angiotensin II
DHFR	dihydrofolate reductase
eNOS	endothelial NO synthase
ESR	electron spin resonance
HAEC	human aortic endothelial cell
hph-1	hyperphenylalaninemia 1
NOX	NADPH oxidase
VEGF	vascular endothelial growth factor

in size.[3] There have been no oral medicines available to treat aneurysms smaller than 5.5 cm, resulting in silent growth of the aneurysms and lethal ruptures. Although the detailed molecular mechanisms underlying AAA remain to be fully understood, a causal role of oxidative stress has been established in the formation of AAA and other types of aortic aneurysms.[4–13] Inflammatory and vascular cells are activated by oxidative stress to produce atrix metallopeptidases, resulting in matrix degradation, weakening of medial layer, and formation of AAA.

We have previously shown that an endothelial cell–specific DHFR (dihydrofolate reductase) deficiency underlies Ang II (angiotensin II)–induced eNOS (endothelial NO synthase) uncoupling and eNOS uncoupling-dependent formation of AAA in Ang II–infused hph-1 (hyperphenylalaninemia 1) mice and apoE null mice.[7–9,14] In hph-1 and apoE null mice, Ang II infusion augments eNOS uncoupling via downregulation of DHFR.[7–9,14] Folic acid prevents progressive uncoupling of eNOS and vascular remodeling via restoration of DHFR function, resulting in completely normalized blood pressure in wild-type mice and abrogated AAA formation in Ang II–infused hph-1 mice and apoE null mice.[7,8] Utilizing double knockout strategies, we have further shown that DHFR deficiency lies downstream of NOX (NADPH oxidase) isoforms 1, 2, or 4 in Ang II–infused hph-1 mice,

in line with our previous findings that hydrogen peroxide (H_2O_2) produced by NOX induces DHFR deficiency.[9,15] Mice knockout of DHFR displayed phenotypes of more severe vascular remodeling and exaggerated AAA and hypertension via mitochondrial dysfunction.[14]

microRNAs are small, endogenous, single-stranded noncoding RNA molecules of 18 to 22 nucleotides which constitute a novel class of gene regulators.[16,17] They bind to the 3′-untranslated regions of specific messenger RNAs to induce their degradation or translational repression via an imperfect complement in animal cells or perfect complement in plant cells.[16,17] MicroRNA-192-5p was reported to decrease DHFR protein expression via translational arrest.[18] Yang et al[19] reported that levels of microRNA-192-5p are reduced in medulloblastoma cells and that DHFR is a target of microRNA-192-5p as evidenced by dual-luciferase microRNA target reporter assay indicating reduced DHFR promoter activity with cotransfection of microRNA-192-5p mimics. Overexpression of microRNA-192 by microRNA-192 mimics suppressed cellular viability and proliferation, and increased cell cycle arrest via downregulation of DHFR.[19] However, whether or not microRNA-192 plays a role in AAA formation via direct targeting of DHFR in endothelial cells has never been examined.

In the present study, we investigated a potential intermediate role of microRNA-192-5p in NOX-dependent downregulation of DHFR, and subsequent formation of AAA. The expression level of microRNA-192-5p was substantially upregulated in the aortic aneurysmal tissues of human patients with AAA (n=15 for both patients with AAA and controls), H_2O_2 treated HAECs (human aortic endothelial cells) and Ang II–treated hph-1 mice, whereas it was decreased in double mutants of hph-1–NOX1, hph-1–NOX2, hph-1–p47phox (neutrophil cytosol factor 1), and hph-1–NOX4 mice. In vivo treatment with microRNA-192-5p–specific inhibitors markedly restored DHFR mRNA and protein levels, decreased superoxide production, recoupled eNOS, restored NO bioavailability, and attenuated AAA formation. These results indicate that targeting microRNA-192-5p may serve as a novel

therapeutic approach for the treatment or prevention of AAA.

METHODS

Data, analytic methods, and study materials will be made available to other researchers on publication of this research article. These will not be stored online or publicly but can be shared electronically or physically on request (see Materials in the Data Supplement). Besides sections below, other methods paragraphs have been included in the Data Supplement.[7,9,10,12,14,20–22]

Human AAA Samples

The aortic aneurysmal tissue samples of human AAA were obtained from the National Institutes of Health National Disease Research Interchange program with approved Institutional Review Board protocol, and the control subjects were those of donors died of sudden causes but without aneurysms (age: 73.1±11.4 years old for control versus 72.5±10.8 years old for AAA; 11 males and 4 females for control versus 10 males and 5 females for AAA).

Cell Culture and MicroRNA Inhibitors Transfection

HAECs of passages 3 to 7 donated from 2 males (aged 49 and 50 years; Lonza; Walkersville, MD) were cultured in EGM2 media supplemented with 10% (v/v) FBS and supplements (Hydrocortisone, Human Fibroblast Growth Factor-basic, VEGF [vascular endothelial growth factor], R3-insulin-like growth factor-1, Ascorbic Acid, Human Epidermal Growth Factor, GA-1000 and Heparin, all reagents from Lonza). Cells were grown in a humidified atmosphere at 5% CO_2 and 37 °C. Transfection of microRNA inhibitors and negative controls (50 µmol/L; 100 pmol/well in 6-well plate, Life Technologies Corporation, Grand Island, NY 14072) into HAECs was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturers' instructions. Forty-eight hours before cells were stimulated with 100 µmol/L H_2O_2 for 24 h. Cells were then harvested for subsequent analyses of hsa–microRNA-192-5p expression, and DHFR mRNA and protein expression.

RNA Extraction, MicroRNA-Specific cDNA Synthesis, and Quantitative Real-Time Polymerase Chain Reaction of MicroRNA

Total RNAs were extracted from HAECs using TRIzol (Invitrogen, Corp, Carlsbad, CA) according to the manufacturer's instructions. The first-strand cDNA was synthesized from RNA samples using the Mir-X miRNA First-Strand Synthesis kit (Clontech Laboratories, Inc, A Takara Bio Company, Mountain View, CA) according to the manufacturer's instructions. Primers were designed on the basis of miRBase sequence (homo sapiens–microRNA-192-5p, MIMAT0000222, miRBase). Variability in the initial quantities of cDNA was normalized relative to the abundance of U6 after amplification (supplied by Clontech Laboratories, Inc), and the data were expressed as fold changes. Quantitative real-time polymerase chain reaction of microRNAs was conducted with the SYBR quantitative real-time polymerase chain reaction kit (Clontech Laboratories, Inc, A Takara Bio Company, Mountain View, CA) according to the manufacturer's instructions.

