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Prenatal Nicotine Exposure Raises Male Blood Pressure via FTO-Mediated NOX2/ROS Signaling

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Abstract

BACKGROUND: Cigarette smoking/nicotine exposure in pregnancy shows an increased risk of hypertension in offspring, but the mechanisms are unclear. This study tested the hypothesis that m6A RNA hypomethylation epigenetically regulates vascular NADPH oxidase (NOX) and reactive oxygen species (ROS) production, contributing to the fetal programming of a hypertensive phenotype in nicotine-exposed offspring.

METHODS: Pregnant rats were exposed to episodic chronic intermittent nicotine aerosol (CINA) or saline aerosol control from gestational day 4 to day 21, and experiments were performed in 6-month–old adult offspring.

RESULTS: Antenatal CINA exposure augmented Angiotensin II-stimulated blood pressure (BP) response in male, but not female offspring. Moreover, CINA increased vascular NOX2 expression and superoxide production exclusively in male offspring. Inhibition of NOX2 with gp91ds-tat, both *ex vivo* and *in vivo*, mitigated the CINA-induced elevation in superoxide production and BP response. Notably, CINA enhanced the expression of vascular m6A demethylase FTO, while reducing the total vascular m6A abundance and specific m6A methylation of the NOX2 gene. Additionally, *ex-vivo* inhibition of FTO with FB23-2 attenuated CINA-induced increases in vascular NOX2 expression. In vitro experiments using human umbilical vein endothelial cells

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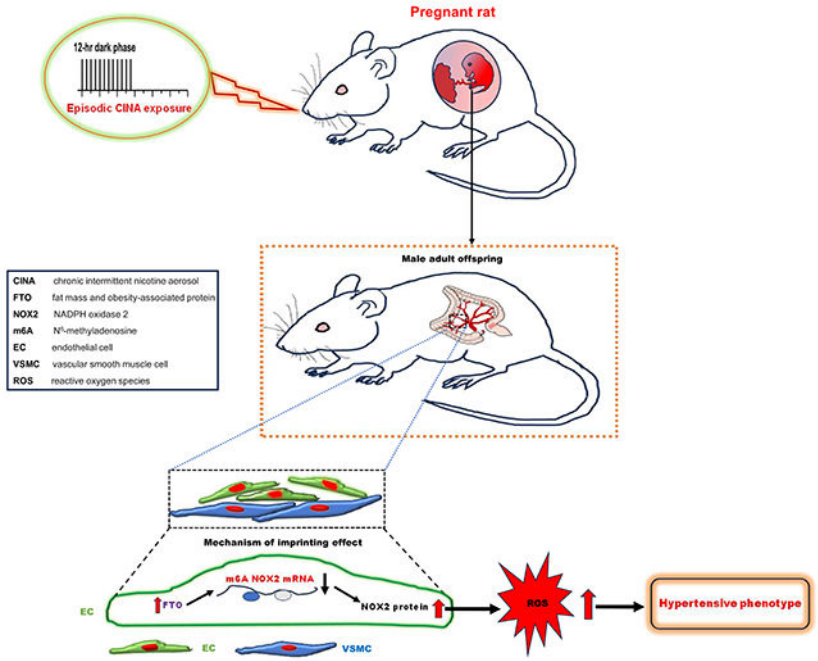
Conflict of interest
The authors declare that they have no conflict of interest.

demonstrated that nicotine dose-dependently upregulated FTO and NOX2 protein abundance, which were reversed by treatment with the FTO inhibitor FB23-2 or FTO knockdown using siRNAs.

CONCLUSIONS: This study uncovers a new mechanism: m6A demethylase FTO-mediated epigenetic upregulation of vascular NOX2 signaling in CINA-induced hypertensive phenotype. This insight could lead to a therapeutic target for preventing and treating developmental hypertension programming.

Graphical Abstract



Keywords

Prenatal nicotine exposure; fetal programming hypertension; m6A demethylase FTO; RNA hypomethylation; NOX2/ROS signaling

Introduction

Maternal cigarette smoking during gestation is a prevalent environmental insult to fetal development [1]. In addition to conventional cigarettes, non-tobacco products like heat-not-burn tobacco heating systems [2,3] and electronic nicotine delivery system (e-cigarette) [4,5] have gained global traction in the last decade. Consequently, nicotine abuse from either traditional smoking or e-cigarette vaping emerges as a mounting health concern in modern life. Cigarette smoking/nicotine exposure significantly escalates cardiovascular disease risk. Both epidemiologic and animal studies indicate that maternal cigarette smoking/nicotine use during pregnancy is associated with an increased risk of hypertension and cardiovascular disease later in life [6–8]. However, the mechanisms underlying antenatal nicotine-induced programming outcomes remain largely unknown, and the specific

pathophysiologic pathways leading to the development of hypertensive phenotype have not yet been identified.

