Journal of the American Heart Association

ORIGINAL RESEARCH

Gestational Hypoxia Impaired Endothelial Nitric Oxide Synthesis Via miR-155-5p/NADPH Oxidase/Reactive Oxygen Species Axis in Male Offspring Vessels

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BACKGROUND: Nitric oxide (NO) is the most important vasodilator secreted by vascular endothelial cells, and its abnormal synthesis is involved in the development of cardiovascular disease. The prenatal period is a critical time for development and largely determines lifelong vascular health in offspring. Given the high incidence and severity of gestational hypoxia in mid-late pregnancy, it is urgent to further explore whether it affects the long-term synthesis of NO in offspring vascular endothelial cells.

METHODS AND RESULTS: Pregnant Sprague–Dawley rats were housed in a normoxic or hypoxic (10.5% O_2) chamber from gestation days 10 to 20. The thoracic aortas of fetal and adult male offspring were isolated for experiments. Gestational hypoxia significantly reduces the NO-dependent vasodilation mediated by acetylcholine in both the fetal and adult offspring thoracic aorta rings. Meanwhile, acetylcholine-induced NO synthesis is impaired in vascular endothelial cells from hypoxic offspring thoracic aortas. We demonstrate that gestational hypoxic offspring exhibit a reduced endothelial NO synthesis capacity, primarily due to increased expression of NADPH oxidase 2 and enhanced reactive oxygen species. Additionally, gestational hypoxic offspring show elevated levels of miR-155-5p in vascular endothelial cells, which is associated with increased expression of NADPH oxidase 2 and reactive oxygen species generation, as well as impaired NO synthesis.

CONCLUSIONS: The present study is the first to demonstrate that gestational hypoxia impairs endothelial NO synthesis via the miR-155-5p/NADPH oxidase 2/reactive oxygen species axis in offspring vessels. These novel findings indicate that the detrimental effects of gestational hypoxia on fetal vascular function can persist into adulthood, providing new insights into the development of vascular diseases.

Key Words: gestational hypoxia ■ male offspring ■ miR-155-5p ■ NO synthesis ■ NOX2

A large number of epidemiological investigations have shown that the occurrence of many adult diseases is related to the intrauterine growth and development of the fetus.[1–3] The concept of fetal origin of adult diseases has been widely confirmed by many studies in the past 20 years.[3–5] In recent years, a large amount of animal experiments and clinical data have demonstrated that various adverse factors during pregnancy including maternal, nutritional, and environmental factors have different degrees of impact on fetal development, laying the foundation for cardiovascular diseases in adulthood.[5–8] Hypoxia, characterized by

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This article was sent to Julie K. Freed, MD, PhD, Associate Editor, for review by expert referees, editorial decision, and final disposition.

Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.123.032079

For Sources of Funding and Disclosures, see page 14.

JAHA is available at: www.ahajournals.org/journal/jaha

RESEARCH PERSPECTIVE

What Is New?

- Gestational hypoxia damages vascular endothelial nitrous oxide synthesis in adult male offspring.
- Nitrous oxide synthesis disorder is associated with the miR-155-5p/NADPH oxidase 2/reactive oxygen species axis in hypoxic adult male offspring.

What Question Should Be Addressed Next?

- How does miR-155-5p upregulate the expression of NADPH oxidase 2 in hypoxic offspring vascular endothelial cells?

Nonstandard Abbreviations and Acronyms

HY	gestational hypoxia group
NO	nitric oxide
NOX2	NADPH oxidase 2
ROS	reactive oxygen species
SNP	sodium nitroprusside
TA	thoracic aorta
VECs	vascular endothelial cells

insufficient oxygen supply, is one of the most common and severe stresses on the internal balance mechanism of organisms. Compared with adults, fetuses in the uterus live in a low-oxygen environment and experience physiological hypoxia. Although physiological hypoxia is crucial for embryo and fetal development, pathological hypoxia during pregnancy resulting from a lower intrauterine oxygen environment has been found to have adverse effects on intrauterine fetal development. Currently, gestational hypoxia is considered the most common and critical adverse factor affecting fetal development. This is not only due to the higher oxygen demand in mid-late gestation but also largely attributed to various pathological factors, such as maternal factors (preeclampsia and diabetes, etc.), placenta and umbilical cord factors, and adverse environmental factors during pregnancy (eg, smoking and passive smoking).[9,10] To date, numerous animal experiments and clinical statistics have shown that gestational hypoxia not only restricts intrauterine fetal growth and development but also increases the risk of cardiovascular diseases in offspring.[9,11] Offspring experiencing hypoxia during pregnancy often exhibit impaired vascular constriction and dilation functions.[11–13]

Vascular endothelial cells (VECs) cover the inner surface of blood vessels and are capable of secreting various vascular regulatory factors, participating in the regulation of vascular function and blood pressure in the body.[14,15] Nitric oxide (NO) is the most important vasodilator secreted by VECs, and its abnormal synthesis and secretion is involved in the occurrence and development of various cardiovascular diseases such as hypertension and atherosclerosis.[15,16] Typically, endothelial NO synthesis is mainly accomplished through the Ca^{2+}-dependent pathway activation of endothelial nitric oxide synthase (eNOS).[17,18] Acetylcholine is a common vasodilator substance in the body, which can activate VECs to synthesize NO, thereby inducing NO-dependent vasodilation. It is often used to assess the ability of VECs to synthesize NO. After binding to acetylcholine receptors on the cell membrane, acetylcholine can activate nearby Ca^{2+} channels (mainly TRP [transient receptor potential] Ca^{2+} channels) to mediate extracellular Ca^{2+} influx on one hand.[18] On the other hand, acetylcholine can stimulate phospholipase C through G protein-coupled transmembrane receptors on the cell membrane, hydrolyzing phosphatidylinositol 4,5-bisphosphate to produce the second messenger inositol 1,4,5-trisphosphate. Inositol 1,4,5-trisphosphate binds to its receptors on the endoplasmic reticulum, causing the release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm.[19,20] The influx of extracellular Ca^{2+} and the release of endoplasmic reticulum Ca^{2+} significantly increase the intracellular Ca^{2+} ($[Ca^{2+}]i$) level. High concentrations of $[Ca^{2+}]i$ bind to CaMs (calmodulins), activating Ca^{2+}/CaMs-dependent kinase II to phosphorylate eNOS, thereby enhancing eNOS activity and catalyzing the production of L-arginine to NO.[21,22] The NO generated by endothelial cells diffuses to the adjacent vascular smooth muscle layer, activating the guanylate cyclase/guanosine 3',5'-cyclic monophosphate pathway to mediate vasodilation. NO plays a pivotal role in maintaining cardiovascular homeostasis. Recent studies have shown that a decrease in endogenous NO synthesis or bioavailability, resulting in reduced production and impaired signaling, is associated with various cardiovascular diseases.[23,24] There is substantial evidence that the synthesis, stability, and bioavailability of endothelial NO is regulated not only by key factors in the aforementioned pathways but also by the level of intracellular oxidative stress, particularly by NOXs (NADPH oxidases) and the subsequent generation of reactive oxygen species (ROS).[25–27]

Currently, although some animal experimental studies have demonstrated that gestational hypoxia can lead to vascular dysfunction in offspring, the effects and mechanisms of gestational hypoxia on the synthesis of vascular endothelial NO in offspring remain unclear. Given the high incidence and severity of

gestational hypoxia in mid-late pregnancy, it is imperative to further investigate whether it affects the long-term synthesis of NO in fetal and offspring VECs. This study aims to investigate and elucidate the long-term effects and pathological mechanisms of gestational hypoxia in mid-late pregnancy on the synthesis of vascular endothelial NO in fetal and offspring. The findings of this study will not only provide important insights into the early developmental origins of fetal-origin vascular diseases but also contribute to new theoretical knowledge for early prevention and treatment of such diseases, as well as offer new perspectives for clinical interventions to mitigate the negative impacts of gestational hypoxia.

METHODS

According to the Transparency and Openness Promotion Guidelines, data of this study are available from the corresponding author upon reasonable request. The authors declare that all supporting data are available within the article and the Data Supplement or from the corresponding author on request.

Experimental Animals

Pregnant Sprague–Dawley rats were purchased from the Animal Center of Soochow University and housed with standard rat food and tap water in a temperature-controlled room with a 10:14-hour light–dark cycle. The pregnant rats were randomly divided into 2 groups: the control group and the gestational hypoxia group (HY), from gestational days 10 to 20 (N=30 per group). The HY group was housed in a hypoxic cabin (10.5% oxygen), and the control group was housed in a normoxic cabin (21% oxygen). Hypoxia was achieved and maintained by using a mixture of nitrogen gas and air in individual chambers. The flow of nitrogen was adjusted to bring the oxygen percentage to 10.5%, which was continuously monitored with an oxygen analyzer (Hangtian Pengcheng Instrument, China). All animals were fed with standard rat food and tap water throughout pregnancy. At term (gestational day 20), some pregnant rats (N=15 per group) were euthanized with isoflurane inhalation (3%). A total of 8 to 12 pups per litter were included in the statistics and the male fetal thoracic aorta (TA) was immediately isolated for studies. The remaining pregnant rats (N=15 per group) were moved to a normal environment and allowed to give birth naturally for offspring experiments. After a month of nursing, male pups were separated and provided with normal food and water and were tested at 8 and 16 weeks old. The Animal Care and Use Committee of Soochow University approved this study, and all animal procedures followed the Guidelines for the Care and Use of Laboratory Animals.