In Vivo Treatment of hph-1 Mice With mus musculus–MicroRNA-192-5p Inhibitors

The locked nucleic acid–mmu (mus musculus)–microRNA-192-5p inhibitors were synthesized by Exiqon (now a QIAGEN company, Germantown, MD), and used to inject (30 mg/kg each time) into hph-1 mice subcutaneously on the first day and the third day after implantation of Ang II pumps. The locked nucleic acid–negative control was injected into the animals as a control group. Mmu–microRNA-192-5p mirVana microRNA inhibitor and negative control for in vivo experimentation were synthesized by Thermo Fisher Scientific (Grand Island, NY). mmu–microRNA-192-5p mirVana microRNA inhibitor or negative control was injected intravenously into hph-1 mice via tail vein on the first day and the third day (4.0 mg/kg each time) after implantation of Ang II pumps.

Electron Spin Resonance Determination of Superoxide Levels

As previously described,[7–10,12,14,15,20,21,23] freshly isolated aortas were homogenized on ice in lysis buffer supplemented with protease inhibitor cocktail (1:100) and centrifuged at 12 000g for 15 minutes. Protein content of the supernatant was determined using a protein assay kit (Bio-Rad, Irvine, CA). Five micrograms of proteins were mixed with ice-cold and nitrogen bubbled Krebs HEPES Buffer containing diethyldithiocarbamic acid (5 µmoL/L), deferoxamine (25 µmoL/L), and the freshly prepared superoxide specific spin trap methoxycarbonyl- 2,2,5,5-tetra-methylpyrrolidine (500 µmoL/L, Axxora, San Diego, CA). The mixture was then loaded into a glass capillary (Kimble, Dover, OH), and measured using electron spin resonance spectrometer (eScan, Bruker, Billerica, MA) for superoxide production. A second measurement was taken with the addition of polyethylene glycol-superoxide dismutase (100 U/mL). To assess eNOS uncoupling activity, a third measurement was made with the addition of L-NAME (NG-nitro-L-arginine methyl ester, NOS inhibitor) (100 µmoL/L). The ESR settings used were: center field, 3480; sweep width, 9 G; microwave frequency, 9.78 GHz; microwave power, 21.02 mW; modulation amplitude, 2.47 G; 512 points of resolution; receiver gain, 1000.

ESR Determination of NO Bioavailability

Aortic NO bioavailability was determined by ESR as we previously described.[7–10,12,14,15,20,21,23–25] In brief, freshly isolated aortas were cut into 2 mm rings and then incubated in freshly prepared NO specific spin trap Fe^{2+}(diethyl dithiocarbamate)$_2$ (0.5 mmol/L) colloid in nitrogen bubbled, modified KHB at 37 °C for 60 minutes, in the presence of calcium ionophore A23187 (10 µmol/L). The aortic rings were snap-frozen in liquid nitrogen and loaded into a finger Dewar for measurement with ESR spectrophotometer (eScan, Bruker, Billerica, MA). 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.

Statistical Analysis

All statistical analyses were performed using the Prism software. Comparison between 2 groups was performed using the Student t test. Comparisons between multiple groups were done using 1-way ANOVA, followed by the Newman-Keuls post hoc test. Comparisons of the incidence rates of AAA among different animal groups were performed using χ^2 test. Statistical significance was set at $P < 0.05$. All grouped data are presented as Mean±SEM.

RESULTS

Hydrogen Peroxide Downregulated DHFR Expression While Upregulating MicroRNA-192-5p Expression

In the present study, we examined an intermediate role of microRNA-192-5p in NOX-dependent modulation of DHFR to impact AAA formation. First, we examined expression of microRNA-192-5p in aortic aneurysmal tissues of human AAA. Intriguingly, the expression of homo sapiens–microRNA-192-5p was substantially upregulated in the aortic aneurysmal tissues of human patients with AAA comparing to donor controls (Figure 1A). The aortic aneurysmal tissue samples of human AAA and the control non-AAA subjects were obtained from the National Institutes of Health National Disease Research Interchange program with approved Institutional Review Board protocol (age: 73.1±11.4 years old for control versus 72.5±10.8 years old for AAA; 11 males and 4 females for control versus 10 males and 5 females for AAA). In H_2O_2 (100 μM, 24 h) treated HAECs, homo sapiens–microRNA-192-5p expression was also significantly upregulated (Figure 1B).

Interestingly, DHFR is a putative target of microRNA-192-5p by TargetScan (http://www.targetscan.org/; Figure 1C) analysis. Of note, microRNA-192-5p was shown to decrease DHFR protein abundance in human colon cancer cell lines,[18] and inhibit medulloblastoma cell proliferation by binding to DHFR 3-untranslated region.[19] We, therefore, hypothesize that microRNA-192-5p, with increased expression in both aortic aneurysmal tissues of human patients with AAA and H_2O_2-treated HAECs, might mediate H_2O_2 downregulation of DHFR in vivo to induce AAA formation (see below).

Silencing of MicroRNA-192-5p With Specific Inhibitors Restored DHFR Expression in Endothelial Cells

To examine whether microRNA-192-5p downregulates DHFR in endothelial cells, HAECs were exposed to H_2O_2 after transfection of microRNA-192-5p inhibitors for 48 hours. As shown in Figure 2A, microRNA-192-5p expression levels were reduced in microRNA-192-5p–specific inhibitor–treated HAECs. Furthermore, homo sapiens–microRNA-192-5p–specific inhibitor markedly restored DHFR mRNA (Figure 2B) and protein expression (Figure 2C and 2D), indicating an intermediate role of microRNA-192-5p in H_2O_2 induced DHFR deficiency.

MicroRNA-192-5p Expression in Ang II–Infused hph-1 Mice: NOX-Dependent Upregulation

Of note, microRNA-192-5p is highly conserved among species, especially in the seed region (red part) that is
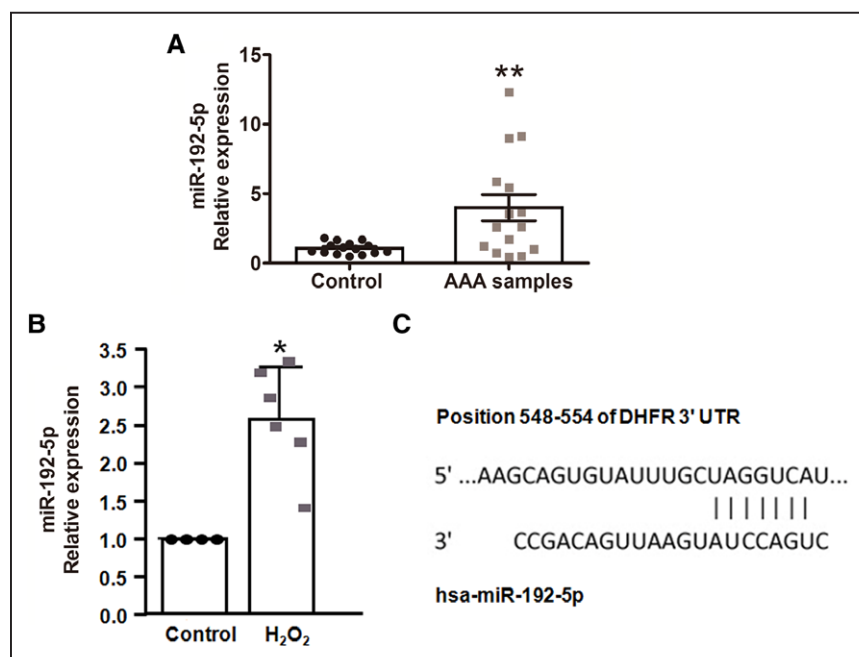
Figure 1. MicroRNA-192-5p, which is predicted to target dihydrofolate reductase, is upregulated in human abdominal aortic aneurysm and hydrogen peroxide treated human aortic endothelial cells.