NADPH oxidase (NOX) is a key enzyme involved in the production of reactive oxygen species (ROS) [9]. NOX1, NOX2 and NOX4 are primary membrane-bound catalytic subunits in the vasculature [9]. Increased vascular NOX expression and ROS production have been observed in various animal models of hypertension [10, 11]. Previous studies link NOX-derived ROS to offspring's cardiovascular dysfunction. Our recent studies show perinatal nicotine exposure escalates vascular ROS production, raising blood pressure in adult offspring [12]. However, the causal role of nicotine-induced NOX-derived ROS in hypertensive programming during adulthood, along with underlying molecular mechanisms, remains unclear. Epigenetic modifications (DNA methylation, histone modification, non-coding RNAs) profoundly impact cardiovascular development. Recently, RNA alterations emerged as epigenetic players, particularly N^6-methyladenosine (m6A), a common mRNA modification [13–15]. This reversible m6A RNA methylation is regulated by writers (METTL3, METTL14, WTAP), erasers (FTO, ALKBH5), and readers (YTHDF1-3). Expression levels and potentially post-translational modifications of writers, erasers and readers can constantly sculpt the RNA methylome [16]. m6A regulates pivotal biological processes, including embryonic development [17,18]. Notably, recent studies have demonstrated that RNA modification contribute to paternal hereditary data in high-fat-diet-induced metabolic disorders in offspring [19]. However, m6A's role in vascular NOX expression remains uncharted. It's unclear if nicotine exposure alters m6A levels, influencing epitranscriptomic vascular function in fetal programming of hypertensive phenotype.

In the present study, we employed a clinically relevant rat model of episodic chronic intermittent nicotine aerosol (CINA) inhalation during pregnancy to examine the hypothesis that antenatal nicotine aerosol exposure enhances vascular NOX2-derived ROS through a mechanism of m6A RNA hypomethylation, resulting in the development of a hypertensive phenotype later in life.

Materials and Methods

The authors declare that all supporting data are available within the article (and in the Supplemental Material). The data that support the results of this study were available from the corresponding author upon reasonable request. Besides sections below, the expanded methods and material can be found in the Supplemental Material.

Results

1. Prenatal CINA exposure increases angiotensin II (Ang II)-induced blood pressure (BP) response in adult male offspring

To determine whether fetal nicotine exposure induces programming of hypertensive phenotype in offspring, pregnant rats were exposed to CINA and BP was measured in adult offspring at the age of 6-month-old. As shown in Figure S1, CINA exposure had no effects on the body weight, baseline BP and heart rate (HR) in both male and female

offspring. However, when the offspring were challenged with Ang II (Figure 1 and Figure S2), Ang II produced a dose-dependent increase in arterial BP and a decrease in HR. In male offspring, maternal CINA exposure significantly enhanced Ang II-induced changes of MAP (ΔMAP) (Figure 1A) but not HR (Figure 1C), as compared to the saline control. In contrast to male offspring, Ang II–induced dose-dependent changes of MAP (Figure 1B) and HR (Figure 1D) were not significant differences between the CINA exposed and saline groups in female offspring. Interestingly, there were no significant differences in the norepinephrine (NE)-induced dose-dependent changes of MAP and HR between the group exposed to CINA and the saline group in both adult male and female offspring (Figure S3).

2. Prenatal CINA exposure increases vascular NOX2-derived ROS production and inhibition of NOX2 attenuates the CINA-mediated effects

To explore whether CINA exposure increases vascular ROS production, dihydroethidium (DHE) staining fluorescence was determined in mesenteric arteries (MAs) using confocal microscopy analysis. As shown in Figure 2A and 2B, in male offspring, DHE staining densities (ROS productions) in both vascular smooth muscle cell (VSMC) section and endothelial cell (EC) section were significantly increased in the CINA exposed group as compared with the saline group. The DHE fluorescent signal in the mesenteric artery section was nearly completely eliminated through coincubation with 500 U/mL polyethylene glycol–superoxide dismutase (PEG-SOD), underscoring the specificity of superoxide detection (Figure 2A). In contrast, there were no significant differences of DHE staining densities in both VSMC and EC sections between the CINA exposed group and the saline control group in female offspring (Figure S4). In order to determine the role of vascular NOX2 in CINA-mediated ROS in male offspring, we measured the ROS production after the treatment with a selective NOX2 inhibitor peptide, gp91ds-tat [20, 21]. As shown in Figure 2A and 2B, gp91ds-tat had no effects on the control group, but blocked CINA-induced increases in ROS productions in both VSMC and EC sections.