Hematoxylin and Eosin Staining

The histological changes of TAs were observed by hematoxylin and eosin staining. Briefly, TAs specimens from fetal and adult offspring were immediately collected and fixed with 4% paraformaldehyde dissolved in PBS. After fixation, the TA samples were decalcified in 5% hydrochloric acid and embedded in paraffin. Then, TA specimens were sectioned into slices of 2 to 4 μm using a microtome (Leica, CM1850) and stained with hematoxylin and eosin (#C0105S, Beyotime, China). Imaging was carried out using a Nikon E600 microscope attached with a digital camera DMX1200. The images of 10 TAs from both the outside and lumen diameters in each group were taken to measure wall thickness using Image-Pro 6.0 software.

Measurement of Vascular Relaxation

Fetal and adult offspring were euthanized with isoflurane inhalation (3%). TAs were then rapidly excised and placed in a physiological saline solution containing the following concentrations (mmol/L): NaCl (142.0), KH_2PO_4 (1.2), $MgSO_4$ (1.7), $CaCl_2{\cdot}2H_2O$ (2.8), KCl (4.7), $NaHCO_3$ (14.9), EDTA (0.02), glucose (5.0), and HEPES (10.0), with a pH of 7.4 at 4 °C. The solution was continuously gassed with 5% CO_2 in O_2. TAs were then cut into ring segments (2–3 mm in length) and carefully threaded onto stainless steel wires. The ring segments were mounted on an M series Myograph System (Radnoti LLC, Covina, CA) in a chamber filled with physiological saline solution bubbled with 95% O_2 and 5% CO_2. Equilibration and normalization procedures were performed as described in our previous studies.[8,28] The viability and integrity of the TA rings were first assessed by repeatedly exposing them to 120 mmol/L potassium chloride before adding drugs. After being washed 3 times with physiological saline solution, the TA rings were allowed to stabilize for 1 hour. Once stabilized, serotonin hydrochloride (5-HT, 10^{-4} mol/L) was added to induce a stable contraction, which served as a platform for testing vascular relaxation. When the 5-HT-induced contraction reached stability, cumulative concentrations of acetylcholine (acetylcholine, 10^{-11}–10^{-4} mol/L) or sodium nitroprusside (SNP, 10^{-11}–10^{-4} mol/L) were added. The mN values of the stable contractions induced by 5-HT were recorded, and the value (ΔmN) relative to the baseline force was calculated. Changes in tension caused by acetylcholine or SNP were expressed as a percentage of the ΔmN value induced by 5-HT. Dose–response curves for relaxation were obtained by cumulatively adding acetylcholine or SNP, respectively. The TA rings were allowed to fully recover and then incubated with the nonselective NOS inhibitor (Nω-nitro-L-arginine methyl ester, 10^{-5} mol/L), vas2870 (an inhibitor of NOX2, 10^{-5} mol/L), or tempol (an SOD [superoxide dismutase]

mimetic, 10^{-5} mol/L) for 30 minutes before the diastolic response with acetylcholine using cumulative concentrations. The experiments described here were conducted on both fetal and adult offspring TA rings. All drugs were purchased from Sigma-Aldrich.

Endothelial Cell Isolation and Treatment

Primary VECs were obtained from the TAs of 16-week-old male offspring, as described in a previous study.[29] Briefly, the TA was rapidly excised and placed in PBS. Denuded TAs were opened lengthwise and spread out in a petri dish. An enzyme solution consisting of 0.2% collagenase type 2 was added to the intima surface of the vessels. After 10 minutes of enzyme digestion, the endothelial layer cells were gently scraped from the surface of the vascular lumen using a pair of sterilized tweezers. The cells were then precipitated, resuspended, and cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37 °C with 5% CO_2 and 95% air in a humidified incubator. The primary cells were confirmed as VECs by staining with CD31 (a specific biomarker of VECs; #131R-2, Sigma-Aldrich). Cultures were passaged every 2 to 3 days in a 10 cm^2 dish and used in experiments between passages 3 and 5. For the miR-155-5p overexpression or suppression assay, VECs were transfected with the miR-155-5p mimics or inhibitor using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The sequences of the inhibitor and mimics for miR-155-5p, synthesized by Genepharma (GenePharma, China), are listed in the Table. After transfection, the cells were cultured in standard media for another 48 hours. At the end, the cells were collected for mRNA, protein, and other analyses.

Determination of ROS Levels

ROS production in VECs was detected by staining with fluorescently labeled dihydroethidium (#S0063, Beyotime, China). Dihydroethidium is a commonly used fluorescent probe for detecting ROS in live cells. After being taken up by live cells, dihydroethidium can undergo dehydrogenation under the action of ROS in the cells, producing ethidium. Ethidium can bind to RNA or DNA and emit red fluorescence. When ROS generation in the cells is high, more ethidium is produced, resulting in stronger red fluorescence. Conversely, when the ROS generation is low, the fluorescence is weaker. The cultured VECs were incubated with dihydroethidium (10^{-5} mol/L) in a light-protected humidified chamber at 37 °C for 30 min, and then the cells were washed 3 times with PBS and fixed with 4% paraformaldehyde for 15 minutes. Finally, the fluorescence intensity was measured using a fluorescent microscope (535 and 610 nm were set for excitation and emission, respectively). Images were acquired, and ROS levels were quantitatively calculated using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD).

Measurement of NO Release and Intracellular Ca^{2+} Levels

Confocal NO and Ca^{2+} imaging of VECs was performed as previously described.[11,30] Briefly, isolated VECs were incubated with 3-amino,4-aminomethy l-2′,7′-difluorescein, diacetate (10^{-6} mol/L; #S0019, Beyotime, China) or Fluo-3 AM (10^{-6} mol/L; Invitrogen) in Tyrode's solution (containing mmol/L: NaCl, 135.0; KCl, 4.7; $MgCl_2$, 1.0; $CaCl_2$, 2.0; HEPES, 10.0; $NaH_2PO_4{\cdot}2H_2O$, 1.2; and glucose, 10.0; pH 7.4) for 20 minutes at room temperature. After loading, VECs were washed 3 times with Tyrode's solution and transferred to a recording chamber. Representative single cell images and traces of NO and Ca^{2+} responses to Acetylcholine (10^{-4} mol/L) were measured using a total internal reflection fluorescence microscopy electron-multiplying charge-coupled device imaging system. Vas2870 (10^{-4} mol/L) or tempol (10^{-4} mol/L) was used to pretreat VECs for 30 minutes before the application of acetylcholine. We measured and monitored NO and Ca^{2+} transients induced by acetylcholine per single cell in the 2 groups. All images were processed and analyzed using Fiji software (Olympus, Japan). The fluorescence intensity was calculated as F/F0, where F0 is the fluorescence intensity during a period from the beginning of the recording when there was no acetylcholine, and F is the fluorescence intensity for the region of interest.

Quantitative Reverse Transcription Polymerase Chain Reaction

The total RNA in VECs was extracted using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. After RNA extraction, all RNA samples were reverse transcribed using the first-strand cDNA Synthesis Kit (Takara, Dalian, China). Then, quantitative polymerase chain reaction (PCR) was conducted using the iQ5 Real-Time PCR Detection System (Bio-Rad) with SYBR Green PCR Master Mix (Applied Biosystems, USA) for mRNA analysis. The relative mRNA levels of genes were calculated using the ΔΔCt method with GAPDH as the normalization control. For miRNA analysis, miRNAs from VECs were extracted using the miRcute miRNA isolation kit (Tiangen, Beijing, China). The miRcute plus miRNA first-strand cDNA kit (Tiangen, Beijing, China) was used for miRNA reverse transcription. Quantitative PCR of miRNAs was performed to determine their relative levels using the miRcute plus miRNA qPCR kit (SYBR Green) and analyzed on the iQ5 Real-Time PCR Detection System