The RNAs were isolated from aortic samples of human patients with abdominal aortic aneurysm and control subjects (n=15 for each group). **A**, MicroRNA-192-5p was upregulated in aortic samples of human patients with abdominal aortic aneurysm comparing to the controls. Data are presented as Mean±SEM (n=15), **$P < 0.01$ vs control. Human aortic endothelial cells were treated with hydrogen peroxide (H_2O_2, 100 μmol/L, 24 h) and the RNAs were isolated. **B**, homo sapiens–miR-192-5p expression was upregulated in H_2O_2 stimulated human aortic endothelial cells. Data are presented as Mean±SEM (n=4−6), *$P < 0.05$ vs control. TargetScan was used to explore the putative relationship between human MicroRNA-192-5p and dihydrofolate reductase. **C**, dihydrofolate reductase is predicted to be the putative target of MicroRNA-192-5p by TargetScan. AAA indicates abdominal aortic aneurysm; miR, MicroRNA; UTR, untranslated region.
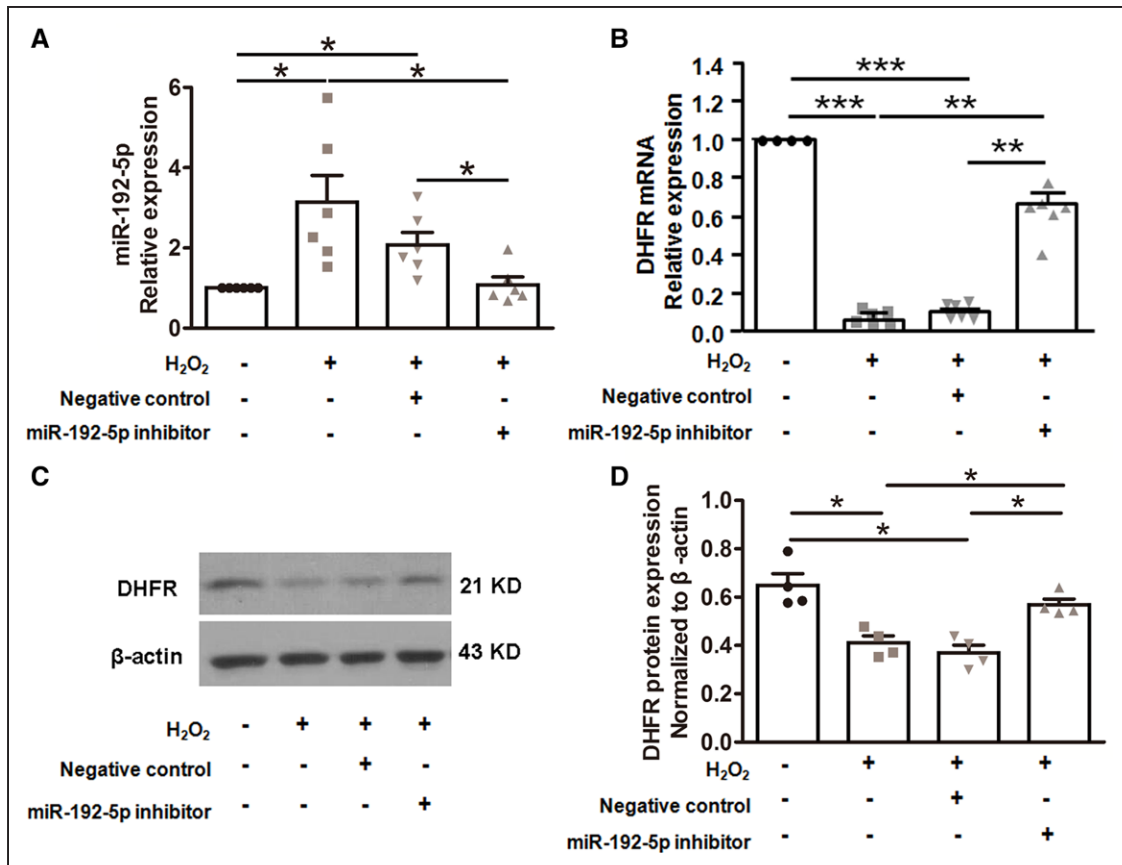
Figure 2. Hsa (homo sapiens)–miR (microRNA)-192-5p–specific inhibitors restore DHFR (dihydrofolate reductase) mRNA and protein expression in hydrogen peroxide stimulated HAECs (human aortic endothelial cells).
HAECs were treated with hydrogen peroxide (H_2O_2, 100 µmol/L, 24 h) in the presence or absence of negative control miR inhibitors or Hsa–miR-192-5p inhibitors, and the RNAs and proteins were isolated for real-time polymerase chain reaction and Western blotting analyses to detect miR-192-5p and DHFR mRNA and protein expression levels. **A**, Hsa–miR-192-5p–specific inhibitors attenuated miR-192-5p expression in H_2O_2 stimulated HAECs. Data are presented as Mean±SEM (n=6), *$P<0.05$. **B**, Hsa–miR-192-5p–specific inhibitors markedly restored DHFR mRNA expression in H_2O_2 stimulated HAECs. Data are presented as Mean±SEM (n=4–6), **$P<0.01$, ***$P<0.001$. **C**, Representative Western blots of endothelial DHFR protein expression with β-actin serving as an internal control. **D**. Hsa–miR-192-5p–specific inhibitors markedly restored DHFR protein expression in H_2O_2 stimulated HAECs. Data are presented as Mean±SEM (n=4), *$P<0.05$.

predicted to target DHFR (Figure S1 in the Data Supplement). Therefore, we explored a potential role of microRNA-192-5p in AAA formation in Ang II–infused hph-1 mice, via predicted downregulation of DHFR in vivo. We have previously shown that DHFR deficiency lies downstream of NOX isoforms 1, 2, or 4 activation in Ang II–infused hph-1 mice, resulting in eNOS uncoupling to induce AAA formation.[9] The incidence of AAA was substantially reduced, with significant difference by χ^2 test, from 79.2% in Ang II–infused hph-1 animals to 11.8%, 15.2%, 7.7%, and 0% in hph-1–NOX1, hph-1–NOX2, hph-1–p47phox, and hph-1–NOX4 double mutant animals, respectively (Figure 3A and 3B; combined data from the present study and the previous work[9]). The microRNA-192-5p expression, determined by quantitative real-time polymerase chain reaction analysis, was significantly upregulated in Ang II–infused hph-1 mice compared with wild-type mice, which was abrogated in hph-1–NOX1 (Figure 3C), hph-1–NOX2 (Figure 3D), hph-1–p47phox (Figure 3E), and hph-1–NOX4 (Figure 3F) double mutant

mice, indicating a downstream role of microRNA-192-5p following NOX activation.