To determine whether the enhanced NOX2-drived ROS plays a key role in Ang II-induced BP response in CINA male offspring, we examined the effect of NOX2 inhibitor gp91ds-tat on Ang II-induced BP response. As shown in Figure 2C–E, gp91ds-tat had no significant effects on Ang II-induced BP responses in the control offspring (Figure 2C), but significantly attenuated Ang II-induced BP responses in CINA-exposed offspring (Figure 2D). However, the treatment with NOX2 inhibitor did not completely eliminate the differences of Ang II-induced BP responses between the saline control and CINA-exposed groups (Figure 2E).

3. Prenatal CINA exposure enhances vascular NOX2 protein abundance in adult male offspring

The protein abundance of NOX1, NOX2 and NOX4 subunits in MAs from offspring was examined by Western blotting analysis. As shown in Figure 3, antenatal CINA exposure increased vascular NOX2 protein abundance in male (Figure 3A), but not in female (Figure 3B) offspring, as compared to the saline control. In contrast, mRNA abundance of NOX2 was not altered by CINA exposure in both male (Figure 3C) and female (Figure 3D) offspring. In addition, NOX2 immunofluorescent staining in MAs showed that NOX2 gene

was expressed in both VSMC and endothelial layers (Figure 3E). Antenatal CINA exposure significantly enhanced NOX2 fluorescent densities in both endothelial (Figure 3F, left panel) and VSMC (Figure 3F, right panel) sections of MAs in male offspring. In contrast to NOX2, CINA exposure had no significant effects on NOX1 (Figure S5A) and NOX4 (Figure S5B) protein expression.

4. Effects of prenatal CINA exposure on DNA methylation/RNA methylation and their role in the regulation of vascular NOX2 expression

In this study, we measured CpG methylation levels at NOX2 gene promoter to see whether NOX2 over-expression is associated with changes of the promoter DNA methylation. As shown in Figure S6, there were no significant differences of CpG methylation levels at NOX2 promoter regions between the CINA-exposed group and saline group in either male (Figure S6B) or female (Figure S6C) offspring.

We further determined whether antenatal CINA exposure altered RNA methylation (m6A methylation) levels. As shown in Figure 4A, mRNA levels of m6A methylation-associated enzymes were measured in MAs in both male (Figure 4A, left panel) and female (Figure 4A, right panel) offspring. CINA exposure had no effects on ALKBH5, METTL3, METTL14, and WTAP gene expressions in both male and female offspring, but selectively enhanced vascular FTO mRNA levels in male, but not in female offspring, as compared to the saline control (Figure 4A). The Western blot analysis showed that CINA exposure increased FTO protein abundance in male (Figure 4B), but not in female (Figure 4C) offspring. In addition, the immunofluorescent staining (Figure 4D) showed that FTO gene was expressed in both VSMC and endothelium sections of MAs. CINA exposure enhanced the immunofluorescent densities of FTO expression in both VSMC (Figure 4D, upper panel) and endothelium (Figure 4D, lower panel) layers in male offspring, as compared to the saline controls.

As shown in Figure 4E, antenatal CINA exposure significantly decreased vascular levels of total m6A methylation in male (Figure 4E, left panel), but not in female (Figure 4E, right panel) offspring. From the sequence-based RNA adenosine methylation site predictor (SRAMP) (http://www.cuilab.cn/sramp) [22], two potential high confidence m6A sites (site 1092 and site 397) are identified in the protein coding sequence (CDS) region of rat NOX2 mRNA (NM_023965.1) (Figure S7). To see whether the NOX2 over-expression was regulated by m6A modification, the m6A levels in the NOX2 mRNA was measured by immunoprecipitation of m6A-modified RNA (MeRIP) followed by RT-qPCR (MeRIP-qPCR). MeRIP-qPCR analysis (Figure 4F) showed that CINA exposure selectively attenuated vascular m6A levels at the site of 1902 but not 397 in the CDS region of NOX2, as compared to the saline group of male offspring.

5. The causal role of FTO in the regulation of vascular NOX2 expression *ex vivo* in prenatal CINA exposed model and *in vitro* in nicotine exposed human umbilical vein endothelial cells (HUVECs) model.

To determine whether inhibition of m6A demethylase FTO attenuates vascular NOX2 expression, MAs isolated from both saline control and CINA-exposed male offspring were treated with a selective FTO inhibitor FB23-2 [20,21] *ex vivo* and the NOX2 protein

abundance was measured by Western blotting analysis. As shown in Figure 5A, treatment with vehicle control did not affect CINA exposure-mediated NOX2 over-expression. However, treatment with FB23-2 eliminated the differences of vascular NOX2 protein expression between the saline control and CINA-exposed groups (Figure 5B).