Table. Primers Used in This Study

Primer/siRNA	Nucleotide sequence (5′ to 3′)	
qRT-PCR primers	Sense	Antisense
SOD1	GTACCACTGCAGGACCTCAT	CCACCTTTGCCCAAGTCATC
SOD2	CCGTGGTGGGTGTTTTGTAT	CGTCCAAGCAATTCAAGCCT
SOD3	CGCCTCCAGTCATCCTAGAG	AAAGTGTCCTGGTCTCCGAG
NOX1	GTCGCACAAGGACTTCACTC	GCAAGTGTCAACCAGCAAGA
NOX2	TGCACATCTGTTCAACGTGG	AACCGAGTCACAGCCACATA
NOX4	TGTCTGCTTGTTTGGCTGTC	TGCAGTTGAGGTTCAGGACA
Endothelial nitric oxide synthase	CTGTCTACACCTACCACGAA	TGCTTCTCATAGGCCTTCAT
M1	ACAGTGACAGGCAACCTACT	CAGAAGATTCATGACAGAGG
M2	GGCTATTACCAGTCCTTACA	GGCCAGTAACCAATCACAGT
M3	CATCTGGCAAGTGGTCTTC	AGAGGTCGCAGGCTAAGTT
M4	CATTGCTACAGTGACTGGCT	ATTGCTCACCACATAGTCCA
M5	TCAGCCTGATGACCATTGTC	AGAAGGTTCATGACAGAAGC
TRPV1	CTGCAAGCCAGGTAACTCTTACA	CTGCAGCTTCCAGATGTTCTT
TRPV2	TGGAACAGCAAGTACCTCACTG	ACTGCAGGCTCCTCTTCTCTAT
TRPV3	CATCAGTGTAGACGCATGGC	GACTTCCAGACTCAGAACTGCTG
TRPV4	ATTGGCTATGCCTCAGCTCT	CAGCAGGAGAATGAAGACCA
TRPA1	ATGAAGCGCAGCTTGAGG	GCGTGATGCAGAAGACAGAG
TRPM2	CCTCATCTCCAATAAGCCTGAG	GGATACAGCAGCTGTGTGGAG
A-kinase anchoring protein 150	GTGATGGTGAGAGATGGTAAGGA	CAGTAGAAGTCACTGGCTGAGG
TRPC1	AGAGATGAGAACTACCAGAAGGTG	CAAATCCCTTATTTCATTTCTGAA
TRPC2	AGTTCTCCTTCTTCAGCAGCAGT	GAGAACTTCAGCAGGTCATATCGG
TRPC3	TCTCGCACGCTCAATGTC	AGAAGTCGTCATCGCGCA
TRPC4	AGCAAGCTCTCAACAATACAGTCA	AGAGGCTGAGATTCTTTCTCTTGT
TRPC5	AGAGGTCGCAGGCTAAGTT	GGCCATGGTTCATAGCAAT
TRPC6	AGAGGCTGAGATTCTTTCTCTTGT	CCTCCACAATCCGTACATAACCTT
TRPC7	TCCTGCCTACATGTTCAATGAGA	AGTTCTCCTTCTTCAGCAGCAGT
IP3R1	GTGGGCTACCTGTTCTTCAAG	ATGAGCAGTGTCTCACACGTGT
IP3R2	GCAACAACTACCGGATCGTC	AGGAAGGTGTGGGCTAAGTC
IP3R3	CTGACAGAGGAGACCAAGCA	GAACACTGCCAGGTTGAAGG
CaM1	TGAAAGACACAGATAGCGAAGAA	TGCTTCTCTGATCATTTCGTCTA
CaM2	ATACAGACAGCGAGGAGGAGATA	GATCATCTCATCCACTTCCTCAT
CaM3	GGATGGAGATGGCACCATTA	GGGAAGTCAATGGTCCCATT
miR-155-5p	CCTGGAGAAACCTGCCAAGTA	TTACTCCTTGGAGGCCATGTA
miR-141-3p	GCCTAACACTGTCTGGTAAAGATGG	TTACTCCTTGGAGGCCATGTA
miR-320-3p	AAAAGCTGGGTTGAGAGGGC	TTACTCCTTGGAGGCCATGTA
miR-532-3p	ATTACCTCCCACACCCAAGGCTT	TTACTCCTTGGAGGCCATGTA
miR-652-3p	AATGGCGCCACTAGGGTTGTG	TTACTCCTTGGAGGCCATGTA
miR-155-5p inhibitor	ACCCCUAUCACAAUUAGCAUUAA	-
miR-155-5p mimics	UUAAUGCUAAUUGUGAUAGGGGU	CCCUAUCACAAUUAGCAUUAAUU

CaM indicates calmodulin; IP3, inositol 1,4,5-trisphosphate; NOX, NADPH oxidase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; siRNA, small interfering RNA; SOD, superoxide dismutase; and TRP, transient receptor potential.

(Bio-Rad). The expression of U6 was used as the normalization control for each miRNA sample. The primers used in this study are listed in Table. All experiments were repeated 3 times using independently prepared cells and were performed as previously described.[30,31]

Western Blotting

Cell lysis and total protein were extracted using RIPA buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 10 mmol/L EDTA) supplemented with Protease Inhibitor Cocktail

Tablet (#sc-29130, Santa Cruz Biotechnology). The protein concentration of each sample was determined using the Bradford Protein Assay Kit (#P0006, Beyotime, China). Then, 20 μg of protein extracts from each sample were loaded onto 10% SDS-PAGE gels and electrotransferred to polyvinylidene difluoride membranes. After blocking with 5% nonfat milk, the membranes were incubated with specific primary antibodies against NOX2 (SAB4300724, 1:1000, Sigma) and β-actin (A5441, 1:3000, Sigma). The membranes were subsequently incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (#7074/6, Cell Signaling Technology, USA) for 1 hour at room temperature. Finally, signals were visualized using a chemiluminescence and imaging system (EC3-Imaging-System, USA), and the ratio of band intensity to β-actin was analyzed using the ImageJ software to quantify the relative NOX2 protein expression. All experiments were repeated 3 times with independently prepared cells.[30]

Statistical Analysis

Data were expressed as mean±SEM. Concentration-dependent agonist response data were analyzed by 2-way ANOVA followed by Bonferroni post hoc test using GraphPad Prism version 7 (GraphPad Software, San Diego, CA). Statistical significance was evaluated for other data using 1-way ANOVA followed by Tukey's multiple comparison test or Student's *t* test or repeated measures analysis of variance using the Greenhouse–Geisser correction. The correlations of TA rings within the same animal were reconciled using Pearson's correlation coefficient. Results with a $P<0.05$ were considered statistically significant.

RESULTS

Acetylcholine-Induced Vascular Relaxation in Fetal TA Rings

The gestational hypoxia model in rats was constructed as shown in Figure 1A. Histological analysis showed similar vascular thickness in the fetal TAs between the 2 groups (Figure 1B). There was no significant difference in 5-HT-induced maximal contraction (Figure S1A). Acetylcholine-induced vasodilation was expressed as a percentage of maximal contraction induced by 5-HT (10^{-4} mol/L). In our preliminary studies, we observed that the detrimental effects of gestational hypoxia on acetylcholine-induced relaxation were more pronounced in male fetuses compared with female fetuses, as shown in Figure 1C and Figure S1B. Therefore, this study focused exclusively on male offspring. In male fetal TA rings, the acetylcholine-induced vascular relaxation was significantly reduced in the HY group (Figure 1C; 10^{-6}–10^{-4} mol/L), with maximum dilation percentages of 68.69% in the control group and 44.24% in the HY group. However, there was no significant difference in the EC50 of the acetylcholine-induced vasodilation response between the 2 groups (Figure 1D). Acetylcholine can detect the ability of endothelial cells to synthesize NO, whereas SNP can directly hydrolyze and generate NO and is usually used to test the response of vascular smooth muscle to NO. By comparing the vasodilation responses mediated by acetylcholine and SNP, the ability of endothelial cells to synthesize NO can be indirectly assessed. There was no significant difference in the vasodilation response mediated by SNP between the 2 groups (Figure 1E). Meanwhile, when the TA rings was preincubated with Nω-nitro-L-arginine methyl ester (an eNOS inhibitor), there was no significant difference in Acetylcholine-induced vasodilation response between the 2 groups (Figure 1F). These results together indicate that gestational hypoxia significantly reduces the NO-dependent vasodilation response mediated by acetylcholine in fetal blood vessels.

Acetylcholine-Induced Vascular Relaxation in Adult Offspring TA Rings

Adult male offspring in both groups showed similar external diameter, internal diameter, and thickness of the TAs (Figure 2A), suggesting that gestational hypoxia does not significantly affect the structure and development of offspring blood vessels. Consistent with the results of vascular function experiments in fetal TAs, the vasodilation response mediated by acetylcholine (10^{-6}–10^{-4} mol/L) was also significantly reduced in the hypoxic adult offspring (Figure 2B; Figure S1C), with maximum dilation percentages of 47.36% in the control group and 30.32% in the HY group. There was no significant difference in the EC50 of the acetylcholine-induced vasodilation response between the 2 groups (Figure 2C). Additionally, there was also no significant difference in the SNP-induced vasodilation response between the 2 groups (Figure 2D). Similar results were also observed in 8-week-old male offspring in both groups (Figure S1D and S1E). Furthermore, the difference in the acetylcholine-induced vasodilation response between the 2 groups could be eliminated by Nω-nitro-L-arginine methyl ester (Figure 2E). In conclusion, these results indicate that the adverse effects of gestational hypoxia on the NO-dependent vasodilation response mediated by acetylcholine in fetal blood vessels persist in adult offspring after birth.

NOX2 Expression and ROS Generation in Offspring TA-VECs

To further elucidate the molecular mechanisms underlying the impaired endothelial NO synthesis in gestational hypoxic offspring, we isolated and cultured VECs from offspring TA in vitro (Figure S2A and S2B).