MicroRNA-192-5p Inhibitors Restored DHFR Expression in Ang II–Infused hph-1 Mice

Of note, the mmu–microRNA-192-5p–specific inhibitors were used to examine whether inhibition of microRNA-192-5p restores DHFR expression in Ang II–infused hph-1 mice. As demonstrated in Figure 4A, quantitative real-time polymerase chain reaction analysis of microRNA expression indicated that mmu–microRNA-192-5p–specific inhibitors decreased mmu–microRNA-192-5p expression in endothelial cells isolated from aortas of Ang II–infused hph-1 mice. Ang II–infused hph-1 mice had significant decreased DHFR mRNA (Figure 4B) and protein expression (Figure 4C and 4D) compared with hph-1 mice, while mmu–microRNA-192-5p inhibitors substantially restored DHFR mRNA (Figure 4B) and protein expression (Figure 4C and 4D)
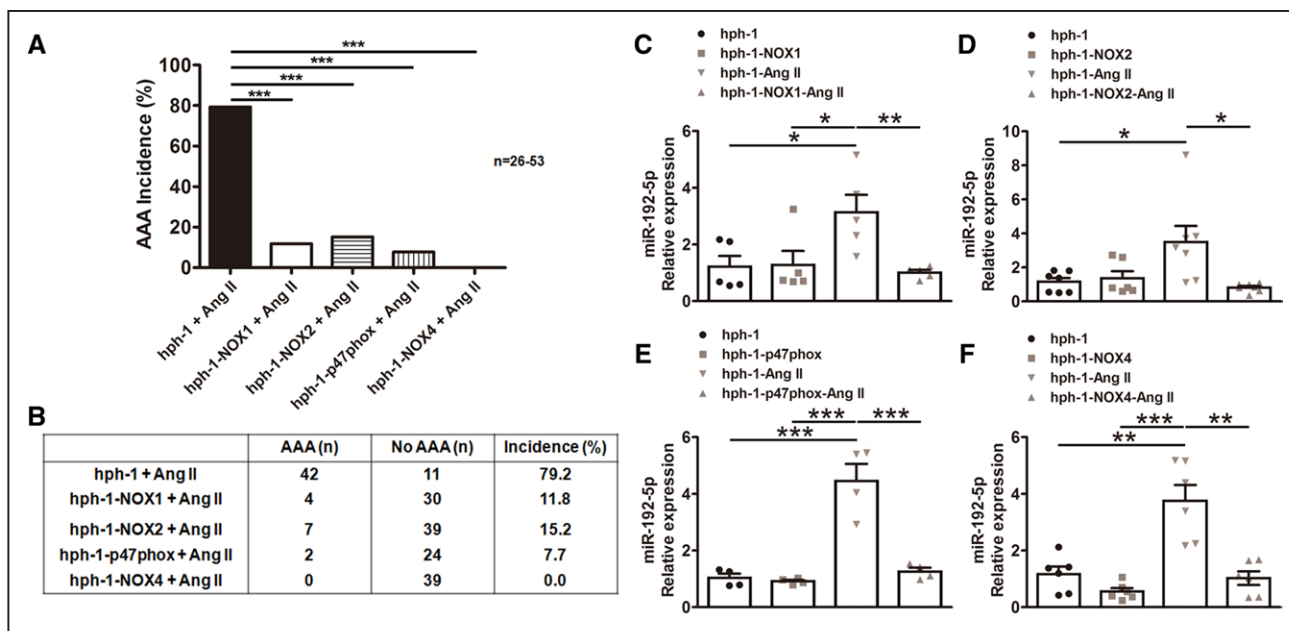
Figure 3. miR (MicroRNA)-192-5p serves as a downstream effector of NOXs (NADPH oxidases) in mediating AAA (abdominal aortic aneurysm) formation.
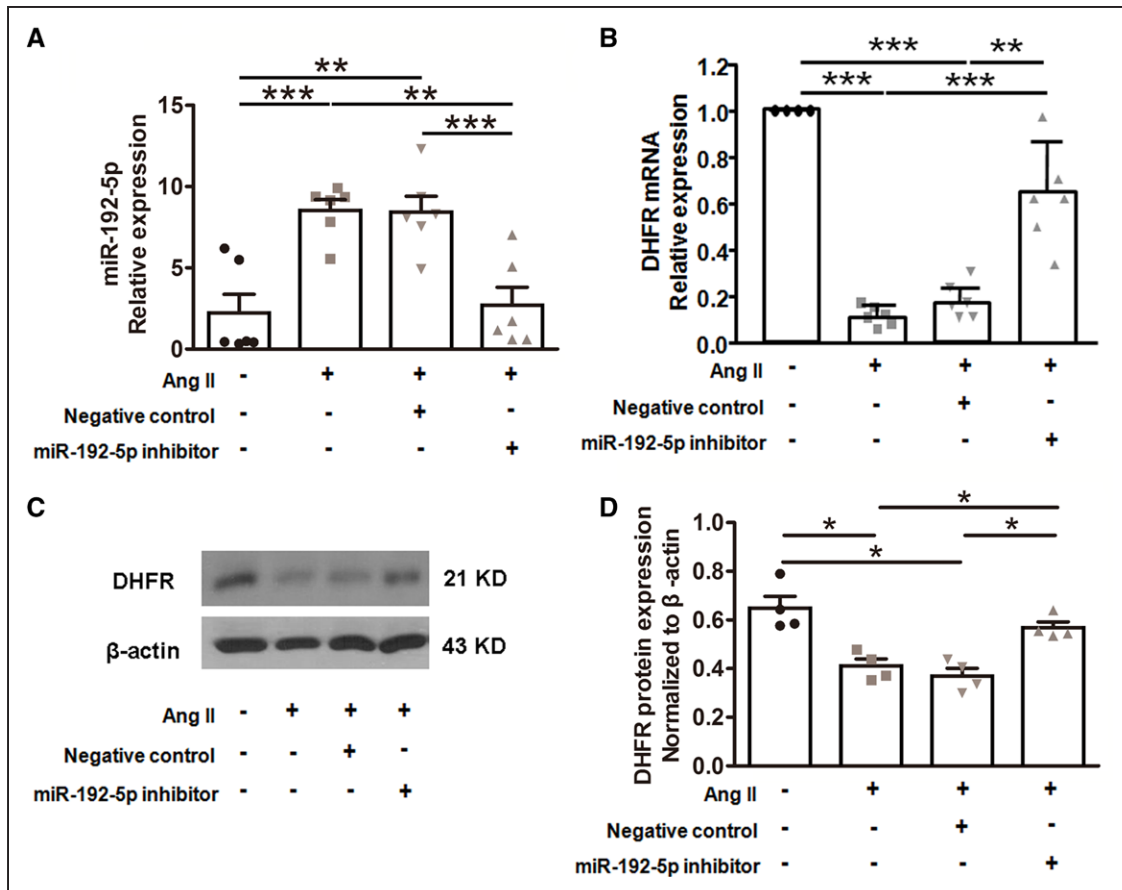Ang II (angiotensin II) was infused into hph-1 (hyperphenylalaninemia 1), hph-1–NOX1, hph-1–NOX2, hph-1–neutrophil cytosol factor 1, and hph-1–NOX4 double mutant animals before phenotyping for AAA and isolation of aortic endothelial cells for detection of miR-192-5p expression levels. **A**, The percentage of AAA in each experimental groups. n=26–53, ***$P<0.001$. **B**, The actual numbers of animals in each experimental groups with and without AAA, combined with our original data reported in Siu et al.[9] The incidence of AAA was greatly reduced from 79.2% in Ang II–infused hph-1 mice to 11.8%, 15.2%, 7.7%, and 0% in hph-1–NOX1, hph-1–NOX2, hph-1–neutrophil cytosol factor 1, and hph-1–NOX4 double mutant animals respectively. **C**, The mmu–miR-192-5p expression levels in hph-1 and hph-1–NOX1 mice with and without Ang II infusion, indicating attenuation of miR-192-5p expression in hph-1-NOX1 double mutant mice in response to Ang II. Data are presented as Mean±SEM (n=5), *$P<0.05$, **$P<0.01$. **D**, The expression levels of mmu–miR-192-5p in hph-1 and hph-1–NOX2 mice with and without Ang II infusion, indicating attenuation of miR-192-5p expression in hph-1-NOX2 double mutant mice in response to Ang II. Data are presented as Mean±SEM (n=6–7), *$P<0.05$. **E**, The expression levels of mmu–miR-192-5p in hph-1 and hph-1–neutrophil cytosol factor 1 mice with and without Ang II infusion, indicating attenuation of miR-192-5p expression in hph-1-p47phox double mutant mice in response to Ang II. Data are presented as Mean±SEM (n=4), ***$P<0.001$. **F**, The expression levels of mmu–miR-192-5p in hph-1 and hph-1–NOX4 mice with and without Ang II infusion, indicating attenuation of miR-192-5p expression in hph-1-NOX4 double mutant mice in response to Ang II. Data are presented as Mean±SEM (n=6), **$P<0.01$, ***$P<0.001$.