To investigate the direct effect of nicotine exposure on NOX2 and FTO expression, human umbilical vein endothelial cells (HUVECs) were treated with different clinically relevant doses of nicotine *in vitro*. Nicotine treatment produced dose-dependent increases in both FTO and NOX2 protein expression in HUVECs. As shown in Figure 6A–6C, protein levels of FTO were not changed by the nicotine exposure at the concentrations of 10^{-9} M and 10^{-8} M (Figure 6A, left panel), but were significantly enhanced as the nicotine concentration was reached at 10^{-7} M (Figure 6A, middle panel) and 10^{-6} M (Figure 6A, right panel). Similarly, nicotine exposure at the concentrations 10^{-9} M and 10^{-8} M (Figure 6B, left panel) had no effect on NOX2 protein expression, but significantly enhanced the protein levels of NOX2 at the concentrations of 10^{-7} M (Figure 6B, middle panel) and 10^{-6} M (Figure 6B, right panel). Furthermore, the nicotine-mediated NOX2 over-expression was positively related with the changes of FTO expression at the concentrations of 10^{-7} M (Figure 6C, left panel) and 10^{-6} M (Figure 6C, right panel).

To explore whether FTO directly regulated NOX2 protein expression, a FTO pharmacologic inhibitor FB23-2 was treated in HUVECs *in vitro* model. As shown in Figure 6D, treatment with FB23-2 (from 0.5 to 10 μM) produced dose-dependent decreases in NOX2 protein expression as compared to the vehicle (DMSO) controls. FB23-2 treatment (from 0.1 to 10 μM) also induced dose-dependent inhibition of FTO protein expression (Figure S8). Furthermore, FB23-2 (5 μM) inhibited the nicotine-mediated NOX2 over-expression and eliminated the differences of NOX2 expression between the control and nicotine exposed groups (Figure 6E). Similarly, knockdown of FTO using its siRNA significantly inhibited NOX2 expression (Figure 6F, left panel) and FTO expression (Figure 6F, right panel) in HUVECs in vitro model. As shown in Figure 6F, nicotine exposure significantly enhanced both NOX2 expression (Figure 6F, left panel) and FTO expression (Figure 6F, right panel) which were blocked by siRNA pretreatment. Pretreatment with the siRNA eliminated the differences of both NOX2 and FTO protein expression between the saline control and nicotine exposed groups.

Discussion

In this study, we employed a pregnant rat model of nicotine aerosol inhalation, closely resembling human smoking patterns in terms of route and timing, to investigate the epigenomic mechanisms driving this programming effect. The major findings are: 1) Antenatal CINA exposure led to a sex-dependent increase in Ang II-induced BP responses in adult male offspring; 2) CINA exposure selectively enhanced vascular NOX2 abundance and ROS production, and inhibition of NOX2 using its inhibitor gp91ds-tat attenuated Ang II-induced BP response in adult males; 3) Antenatal CINA exposure didn't effect CpG methylation in the NOX2 gene promoter, but it did reduce m6A levels at the 1902 site (but not at 397) within the NOX2 gene's coding sequence (CDS) region; 4) Changes in m6A RNA methylation corresponded with increased FTO gene expression; 5) Silencing FTO

expression, both ex vivo and in vitro, directly suppressed NOX2 expression and abolished the differences in vascular NOX2 expression between control and CINA-exposed groups. Collectively, our study provides the first evidence that the m6A demethylase FTO-mediated epigenetic increase in vascular NOX2 signaling significantly contributes to the nicotine-induced fetal programming of the adult hypertensive phenotype.

In the present study, we developed a pregnant rat model of chronic intermittent nicotine aerosol (CINA) exposure to replicate human smokers' daily nicotine fluctuations. Our data (Figure S9) showed that our CINA exposed pregnant rats exhibited blood nicotine and cotinine levels mirroring those of human smokers and e-cigarette users [2,5,23], bolstering the clinical relevance of our animal model. Using this model, we assessed adult offspring BP responses. Our data show that arterial BP responses to Ang II stimulation are significantly enhanced in CINA exposed male but not female offspring as compared to the control offspring, which is consistent to previous studies showing an increased BP response in perinatal nicotine exposed rat model [8]. These findings suggest that nicotine exposure either from inhaled aerosols or subcutaneous delivery during pregnancy induces a sex-dependent fetal programming of adult hypertensive phenotypes.