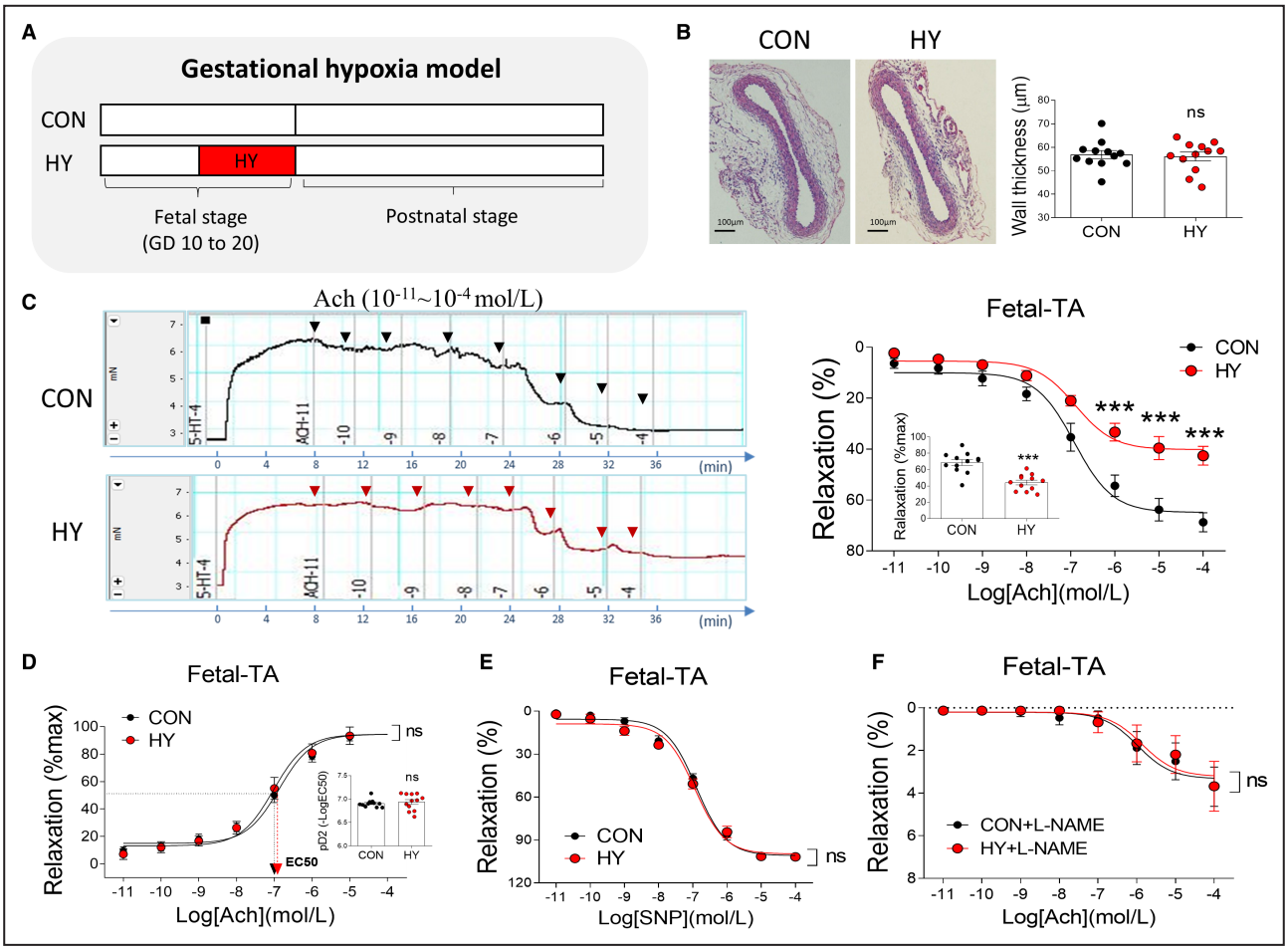

Figure 1. Acetylcholine-induced vascular relaxation in fetal TA rings.
A, Illustration depicting the construction of the gestational hypoxia rat model. **B**, Histological analysis shows that the thoracic aorta (TA) rings isolated from male fetus (N=6, n=12). **C** and **D**, Acetylcholine–mediated relaxation in fetal TA rings (N=6, n=12). **E**, SNP-mediated relaxation in fetal TA rings (N=6, n=12). **F**, Effect of L-NAME on acetylcholine–mediated relaxation in fetal TA rings (N=6, n=12). N represents the number of male fetuses, and n represents the number of fetal TA rings. Two male fetuses were randomly selected from each litter, and 2 TA rings were selected from each male fetus for experiments. Data were presented as means±SEM. Statistical significance was calculated by the Student's *t* test or 2-way ANOVA with Bonferroni post hoc tests. Repeated measures analysis of variance was performed in **C** and **D**. ***P<0.001. Ach indicates acetylcholine; CON, control group; GD, gestational day; HY, gestational hypoxia group; L-NAME, Nω-nitro-L-arginine methyl ester; ns, nonsignificance; pD2, –log[50% effective concentration (EC50)]; and SNP, sodium nitroprusside.

By assessing the expression of key genes involved in NO synthesis, we found no significant differences in the expression of acetylcholine receptors, eNOS, TRP channel components, inositol 1,4,5-trisphosphate receptors, and CaMs in the TA-VECs between the 2 groups (Figure S2C through S2H). Consistent experimental results were obtained in both fetal and offspring TA tissue (Figure S3A and S3B). NOX is the main source of ROS generation, and SOD is the most important antioxidant enzyme responsible for oxidative balance in blood vessels. Among the subtypes of the NOX and SOD families, NOX1/2/4 and SOD1/2/3 are mainly expressed in the vascular system. We detected the expressions of major SODs and NOXs in the offspring TA-VECs (Figure 3A through 3D) and found that the expression levels of NOX2, both in protein and mRNA, were significantly increased in the HY group (Figure 3B through 3D; Figure S3C). Meanwhile, the level of ROS was also significantly increased in the HY group (Figure 3E). These experimental results suggest that gestational hypoxia impairs NO synthesis in offspring endothelial cells, which may be related to increased oxidative stress mediated by NOX2.

Acetylcholine-Induced NO Release and Intracellular Ca^{2+} Levels in Offspring TA-VECs

To investigate whether the elevated NOX2-mediated oxidative stress directly causes impaired NO