comparing to Ang II–infused hph-1 mice in endothelial cells isolated from aortas.

MicroRNA-192-5p Inhibitors Diminished Endothelial Superoxide Production, Recoupled eNOS and Restored NO Bioavailability in Ang II–Infused hph-1 Mice

We have previously shown that endothelial DHFR deficiency leads to a reduction in H_4B bioavailability and consequent eNOS uncoupling to result in development of AAA.[7–10,14] Aortic total superoxide production detected by dihydroethidium staining was markedly increased in Ang II–infused hph-1 mice comparing to untreated hph-1 mice, which was significantly attenuated in Ang II–infused hph-1 mice with mmu–microRNA-192-5p inhibitors (Figure 5A and 5B). In addition, aortas were harvested and subjected to ESR determination of superoxide production in the presence or absence of L-NAME, an inhibitor of NOS. If eNOS is functional and coupled, its inhibition by L-NAME to remove the buffering effect of NO will increase the measured superoxide. However, if eNOS is dysfunctional and uncoupled, it produces superoxide. Therefore, inhibition with L-NAME will reduce measured superoxide. There is an increased superoxide production in Ang II–infused hph-1 mice, and this increase was inhibited by L-NAME, indicating that uncoupled eNOS is the enzymatic source of the superoxide production (Figure 5C), as we previously shown.[7–10,14] L-NAME-sensitive superoxide production, reflective of eNOS uncoupling activity, was completely attenuated by microRNA-192-5p inhibitors (Figure 5C). Notably, NO bioavailability was decreased in Ang II–infused hph-1 mice, which was also substantially restored by microRNA-192-5p inhibitors (Figure 5D). These results show that microRNA-192-5p inhibitors could improve the coupling state of eNOS, reduce superoxide production and restore NO bioavailability in Ang II–infused hph-1 mice via restoration of DHFR expression. Our previous work[7–10,14] and these data again confirm that in addition to vascular smooth muscle cell generation of superoxide in response to Ang II, uncoupled eNOS can serve as a

Figure 4. Mmu–miR (microRNA)-192-5p–specific inhibitors restore DHFR (dihydrofolate reductase) mRNA and protein expressions in Ang II (angiotensin II)–infused hph-1 (hyperphenylalaninemia 1) mice.
Mmu–miR-192-5p–specific inhibitors and negative controls were injected into Ang II–infused hph-1 mice, and aortic endothelial cells were harvested to detect miR-192-5p expression, and DHFR mRNA and protein expression levels. **A**, Mmu–miR-192-5p–specific inhibitors attenuated mmu–miR-192-5p expression in Ang II–infused hph-1 mice. Data are presented as Mean±SEM (n=6), **$P<0.01$, ***$P<0.001$. **B**, Mmu–miR-192-5p–specific inhibitors markedly restored DHFR mRNA in Ang II–infused hph-1 mice. Data are presented as Mean±SEM (n=4–6), **$P<0.01$, ***$P<0.001$. **C**, Representative Western blots of endothelial DHFR protein expression with β-actin serving as an internal control. **D**, Mmu–miR-192-5p–specific inhibitors markedly restored DHFR protein expression in Ang II–infused hph-1 mice. Data are presented as Mean±SEM (n=4), *$P<0.05$.

major contributor of endothelial-specific production of superoxide production to result in AAA formation (also Figure 6 below).