Blood pressure (BP) regulation involves central neural and peripheral vascular resistance pathways. We showed that Ang II produced a dose-dependent increase in BP, associated with a decrease in the heart rate in both CINA and control groups. Notably, CINA exposure significantly enhanced Ang II-induced BP without affecting the heart rate, suggesting intact arterial baroreflex. In contrast to the effect on arterial baroreflex, previous studies have demonstrated that changes of peripheral vascular resistance and vascular tone play an important role in prenatal nicotine exposure-mediated hypertensive response in offspring [8,12]. Reactive oxygen species (ROS) are key mediators in the regulation of vascular reactivity and vascular tone [24, 25]. Excessive ROS contribute to Ang II-induced hypertension [24,26]. Our previous studies have demonstrated that perinatal nicotine exposure develops a hypertensive phenotype and exaggerated ROS production [8,12,27]. Similarly, this study also showed that CINA exposure caused a sex-dependent increase in ROS productions in male offspring's vasculature. It is well known that NOX is a major enzyme in the regulation of ROS productions in vasculatures [11,26, 28]. A recent study demonstrates that e-cigarette vaping exposure increase vascular, oxidative stress via a NOX2-dependent mechanism, resulting in endothelial dysfunction in chronic smokers and increased blood pressure in experimental animals [29]. Our present study showed that prenatal CINA exposure selectively enhanced NOX2 but not NOX1 and NOX4 protein abundance in the vasculature of adult male offspring. Furthermore, treatment with a NOX2 inhibitor, gp91ds-tat [20] eliminated the difference of ROS productions between the CINA exposed group and saline control group and attenuated Ang II-induced BP response in CINA exposed adult male offspring. However, the NOX2 inhibitor did not completely eliminate the differences in Ang II-induced BP responses between the saline control and CINA-exposed groups. These findings suggest that both NOX2/ROS-dependent and -independent signaling pathways contribute to antenatal nicotine-induced hypertension. Indeed, previous studies have shown that, in addition to changes in NOX2/ROS signaling, antennal nicotine exposure also alters the vascular Ang II receptor-mediated signaling pathway, resulting in an increased Ang II-induced BP response in offspring [8].

Our findings indicate that antenatal CINA exposure led to a sex-dependent rise in vascular NOX2 protein expression in adult male offspring, implying epigenetic regulatory involvement in NOX2 programming. While DNA methylation is a key gene expression regulator, we observed that CINA exposure didn't modify CpG methylation patterns at the NOX2 promoter region. This suggests that DNA methylation might not contribute to CINA-induced NOX2 overexpression. Of interesting, our data indicated that antenatal CINA exposure increased vascular NOX2 protein expression without significantly affecting mRNA levels. This suggests a potential post-transcriptional RNA modification role in NOX2 protein regulation. Recently, RNA modifications via changes of RNA methylation pattern have been recognized as a new layer of epigenetic regulation, among which m6A is the most prevalent internal post-transcriptional modification on mammalian mRNA [13–15]. RNA methylation has been implicated in various cardiovascular diseases [16]. The present findings that CINA exposure decreased total m6A RNA levels and selectively attenuated vascular m6A levels at the site of 1902 but not 397 in NOX2 gene's protein coding sequence (CDS) region, suggest that antenatal CINA exposure induces a sex-dependent vascular hypomethylation of NOX2 mRNA in adult male offspring. mRNA methylation by means of m6A is catalyzed by demethylases (FTO and ALKBH5) and methyltransferase complex (METTL3, METTL14 and WTAP) [13–15]. We showed that CINA exposure selectively increased FTO, but not METTL3, METTL14, WTAP and ALKBH5 protein abundance in the vasculature, suggesting that FTO is a key regulator for CINA exposure-mediated m6A RNA demethylation. Furthermore, we have demonstrated that FTO and NOX2 are expressed in both VSM cells and endothelial cells in mesenteric arteries. The finding that treatment of nicotine *in vitro* in HUVEC culture model caused a doses-dependent increase in both FTO and NOX2 expression, suggests that CINA exposure-mediated changes of both FTO and NOX2 expression are directly regulated by nicotine. Of importance, both *ex vivo* and *in vitro* inhibition of FTO using an inhibitor FB23-2 or FTO siRNAs reduced NOX2 expression and eliminated the differences of vascular NOX2 expression between control and CINA-exposed groups. These findings highlight a causal role of FTO-mediated hypomethylation of m6A in the upregulation of NOX2 expression in response to antenatal CINA exposure.

Our previous studies have demonstrated that both prenatal nicotine and e-cigarette exposure lead to fetal growth restriction and a notable decrease in body weights up to 30 days after birth [7,30]. However, in adult offspring, no significant differences in body weights exist between the control and prenatal nicotine-exposed groups [7]. In the current study, while we did not measure birth weights or postnatal body weights, we identified no significant differences in body weights among adult offspring in the control and CINA-exposed groups (Figure S1). These findings suggest the presence of a catch-up growth adaptation mechanism in response to prenatal nicotine exposure in the offspring. Human studies [31,32] suggest significant sex-specific differences in the effects of nicotine on cardiovascular health between males and females. While some studies indicate that nicotine could exert a more pronounced detrimental influence on cardiovascular well-being in males in comparison to females, the findings are not always consistent across all studies. Such variations may depend on factors like age, lifestyle, and others. The present study demonstrated that CINA exposure enhanced Ang II-induced BP response in adult male but not female offspring, revealing a sex dimorphism in fetal programming of hypertensive phenotype. This is