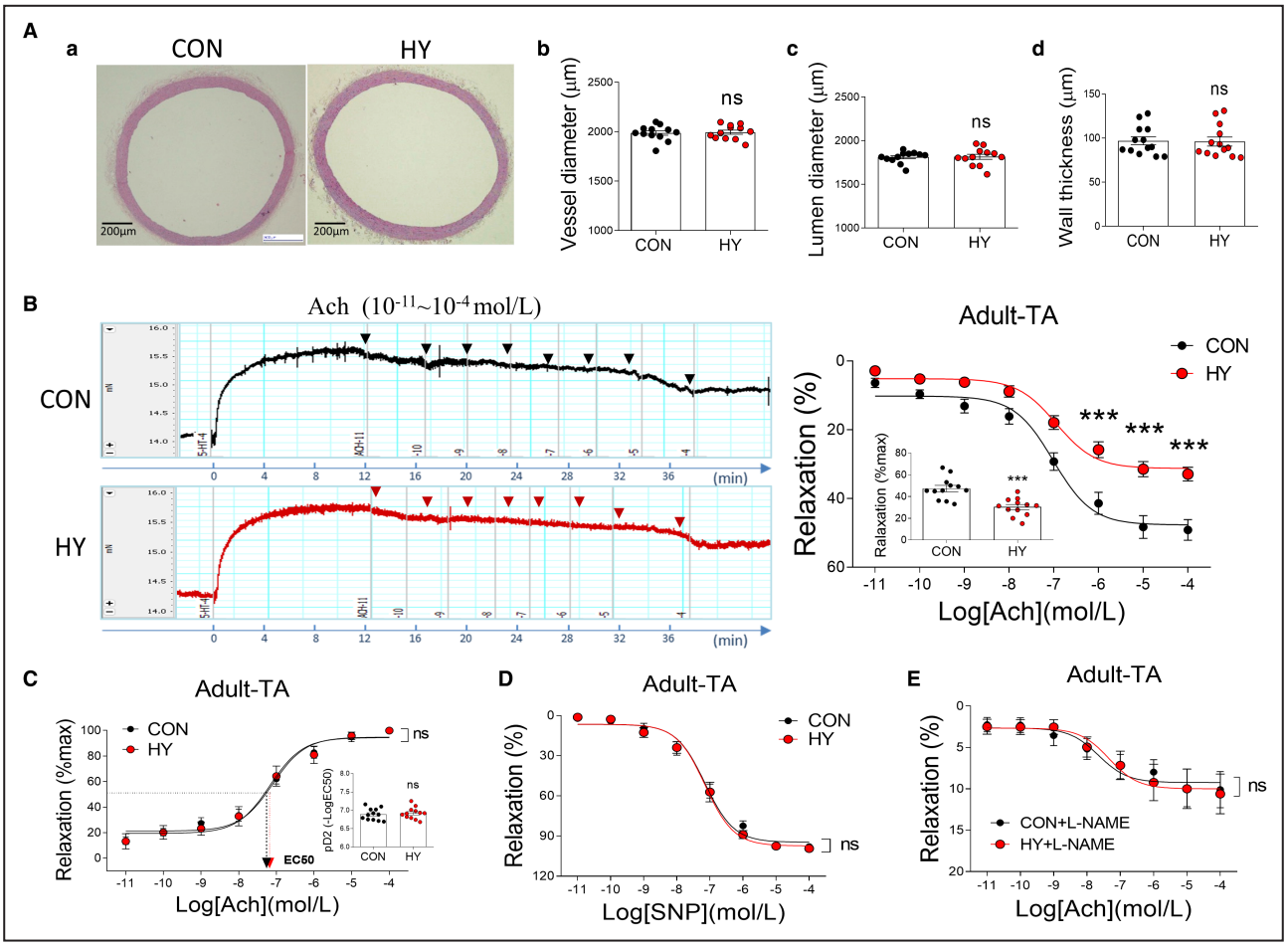

Figure 2. Acetylcholine-induced vascular relaxation in adult offspring TA rings.
A, Histological analysis shows that the TA rings isolated from adult male offspring (N=6, n=12). **B** and **C**, Acetylcholine-mediated relaxation in male offspring TA rings (N=6, n=12). **D**, SNP–mediated relaxation in male offspring TA rings (N=6, n=12). **E**, Effect of L-NAME on acetylcholine-mediated relaxation in male offspring TA rings (N=6, n=12). One male offspring was randomly selected from each litter, and 2 TA rings were selected from each male offspring for experiments. Data were presented as means±SEM. Statistical significance was calculated by the Student's *t* test or 2-way ANOVA with Bonferroni post hoc tests. Repeated measures analysis of variance was performed in **B** and **C**. **P*<0.05, ****P*<0.001. Ach indicates acetylcholine; CON, control group; HY, gestational hypoxia group; L-NAME, Nω-nitro-L-arginine methyl ester; N, number of adult male offspring; n, number of TA rings; ns, nonsignificance; pD2, –log[50% effective concentration (EC50)]; SNP, sodium nitroprusside; and TA, thoracic aorta.

synthesis in hypoxic offspring VECs, we conducted dynamic assessments of acetylcholine-induced NO synthesis and intracellular Ca^{2+} levels in single VECs. Figure 4A shows the changes in fluorescence intensity and traces of acetylcholine-induced intracellular Ca^{2+} levels in single VECs. We calculated the maximum fluorescence intensity as F/F0 (max), where F represents the maximum fluorescence value induced by acetylcholine, and F0 represents the Ca^{2+} fluorescence value before acetylcholine stimulation. The F/F0 (max) did not show any significant difference between the 2 groups, indicating that the impaired NO synthesis in gestational hypoxic offspring VECs is not related to the Ca^{2+} pathway. Figure 4B illustrates the changes in fluorescence intensity and traces of acetylcholine-induced NO synthesis and release. The F/F0(max) of acetylcholine-induced NO release was lower in the HY group (1.198±0.0138 versus 1.112±0.0093; control versus HY; *P*=0.00011). After pretreatment with vas2870 or tempol, no significant differences were observed in acetylcholine-induced NO release between the 2 groups (Figure 4C and 4D). To further investigate the roles of NOX2-mediated oxidative stress, we also measured acetylcholine-induced vascular relaxation in the absence or presence of vas2870 or tempol. Both vas2870 and tempol effectively restored vascular relaxation to acetylcholine in both hypoxic fetal and adult offspring TA rings (Figure 4E through 4H). These results collectively indicate that the elevated oxidative stress mediated by NOX2 is the cause of impaired NO synthesis in gestational hypoxic offspring vessels.

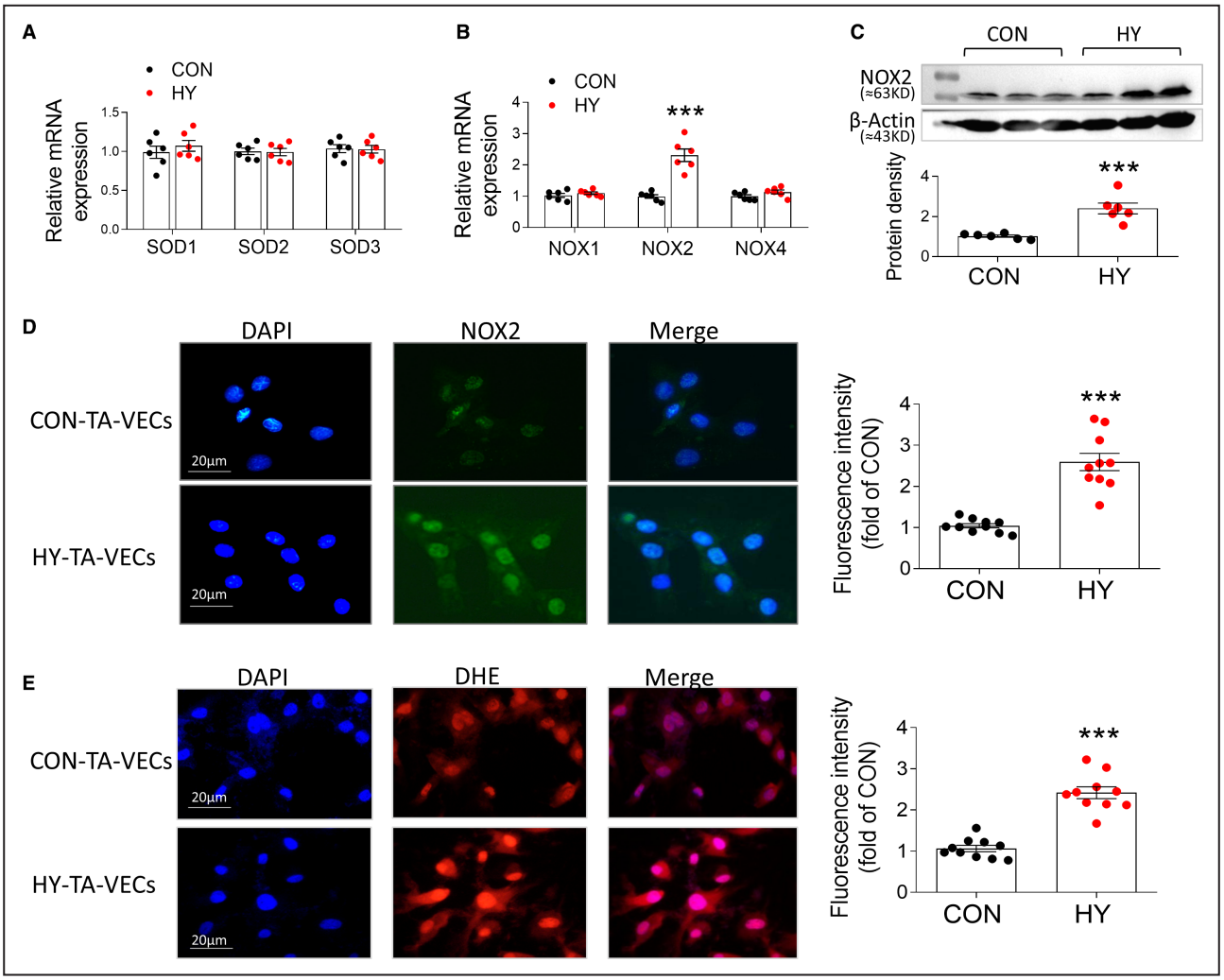

Figure 3. NOX2 expression and ROS levels in TA-VECs.
A and **B**, Relative mRNA expression of SODs and NOXs in isolated primary VECs from male offspring TA (TA-VECs). **C**, Representative figure of NOX2 protein level in TA-VECs. **D**, Immunostaining of NOX2 protein levels in TA-VECs. **E**, dihydroethidium staining shows the ROS levels in TA-VECs. Results from 3 to 6 independent experiments. Data were presented as means±SEM. Statistical significance was calculated by the Student's *t* test or 1-way ANOVA followed by Tukey's multiple comparison test. ***P<0.001. CON indicates control group; HY, gestational hypoxia group; DHE, dihydroethidium; NOX, NADPH oxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TA, thoracic aorta; and VECs, vascular endothelial cells.