MicroRNA-192-5p Inhibitors Attenuated AAA Formation in Ang II–Infused hph-1 Mice

Since microRNA-192-5p expression was increased in Ang II–infused hph-1 mice, we explored a potential intermediate role of microRNA-192-5p in the development of AAA via downregulation of DHFR. The incidence of AAA was greatly reduced from 80.0% in negative control–treated hph-1 animals to 25.0% in microRNA-192-5p–specific inhibitors treated hph-1 animals after Ang II infusion (Figure 6A). At days 0, 7, and 14, abdominal ultrasound was performed to assess dimensions of abdominal aorta. The sizes of abdominal aorta measured by ultrasound were significantly smaller in hph-1 mice treated with mmu–microRNA-192-5p

inhibitors, compared to that of control group (Figure 6B). Postmortem inspection indicated that AAA formation was prevented in Ang II–infused hph-1 mice with in vivo treatment of mmu–microRNA-192-5p inhibitors (Figure 6C). Representative images of hematoxylin and eosin were shown in Figure 6D, which confirmed that mmu–microRNA-192-5p inhibitors abrogated AAA formation in Ang II–infused hph-1 mice. Besides, the external diameters of the AAA were increased in negative control–treated hph-1 animals, which were attenuated in microRNA-192-5p–specific inhibitors treated hph-1 mice after Ang II infusion (Figure 6E). Of note, Ang II infusion induced marked adventitial hypertrophy as well as intrawall thrombosis, which were markedly attenuated by mmu–microRNA-192-5p inhibitors. Verhoeff-van Gieson staining indicated significant degradation and flattening of elastic fibers in Ang II–infused hph-1 mice, which was substantially abrogated by in vivo treatment with microRNA-192-5p inhibitors (Figure 6F).
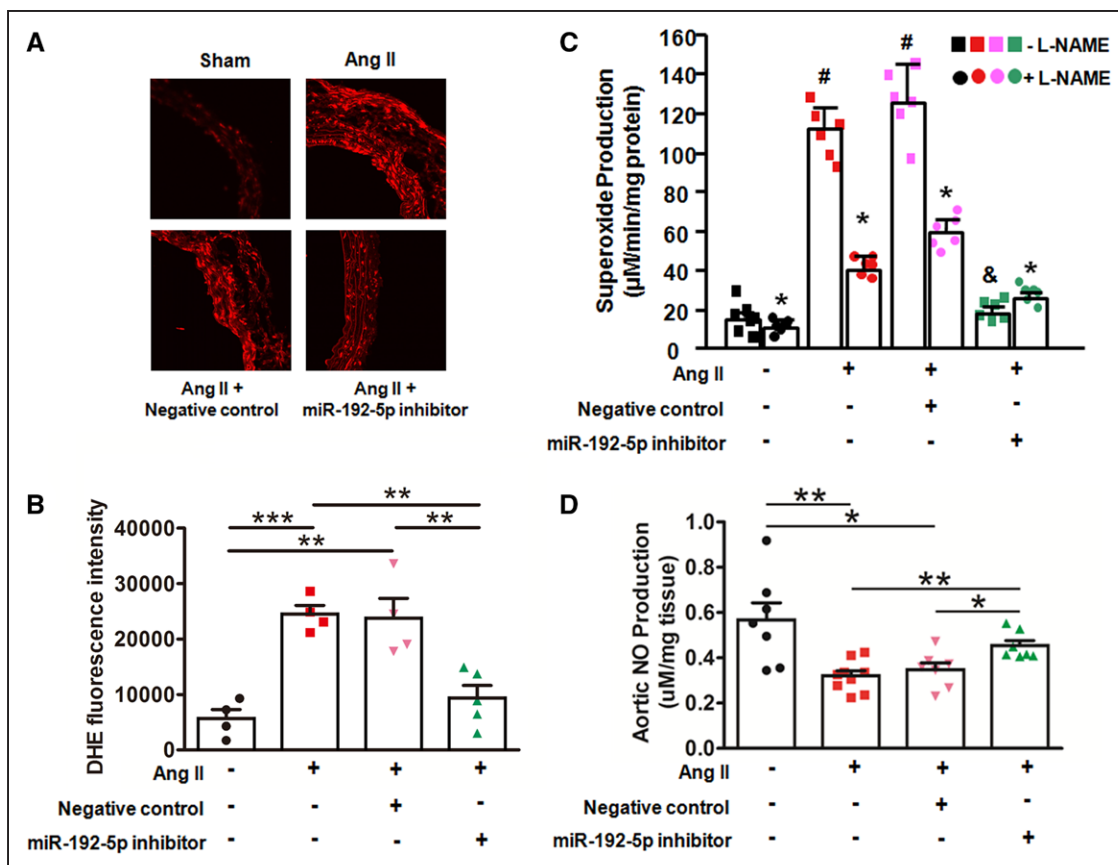
Figure 5. In vivo treatment with mmu−miR (microRNA)-192-5p–specific inhibitors attenuates superoxide production, recouples eNOS (endothelial NO synthase), and restores NO bioavailability.
Mmu−miR-192-5p–specific inhibitors and negative controls were injected into Ang II (angiotensin II)−infused hph-1 (hyperphenylalaninemia 1) mice, and the aortas were freshly harvested for dihydroethidium imaging analysis of superoxide production, electron spin resonance analyses of superoxide production and eNOS uncoupling activity, and electron spin resonance determination of NO bioavailability. **A**, Aortic production of superoxide detected by dihydroethidium imaging was markedly increased in Ang II−infused hph-1 mice, which was substantially attenuated by in vivo treatment with mmu−miR-192-5p–specific inhibitors. **B**, Quantitative analysis of fluorescent intensity of dihydroethidium images, indicating same notion as in **A**. Data are presented as Mean±SEM (n=4–5), **$P<0.01$, ***$P<0.001$. **C**, Total superoxide production determined by electron spin resonance in the presence or absence of L-NAME (NG-nitro-L-arginine methyl ester, NOS inhibitor). There is a very modest eNOS uncoupling (L-NAME–inhibitable superoxide production) activity at baseline in hph-1 mice as we previously published. The marked increase in eNOS uncoupling activity in Ang II−infused hph-1 mice was completely attenuated by in vivo treatment with mmu−miR-192-5p–specific inhibitors. Data are presented as Mean±SEM (n=6–7), *$P<0.05$ vs L-NAME(−) for all corresponding groups, #$P<0.05$ vs sham group without Ang II infusion, &$P<0.05$ vs Ang II and Ang II+negative control groups without L-NAME. **D**. NO bioavailability determined by electron spin resonance was markedly decreased in Ang II−infused hph-1 mice, which was significantly restored by in vivo treatment with mmu−miR-192-5p–specific inhibitors. Data are presented as Mean±SEM (n=7–9), *$P<0.05$, **$P<0.01$.