consistent with previous studies showing male-specific alterations in vascular reactivity and BP response due to prenatal nicotine exposure [8]. While the precise mechanisms driving this sex disparity in antenatal CINA-mediated BP response remain unclear, potential factors include inherent sex chromosome distinctions, diverse gene expression in male and female vasculature, or varying fetal reactions to nicotine exposure based on sex. Notably, previous studies suggest that sex hormones' involvement in cardiovascular disease programming in adult offspring [33]. This sets the stage for future exploration into the molecular mechanisms contributing to sex differences in cardiac dysfunction development among offspring exposed to maternal nicotine.

Perspective

Epidemiologic studies indicate that cigarette smoking during pregnancy increases the risk of hypertensive outcomes in offspring due to nicotine exposure [6]. Indeed, animal studies in ours [8,27] and others [34] support this link, demonstrating that fetal nicotine exposure leads to adult hypertension. This study introduces a novel approach using a unique pregnant rat model of chronic intermittent nicotine aerosol (CINA) exposure that mimics smokers' nicotine levels. The present study reaffirms prenatal nicotine exposure's role in programming adult hypertension and uncovers an epigenetic mechanism. Specifically, the current study reveals that antenatal CINA exposure epigenetically influences the vascular NOX2 gene via FTO-dependent M6A RNA hypomethylation, leading to excess ROS production, consequential development of hypertensive phenotype in adult offspring in sex-dependent manner. This study underscores the growing importance of epigenetic mechanisms in public health research. Unlike genomic regulation, epigenetic processes are reversible, offering opportunities for postnatal intervention. Based on our current findings that inhibition of FTO/NOX2 signaling pathway rescued the antenatal CINA exposure-mediated NOX2 over-expression and ROS over-production leading to attenuation of the hypertensive response in offspring, these findings could provide insightful molecular targets and therapeutic strategies for prevention or treatment of the fetal programming of hypertension associated with cigarette smoking/nicotine abuse during pregnancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Nonstandard Abbreviations and Acronyms

NOX	NADPH oxidase
ROS	reactive oxygen species
m6A	N^6-methyladenosine

METTL3	methyltransferase-like 3
METTL14	methyltransferase-like 14
WTAP	Wilms' tumor 1-associating protein
FTO	fat mass and obesity-associated protein
ALKBH5	ALKB homolog 5
CINA	chronic intermittent nicotine aerosol
Ang II	angiotensin II
BP	blood pressure
ΔMAP	changes of mean arterial pressure
HR	heart rate
DHE	dihydroethidium
VSMC	vascular smooth muscle cell
EC	endothelial cell
CDS	coding sequence
HUVECs	human umbilical vein endothelial cells
DAPI	4′,6-diamidino-2-phenylindole

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Novelty and Relevance

What Is New?

The present study introduces novel evidence that antenatal CINA exposure triggers epigenetic programming of vascular NOX2 gene and ROS signaling, leading to sex-specific adult hypertensive phenotypes.

The study uniquely identifies m6A demethylase FTO-mediated RNA hypomethylation as a key mechanism driving NOX2/ROS upregulation.

Inhibiting FTO/NOX2 with pharmacological inhibitors and siRNA mitigates prenatal CINA's adverse effects, reducing NOX2-related ROS and blood pressure response.

These findings provide an innovative link between m6A RNA modification and hypertension programming from prenatal nicotine exposure.

What Is Relevant?

This study introduces a clinically relevant pregnant rat model of chronic intermittent nicotine aerosol (CINA) exposure, closely mimicking chronic smokers' patterns. The model's findings have strong clinical implications, highlighting the therapeutic potential of addressing FTO-mediated m6A RNA hypomethylation in the vascular NOX2 gene/ROS signaling pathway. By uncovering these molecular mechanisms, the research opens avenues for targeted interventions that could substantially influence the prevention and treatment of hypertension-related issues stemming from maternal smoking or nicotine exposure during pregnancy.

Clinical/Pathophysiological Implications

Both clinical and epidemiological investigations have linked maternal smoking during pregnancy with a heightened risk of offspring developing hypertension in later life. This study not only confirms these clinical findings but also offers fresh insights into the epigenetic mechanisms governing nicotine-induced fetal programming of adult hypertension. Better understanding of the role of FTO-mediated m6A RNA hypomethylation in regulation of vascular NOX2 gene/ROS signaling opens up new avenues for translational research and potential therapeutic approaches in addressing hypertension resulting from fetal nicotine exposure.