Exogenous miR-155-5p Mimics Upregulated NOX2 Expression and ROS Generation in Offspring TA-VECs

The results indicated that the upregulation of NOX2 expression is a key cause for the impaired NO synthesis in gestational hypoxic offspring VECs. Considering that miRNAs are important regulatory factors for genes such as NOX2, we predicted potential miRNA targets of NOX2 through bioinformatics analysis and previous literature reports. We then measured the expression levels of the predicted potential miRNAs in offspring VECs and found that only the expression level of miR-155-5p significantly increased in gestational hypoxic offspring VECs, whereas the others did not show significant changes (Figure 5A). Additionally, miR-155-5p was also significantly increased in both fetal and offspring TA tissue (Figure S3D). Therefore, we speculate that miR-155-5p is a potential target of NOX2. To confirm that high expression of miR-155-5p is associated with excessive NOX2 levels, we overexpressed miR-155-5p in offspring VECs. Transfecting with miR-155-5p mimics significantly increased the expression level of miR-155-5p compared with controls or negative control miRNA (Figure 5B), indicating that these mimics function properly. We then measured the mRNA and protein levels of NOX2 in offspring VECs and found that miR-155-5p mimics significantly increased the expression of NOX2 (Figure 5C through 5E). Consistent with the increased NOX2 expression,

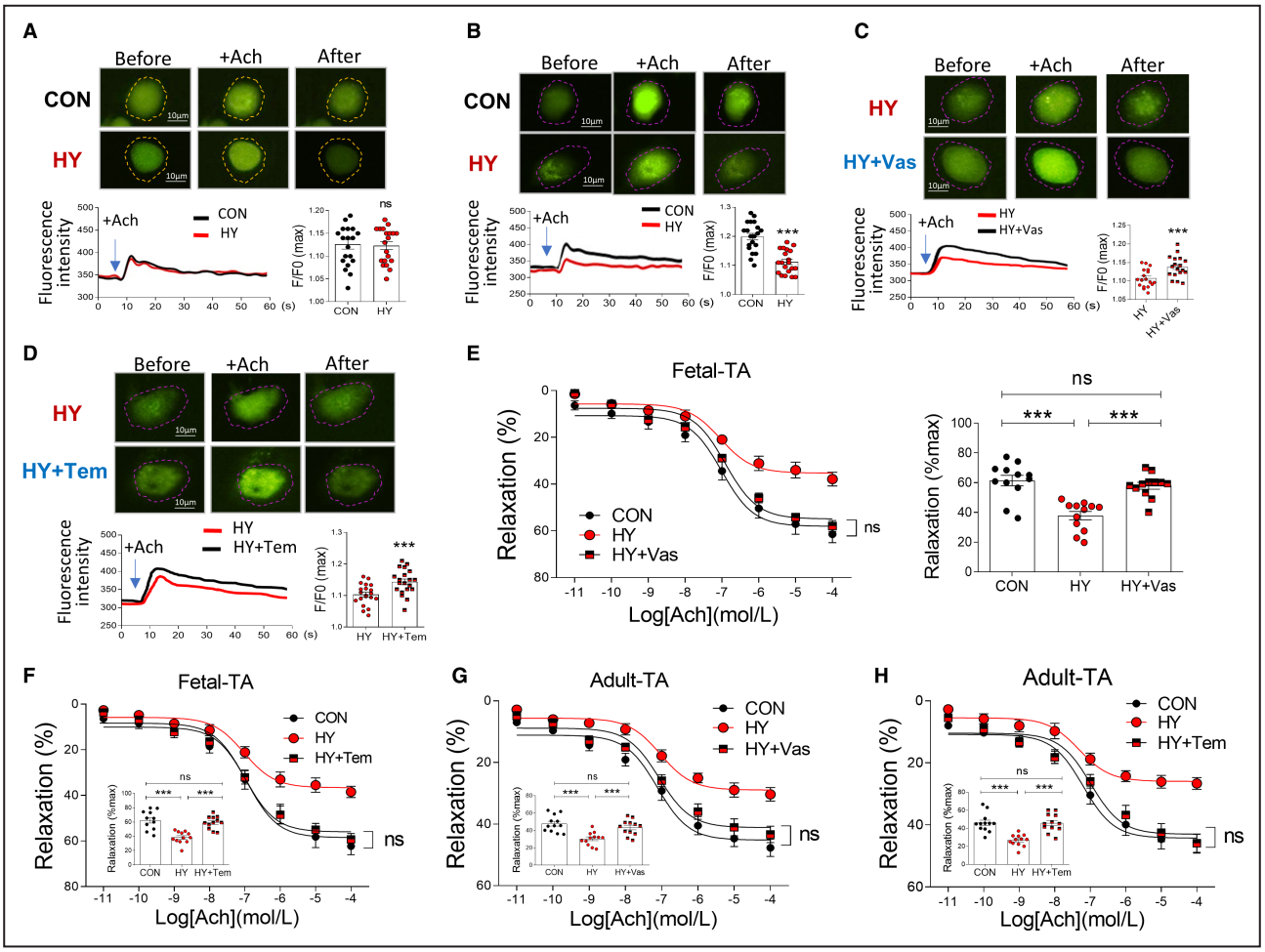

Figure 4. Acetylcholine-induced NO release and intracellular Ca^{2+} levels in offspring TA-VECs.
A and **B**, The transients of acetylcholine-induced intracellular Ca^{2+} level (**A**) and NO release (**B**) in offspring TA-VECs with TIRFM-EMCCD imaging system. **C** and **D**, The transients of acetylcholine-induced NO release in pretreated with vas2870 (Vas; **C**) or the superoxide dismutase mimetic tempol (Tem; **D**) in TA-VECs. Representative single-cell images (upper), traces (lower left), and summarized data (lower right) of NO and intracellular Ca^{2+} levels measured with the NO indicator (DAF-FM) and fluorescence Ca^{2+} indicator (Fluo-3), respectively. The fractional fluorescence intensity was calculated as F/F0, where F is the fluorescence intensity for the region of interest, and F0 is the fluorescence intensity during a period from the beginning of the recording before adding acetylcholine (n=20 cells from 6 rats per group, about 3 to 4 cells were obtained from each offspring TAs). **E** and **F**, Concentration-response curves to acetylcholine in either absence or presence of vas2870 (Vas; **E**) or Tempol (Tem; **F**) in fetal TA rings (N=6, n=12). N represents the number of male fetuses, and n represents the number of fetal TA rings. Two male fetuses were randomly selected from each litter, and 2 TA rings were selected from each male fetus for experiments. **G** and **H**, Concentration-response curves to acetylcholine in either absence or presence of Vas2870 (**G**) or tempol (**H**) in adult offspring TA rings (N=6, n=12). One male offspring was randomly selected from each litter, and 2 TA rings were selected from each male offspring for experiments. Data were presented as means±SEM. Comparisons of 2 different samples were performed by Student's *t* test or 2-way ANOVA with Bonferroni post hoc tests. ***P<0.001. Ach indicates acetylcholine; CON, control group; HY, gestational hypoxia group; NO, nitrous oxide; ns, nonsignificance; TA, thoracic aorta; Tem, tempol; and VECs, vascular endothelial cells.

the ROS generation in offspring VECs also significantly increased by miR-155-5p mimics (Figure 5F).

MiR-155-5p Inhibitor Decreased NOX2 Expression and ROS Generation in Offspring TA-VECs

We also investigated the effects of the miR-155-5p inhibitor on the expression of NOX2 and the generation of ROS in offspring VECs. As depicted in Figure 6A, the expression level of miR-155-5p significantly decreased in offspring VECs compared with the negative control inhibitor. Knocking down miR-155-5p resulted in a significant decrease in the expression of NOX2 in offspring VECs (Figure 6B and 6C). Additionally, treatment with the miR-155-5p inhibitor noticeably reduced the generation of ROS in offspring VECs (Figure 6D). These findings indicate that the expression of miR-155-5p affects the levels of NOX2 expression and ROS generation in offspring VECs.