Notably, the sequences of microRNA-192-5p are the same between humans and mice (Figure S1). Therefore, these results indicate that microRNA-192-5p inhibitors maybe be readily used as potential therapeutics for human AAA.

DISCUSSION

The most significant findings in the present study are the first demonstration that microRNA-192-5p plays a critical role in mediating NOX-dependent DHFR deficiency in AAA formation, and that in vivo silencing of microRNA-192-5p expression with specific inhibitors is markedly effective in attenuating AAA formation via preservation of endothelial DHFR expression, coupling activity of eNOS and NO bioavailability in aortic endothelial cells. Our data indicate that H_2O_2 generated from NOXs activates microRNA-192-5p expression to reduce DHFR protein abundance, resulting in eNOS uncoupling-dependent AAA formation. Inhibition of microRNA-192-5p in vivo with specific inhibitors restored endothelial DHFR expression and coupling activity of eNOS to result in attenuation of oxidative stress, restoration of NO bioavailability, and prevention of matrix degradation and adventitial hypertrophy to eliminate AAA formation. Therefore, microRNA-192-5p may serve as a novel target for the treatment of AAA.

AAA is a progressive vascular disease, and several microRNAs have been implicated in the pathogenesis of AAA.[26,27] MicroRNA-33a-5p expression in central
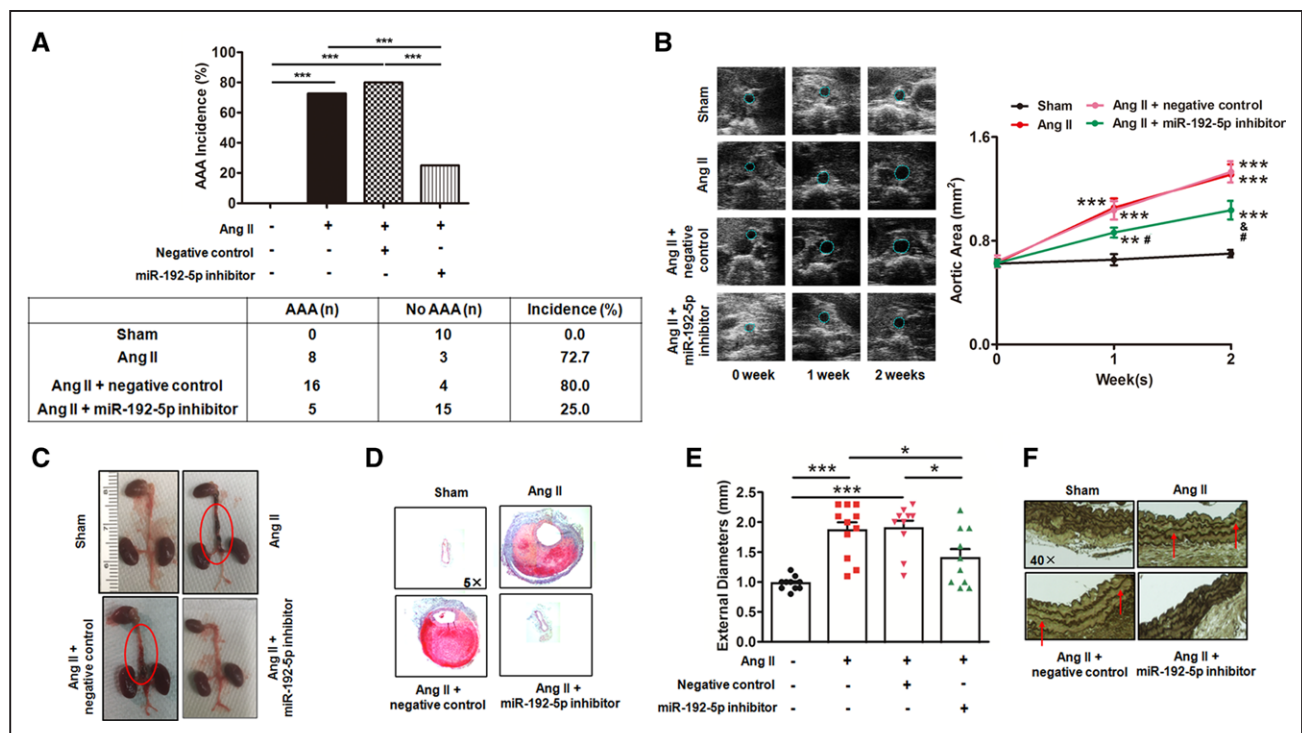
AORTIC ANEURYSM

Figure 6. Mmu−miR (microRNA)-192-5p–specific inhibitors attenuate abdominal aortic aneurysm development in Ang II (angiotensin II)–infused hph-1 (hyperphenylalaninemia 1) mice.
Mmu−miR-192-5p–specific inhibitors and negative controls were injected into Ang II–infused hph-1 mice, before phenotyping of the mice for AAA formation. **A**, The percentages of abdominal aortic aneurysm in Ang II–infused hph-1 mice treated with negative controls and mmu−miR-192-5p–specific inhibitors. The incidence of abdominal aortic aneurysm was greatly reduced from 80.0% in hph-1 animals treated with negative controls, to 25.0% in hph-1 mice treated with mmu−miR-192-5p–specific inhibitors. n=10–20, ***$P<0.001$. **B**, Time-dependent expansion of abdominal aortas defined by ultrasound was attenuated by in vivo treatment with miR-192-5p–specific inhibitors in Ang II–infused hph-1 mice. n=7, **$P<0.01$, ***$P<0.001$ vs sham; #$P<0.05$ vs Ang II; &$P<0.05$ vs Ang II+negative control. **C**, Postmortem inspection indicated that abdominal aortic aneurysm formation was attenuated by in vivo treatment of mmu−miR-192-5p–specific inhibitors in Ang II–infused hph-1 mice. **D**, Ang II infusion into hph-1 mice induced a marked adventitial hypertrophy and intrawall thrombosis, which was attenuated by in vivo treatment with mmu−miR-192-5p–specific inhibitors. **E**, Ang II infusion into hph-1 mice induced enlargement of external diameters of the abdominal aortas, which was attenuated by in vivo treatment with mmu−miR-192-5p–specific inhibitors. Data are presented as Mean±SEM (n=10–11), *$P<0.05$, ***$P<0.001$. **F**, Verhoeff-van Gieson staining indicating significant degradation and flattening of elastic fibers in the aortic medial layers of Ang II–infused hph-1 mice (red arrows), which was reversed by in vivo treatment with mmu−miR-192-5p–specific inhibitors.