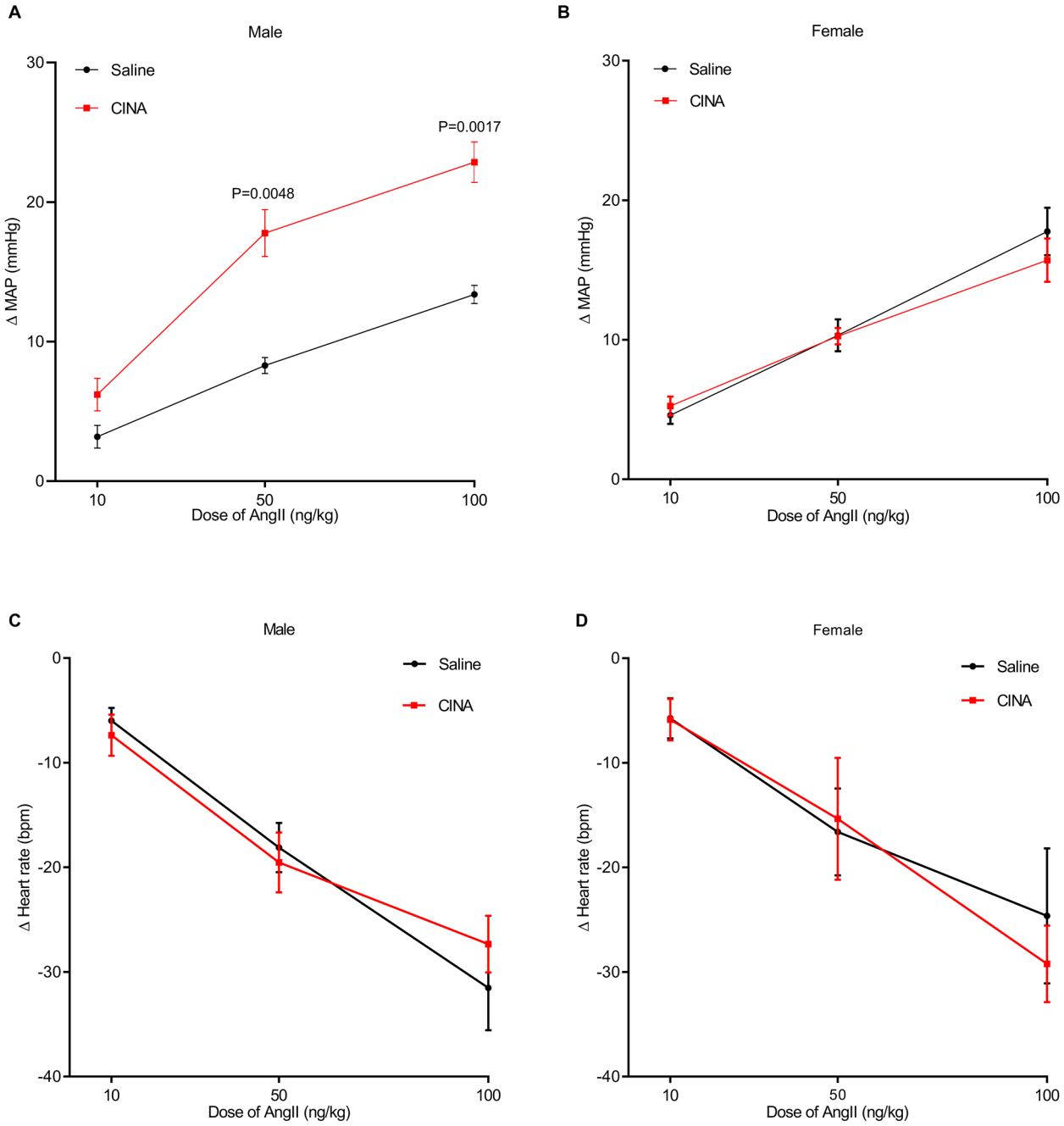

Figure 1. Effects of prenatal CINA on Ang II-induced arterial blood pressure (BP) responses in adult offspring.

A-D, Quantitative data for change (Δ) in mean arterial pressure (MAP) (**A-B**) and heart rate (**C-D**) were determined in adult male and female offspring after intravenous bolus doses of Ang II (10–100 ng/kg) administration. Data were presented as means ± SEM and analyzed by two-way repeated-measures of variance (ANOVA) followed by Bonferroni post-tests. n=6 animals.