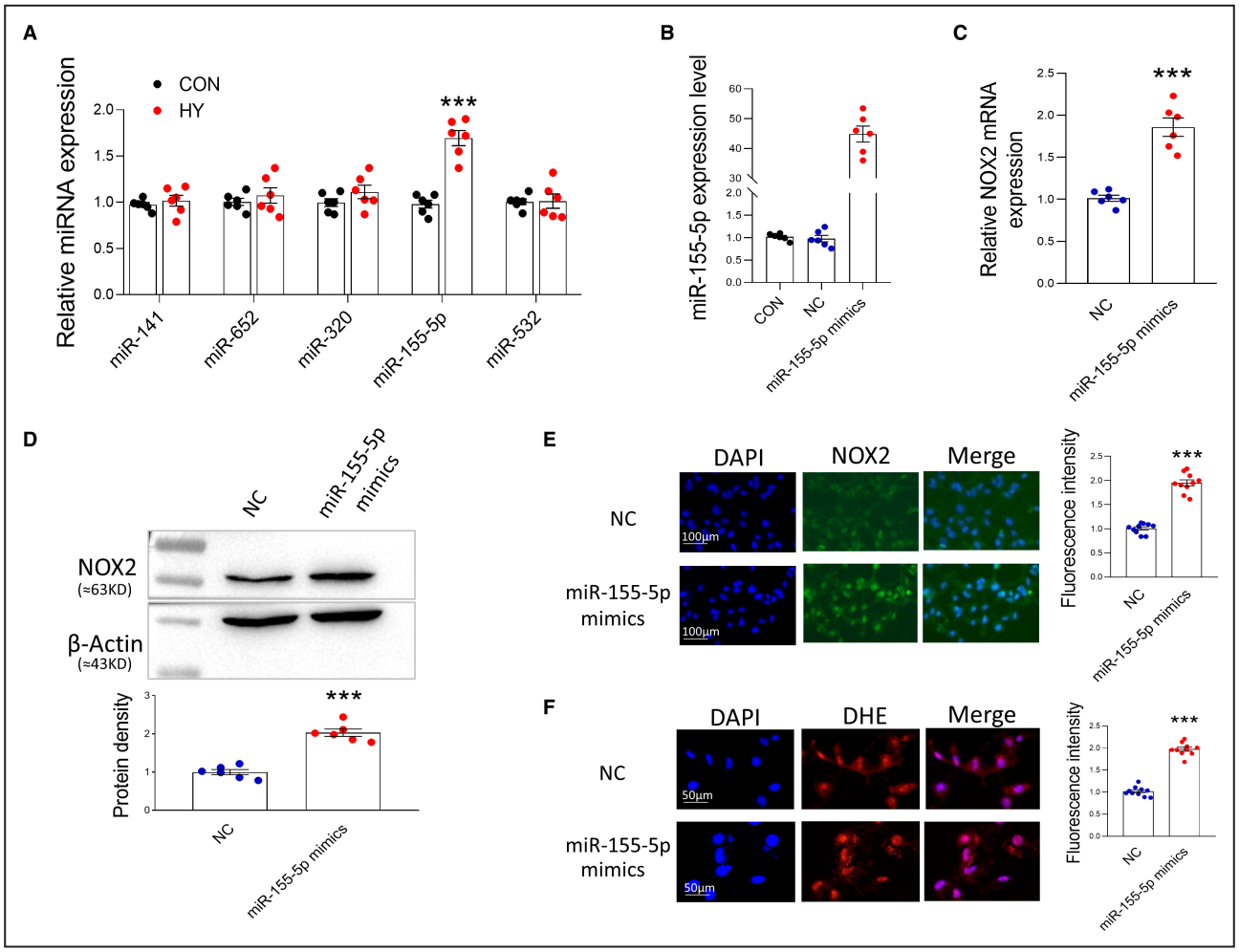

Figure 5. Exogenous miR-155-5p mimics upregulated NOX2 expression and ROS level in offspring TA-VECs.
A, Expression levels of several miRNAs in offspring TA-VECs. **B** and **C**, qRT-PCR analysis was performed to detect the expression level of miR-155-5p and NOX2 in TA-VECs. **D** and **E**, Western blot and immunostaining detect the protein expression of NOX2 after overexpression of miR-155-5p in offspring VECs. **F**, Dihydroethidium staining shows ROS levels after overexpression of miR-155-5p in offspring VECs. Results from 3 to 6 independent experiments. Data were presented as means±SEM. Statistical significance was calculated by the Student's *t* test or 1-way ANOVA followed by Tukey's multiple comparison test. ***$P<0.001$. CON indicates control; DHE, dihydroethidium; HY, gestational hypoxia group; NC, negative control miRNA; NOX2, NADPH oxidase 2; qRT-PCR, quantitative reverse transcription polymerase chain reaction; ROS, reactive oxygen species; TA, thoracic aorta; and VECs, vascular endothelial cells.

To confirm whether miR-155-5p exerts its effects on endothelial NO synthesis, we conducted dynamic assessments of acetylcholine-induced NO synthesis in single VECs from hypoxic offspring in the presence or absence of the miR-155-5p inhibitor. Figure 6E demonstrates that knocking down miR-155-5p significantly restored acetylcholine-induced NO synthesis in hypoxic offspring TA-VECs. In conclusion, we have demonstrated that gestational hypoxia increases the levels of miR-155-5p in offspring TA-VECs. The increased miR-155-5p upregulates the expression of NOX2 and the generation of ROS, ultimately leading to impaired NO synthesis and vascular dysfunction in offspring. Figure 6F summarizes the findings of the current study.

DISCUSSION

The present study employed a rat model to simulate gestational hypoxia during mid-late gestation. It has been discovered, for the first time, that gestational hypoxia has a negative impact on the synthesis of endothelial NO in offspring VECs. Specifically, male offspring exposed to gestational hypoxia exhibited a reduced capacity for NO synthesis in their blood vessels. This reduction can primarily be attributed to an increase in the expression of NOX2 and heightened oxidative stress in fetal VECs induced by gestational hypoxic stress. These alterations in the VECs persisted from birth into adulthood. Furthermore, both gestational hypoxic fetuses and adult offspring displayed

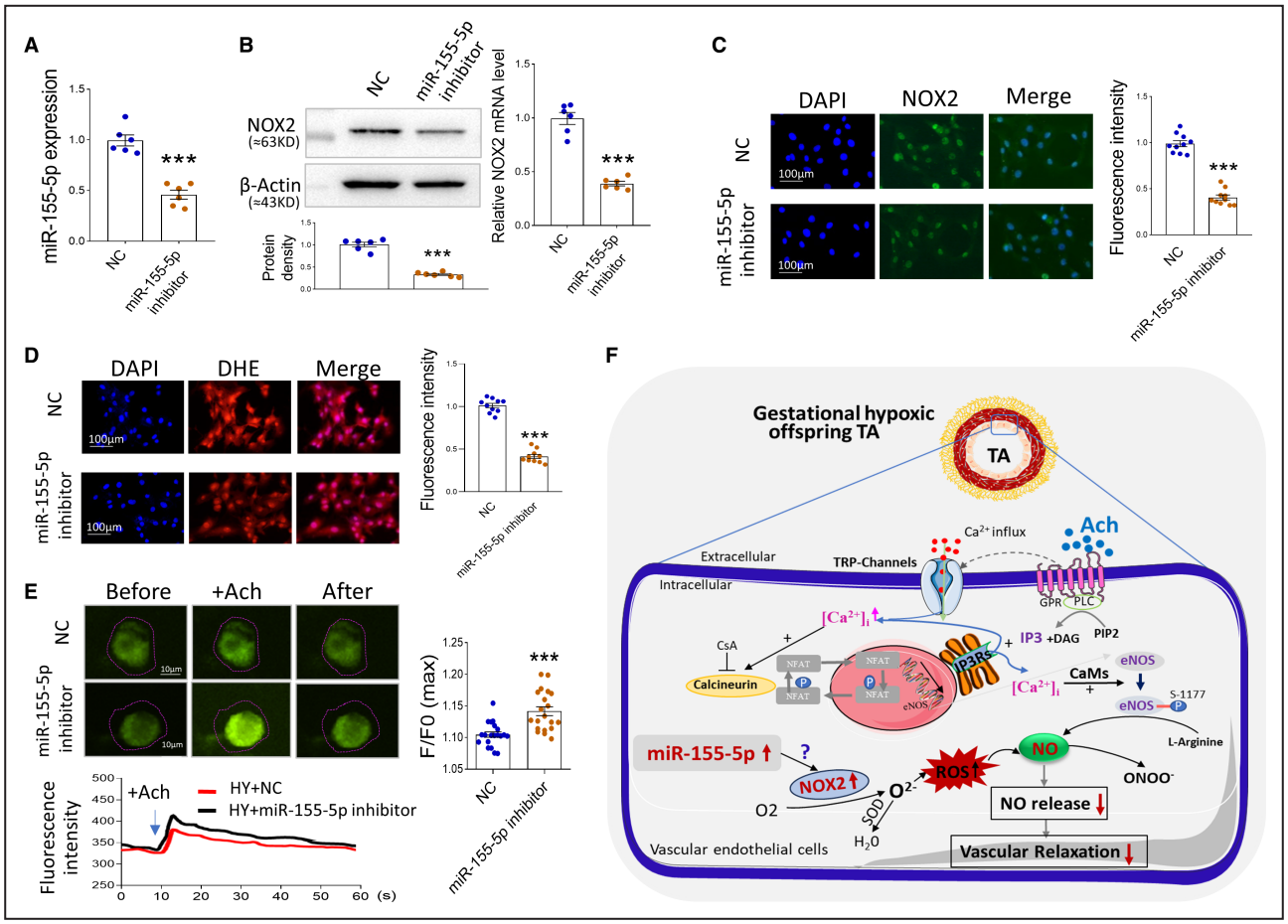

Figure 6. miR-155-5p inhibitor decreased NOX2 expression and ROS level in offspring TA-VECs.
A through **C**, Expression levels of miR-155-5p and NOX2 in TA-VECs after miR-155-5p inhibitor treatment. **D**, Dihydroethidium staining shows ROS levels after miR-155-5p inhibitor treatment. **E**, The transients of acetylcholine-induced NO release after miR-155-5p inhibitor treatment in TA-VECs from gestational hypoxic offspring. **F**, Mechanism diagram of this present study. Results from 3 to 6 independent experiments. Data were presented as means±SEM. Statistical significance was calculated by the Student's *t* test. ***P<0.001. Ach indicates acetylcholine; CaMs, calmodulins; DAG, diglyceride; DHE, dihydroethidium; GPR, G protein-coupled transmembrane receptors; IP3, inositol 1,4,5-trisphosphate; NC, negative control miRNA; NO, nitric oxide; NOX2, NADPH oxidase 2; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; ROS, reactive oxygen species; TA, thoracic aorta; TRP-channels, transient receptor potential Ca^{2+} channels; and VECs, vascular endothelial cells.

elevated levels of miR-155-5p in their blood vessels. This miRNA was associated with an increased expression of NOX2 and was found to indirectly regulate its expression. These groundbreaking findings indicate that the detrimental effects of gestational hypoxia on endothelial function in fetal vasculature can persist into adulthood, providing valuable insights into the development of vascular diseases.