zone of human AAA is higher than marginal zone, and that microRNA-33 deletion attenuated AAA formation in mice via downregulation of matrix metallopeptidase 9 in macrophages, and monocyte chemotactic protein-1 in vascular smooth muscle cells.[28] MicroRNA-155 expression was found significantly increased in AAA biopsies, whereas circulating microRNA-155 levels were also elevated in patients with AAA compared to controls, with a 2.67-fold upregulation at borderline significance.[29] Two immunologically important microRNA-155 target genes, CTLA4 (cytotoxic T-lymphocyte-associated protein) and SMAD (homologies to the *Caenorhabditis elegans* SMA ["small" worm phenotype] and MAD ["Mothers Against Decapentaplegic"] family of genes in Drosophila) 2 were found significantly downregulated within AAA bodies compared with AAA necks, which play an important role in promoting chronic inflammation by enhancing T-cell development and decreasing expression of TGF (transforming growth factor)-β−dependent genes in the nucleus.[29] However, the detailed molecular mechanisms

of microRNAs in human AAA need to be explored further, not to mention that the specific roles and regulations of endothelial microRNAs. Here, our data for the first time demonstrated that microRNA-192-5p was upregulated in aortic aneurysmal tissues of human AAA, and it played a critical role in mediating AAA formation in Ang II–infused hph-1 mice via downregulation of endothelial DHFR and consequent uncoupling of eNOS Inhibition of microRNA-192-5p in vitro and in vivo restored DHFR expression and eNOS coupling activity to result in abrogation of AAA formation.

DHFR deficiency uncouples eNOS to induce hypertension and AAA formation.[7–10,13–15,20,23] Relatively modest DHFR deficiency results in a 2-fold increase in eNOS uncoupling activity and development of hypertension in Ang II–infused wild-type mice, while more severe DHFR deficiency in Ang II–infused hph-1 mice induces a 3-fold eNOS uncoupling activity to lead to AAA formation.[7] We have previously established that augmentation of endothelial DHFR expression and activity is robustly

effective in protecting against development of AAA.[7–10,13,14] Here, we show for the first time that microRNA-192-5p upregulation following NOX activation is able to induce DHFR deficiency in human endothelial cells in vitro and in Ang II–infused hph-1 mice in vivo. Besides, microRNA-192-5p inhibitors restored DHFR mRNA and protein expression in H_2O_2 stimulated endothelial cells and in Ang II–infused hph-1 mice to attenuate eNOS uncoupling activity. Intriguingly, microRNA-192-5p inhibitors reduced the incidence rate of AAA from 80% to 25% and substantially attenuated AAA formation at molecular and histological levels in Ang II–infused hph-1 mice. The microRNA-192-5p inhibitors alleviated vascular remodeling including medial elastin degradation and flattening, as well as adventitial hypertrophy, features shown by our previous studies to characterize AAA formation in Ang II–infused hph-1 mice. Whether regulation of microRNA-192-5p in endothelial cells plays a role in adventitial hypertrophy in other vascular diseases remain to be further investigated. Overall, our data establish an important role of microRNA-192-5p in the formation of AAA in the robust model of Ang II–infused hph-1 mice. Notably, the sequences of microRNA-192-5p are the same between human and mouse. Therefore, microRNA-192-5p inhibitors can be readily used as potential and powerful therapeutics for human AAA.

NOX isoform 1, 2, or 4 lies upstream of DHFR deficiency to induce AAA formation.[9] Activation of NOX by Ang II produces reactive oxygen species to contribute to cardiovascular pathogenesis.[13,30–34] NOX produces reactive oxygen species in response to Ang II in endothelial cells and vascular smooth muscle cells.[33,34] Endothelial NOX-derived H_2O_2 downregulates DHFR expression in response to Ang II.[15] Double mutant mice of hph-1–NOX1, hph-1–NOX2, hph-1–p47phox, and hph-1–NOX4 had preserved DHFR expression and activity in endothelial cells in response to Ang II infusion.[9] Of note, microRNA-192-5p expression was significantly upregulated in Ang II–infused hph-1 mice, which was however abrogated in Ang II–infused hph-1–NOX1, hph-1–NOX2, hph-1–p47phox, and hph-1–NOX4 mice, indicating downstream role of microRNA-192-5p in mediating NOX-dependent DHFR deficiency. mmu–microRNA-192-5p–specific inhibitors restored DHFR mRNA and protein expression in Ang II–infused hph-1 mice. Of note, animal and preliminary human data have shown that microRNA mimics and inhibitors have the great potential to develop into a whole new class of therapeutics for the treatment of cardiovascular diseases.[35–38] MicroRNAs are small RNA molecules with known sequence that is often remarkably conserved between species, such as microRNA-192-5p, in this research. These characteristics make microRNAs excellent drug targets that can be manipulated with mostly on-target effects, which have promoted microRNA-modulating compounds to enter preclinical efficacy and safety studies as well as in clinical trials.[39,40] Anti-microRNAs can be generated based on antisense technologies, and can effectively bind to their cognate microRNA targets with excellent affinity and specificity[39–41]; the mmu–microRNA-192-5p inhibitors used in this study have indeed proved to be highly efficient and effective in vivo in attenuating AAA formation.

In conclusion, our work represents the first evidence that microRNA-192-5p, downstream of activation of NOX isoforms in response to Ang II, mediates H_2O_2-induced endothelial DHFR deficiency, eNOS uncoupling, and consequent AAA formation. Whereas, specific inhibition of microRNA-192-5p in Ang II–infused hph-1 mice is robustly effective in attenuating AAA formation via preservation of endothelial DHFR expression and eNOS coupling activity, and abrogation of sustained oxidative stress, matrix degradation, and vascular remodeling. Since the human and the mouse microRNA-192-5p sequences are identical, these data indicate that microRNA-192-5p inhibitors may be readily used as novel therapeutic options for AAA.

Perspectives

As schematically illustrated in the Graphic Abstract, Ang II induces a rapid and transient activation of endothelial NOX, resulting in oxidative stress and H_2O_2-dependent upregulation of microRNA-192-5p, which in turn leads to decreased DHFR mRNA and protein expression, uncoupling of eNOS, and development of AAA. Inhibition of microRNA-192-5p with microRNA-192-5p inhibitors, however, attenuates AAA formation in Ang II–infused hph-1 mice, via upregulation of DHFR protein expression to restore eNOS coupling activity and NO bioavailability. Indeed, microRNA-192-5p expression is upregulated in aneurysmal aortic samples of human patients with AAA, and the sequences of microRNA-192-5p are identical between humans and mice. These observations strongly support the robustness and translational value of our molecular mechanistic findings. In conclusion, our work in the present study indicates that specifically targeting microRNA-192-5p may be used as a novel therapeutic option for the treatment and prevention of AAA.

Disclosures

None.

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