Sexual dimorphism is commonly observed in many diseases, including differences in prevalence, age of onset, course, and severity. Previous studies have indicated that male offspring typically have a higher prevalence of fetal-origin cardiovascular diseases compared with female offspring.[32,33] Research has shown that prenatal hypoxia can have a sex-dependent impact on cardiovascular system in offspring, with male offspring being more susceptible to vascular dysfunction compared with female offspring.[34–36] In our preliminary studies, we also examined the sex-dependent effects of gestational hypoxia on acetylcholine-induced relaxation in fetal TA. The detrimental effects of gestational hypoxia on acetylcholine-induced relaxation were more pronounced in male fetuses. Therefore, this study focused solely on male offspring. We propose that these discrepancies may be attributed to the sexual dimorphism of the placenta. Rodent models have been extensively used to study placental development, and these studies have demonstrated that the growth and development trajectory of the placenta differs between females and males and also show altered adaptive responses to stressful environments. These placental adaptations are likely to depend on the type of stressor, duration, severity, and the window of exposure during development. Kalisch-Smith et al

suggested that perturbations during early placental development may have a greater impact on the viability and growth of the female fetus, whereas those occurring later in gestation may preferentially affect the male fetus.[37,38]

In our previous study, we discovered that although adult offspring exposed to gestational hypoxia did not develop hypertension, their baseline blood pressure values were slightly elevated.[11,12] Currently, there are only a limited number of studies on the effects of gestational hypoxia on offspring vascular endothelial NO synthesis.[13,39–41] He et al discovered that prenatal acute hypoxia impairs acetylcholine-induced vasodilation in the cerebral arteries of fetal sheep. However, their study did not investigate whether these adverse effects on fetal vessels persist after birth.[13] Tang et al established a prenatal hypoxia rat model from gestational day 4 to 20 and found that prenatal hypoxia impairs acetylcholine-mediated vasodilation in the mesenteric vessels of adult offspring, but the specific mechanisms are not thoroughly investigated.[40] The significance of oxygen throughout the entire pregnancy cannot be ignored. The demand for oxygen is not high during the early stages of embryonic and placental formation, but it significantly increases in the mid-late stages of pregnancy for both the mother and the fetus. Clinical intrauterine fetal hypoxia often occurs during this period. This present study established a rat model of hypoxia during mid-late stage pregnancy based on the actual occurrence of clinical intrauterine fetal hypoxia. TA was chosen as the research material because it is an important capacitance vessel responsible for delivering oxygenated blood to various organs and tissues. Additionally, the fetal TA is visible to the naked eye and easy to isolate, making it the only fetal vessel that can be used for vascular tension experiments.

Acetylcholine was used in the present study to examine the ability of TA endothelial cells to synthesize NO. The differences in relaxation induced by acetylcholine between the 2 groups were not apparent until acetylcholine reached a concentration of 10^{-6} mol/L. We believe that the possible reasons for this phenomenon are as follows. Adverse factors during pregnancy usually have a mild impact on the fetus and offspring, often resulting in functional birth defects. Excessive adverse effects often lead to the inability of the fetus and offspring to survive. NO has significant and undeniable physiological and pathological effects in the vascular system. Mild damage to NO synthesis will undoubtedly have a significant adverse impact on the vascular system. Additionally, the vascular wire myograph system is commonly used for measuring drug-induced in vitro vasodilatory capacity. However, due to the limitations of its measurement range and sensitivity, it is difficult to monitor and record the vasodilation induced by physiological concentration drugs in vitro. Therefore, in this present study, we also tested the vasodilatory capacity induced by high concentrations of Acetylcholine (10^{-6}–10^{-4} mol/L), which far exceeded physiological concentrations. Compared with previous studies, this present study has the following advantages: (1) it includes both fetal and adult offspring vascular research, thereby revealing the long-term effects of gestational hypoxia on offspring vascular endothelial NO synthesis through a longitudinal study from fetal to adult offspring vessels; and (2) it investigates gestational hypoxia at both the vascular tissue and the individual endothelial cell level, thereby elucidating the molecular mechanisms of gestational hypoxia-induced impairment of offspring vascular endothelial NO synthesis at multiple levels.

NOX2 is a key component in the generation of oxidative stress within cells. Multiple studies have confirmed that oxidative stress increases the production of oxygen free radicals, which makes NO more susceptible to oxidation and reduces its stability.[23,24] This present study confirms that the decreased ability of NO synthesis in offspring exposed to gestational hypoxia is attributed to an increase in ROS generation mediated by elevated NOX2 expression. Therefore, this study focuses on revealing the mechanism underlying the upregulation of NOX2 expression in the VECs of gestational hypoxic offspring. Through a literature review, we have identified the following reported mechanisms of NOX2 expression regulation: (1) transcription factors such as NF-κB (nuclear factor-κB) and AP-1 (activator protein 1) can directly bind to the promoter region of the NOX2 gene, regulating its transcription and expression[42,43]; (2) various cytokines and growth factors, such as tumor necrosis factor alpha, interleukin-1β, and epidermal growth factor, can indirectly regulate NOX2 gene expression by activating transcription factors like NF-κB and AP-1[44,45]; (3) DNA methyltransferases can inhibit the transcription and expression of NOX2 by methylating the promoter region of the NOX2 gene[43,46]; and (4) miRNA can inhibit the translation and expression of NOX2 by binding to its mRNA.[47,48] In our preliminary research, we explored the mechanisms underlying the upregulation of NOX2 expression from these aspects and found a correlation between the expression of NOX2 in VECs of gestational hypoxic offspring and the expression of miR-155-5p.

Various miRNAs are directly or indirectly involved in regulating NOX2 expression.[31,49,50] For example, Yang et al demonstrated that miR-106b, miR-148b, and miR-204 could enhance heart function after myocardial infarction in mice by targeting NOX2.[51] Jiang et al showed that overexpression of miR-155-5p can increase NOX2 expression and ROS generation in renal tubular epithelial cells.[51] In this present study, we observed that overexpression or inhibition of miR-155-5p in offspring VECs resulted in corresponding changes in NOX2 expression, ROS production, and acetylcholine-induced

NO synthesis. These experimental findings further support the regulation of NOX2 expression in VECs by miR-155-5p. Unfortunately, we did not uncover the mechanisms by which gestational hypoxia leads to increased expression of miR-155-5p in offspring VECs and how elevated miR-155-5p upregulates NOX2 expression. We attempted to elucidate the mechanism by which miR-155-5p upregulates NOX2 expression, including indirect upregulation of specific transcription factors or cytokines that subsequently affect NOX2 expression, but we lack the corresponding experimental data to support these hypotheses. We consider these questions to be complex, intriguing, and worthy of further investigation, which will be the focus of our future research endeavors.

CONCLUSIONS

In conclusion, this present study elucidates the molecular mechanisms underlying impaired endothelial NO synthesis in offspring exposed to gestational hypoxia. It also emphasizes the important role of miRNA in the early development of vascular problems, contributing to the understanding of the long-term adverse effects of gestational hypoxia on offsprings' vascular health. However, there are some limitations and future directions that should be acknowledged. First, this study includes only male offspring, limiting the applicability of the current findings to male children in clinical settings. Future studies should investigate the sex-dependent effects of gestational hypoxia on offspring and explore the underlying mechanisms. Second, this study focuses only on endothelial NO synthesis in the TA and does not evaluate other types of blood vessels. Further research is needed to determine if the impact of gestational hypoxia on NO synthesis exists in the systemic vasculature of offspring. Third, as mentioned previously, this study does not reveal how gestational hypoxia leads to increased expression of miR-155-5p and the related mechanisms of upregulating NOX2. Additionally, gestational hypoxia commonly occurs in the mid to late stages of pregnancy in clinical practice, and animal experiments have demonstrated that the detrimental effects of gestational hypoxia on endothelial function in fetal vasculature can endure into adulthood. Therefore, further clinical observations on individuals exposed to gestational hypoxia are necessary to validate our research findings.

ARTICLE INFORMATION

Received August 3, 2023; accepted December 8, 2023.

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Acknowledgments

We thank Gemma Pharmaceutical Technology Co., Ltd., Shanghai, China for the excellent technical assistance.

Sources of Funding

This work was supported partly by the Ministry of Science and Technology (2019YFA0802600), National Nature and Science Foundation of China (82271724, 82271712, 81873841, 81741024, and 81401244), Suzhou City Wei Sheng Ren Cai (GSWS2019029) program, General Programs of Jiangsu Commission of Health (M2021087), and Nature and Science Foundation of Jiangsu (BK20221243).

Disclosures

None.

Supplemental Material

Figures S1–S3

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