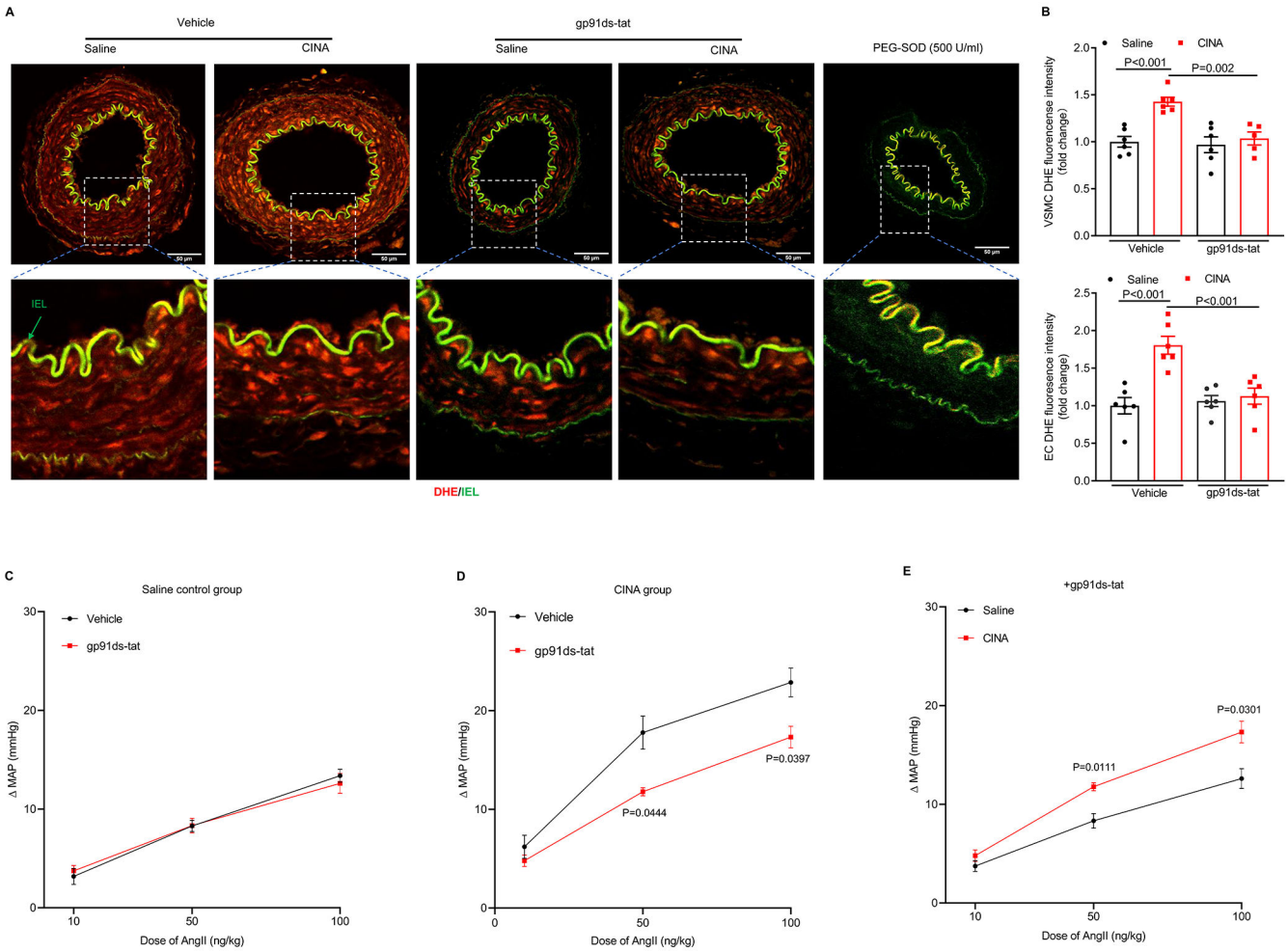

Figure 2. Effects of prenatal CINA on vascular ROS production and the role of vascular NOX2-derived ROS in Ang II-induced BP responses in male offspring.

Vascular superoxide production was measured by dihydroethidium (DHE) staining in mesenteric artery rings isolated from saline control and CINA male offspring in the absence (vehicle) and presence of 50 μmol/L gp91ds-tat. **A**, the representative images showing DHE fluorescence (red color) staining and specificity for superoxide was determined by polyethylene glycol-superoxide dismutase (PEG-SOD, 500 U/mL), IEL indicates internal elastic lamina (green color). **B**, bar graphs showing the quantitative data (means ± SEM) of DHE fluorescence intensity in both vascular smooth muscle cell (VSMC) layer and endothelial cell (EC) layer. **C** and **D**, the Ang II-induced changes (Δ) in mean arterial blood pressure (MAP) in the absence and presence of 1.2 mg/kg gp91ds-tat were measured in the saline (**C**) and CINA-exposed male offspring (**D**). **E**, in the presence of gp91ds-tat, the Ang II-induced ΔMAP in both saline and CINA-exposed offspring. Data were presented as means ± SEM and analyzed by two-way repeated-measures of variance (ANOVA) followed by Bonferroni post-tests. gp91ds-tat vs vehicle, or CINA vs saline control. n=6 animals.

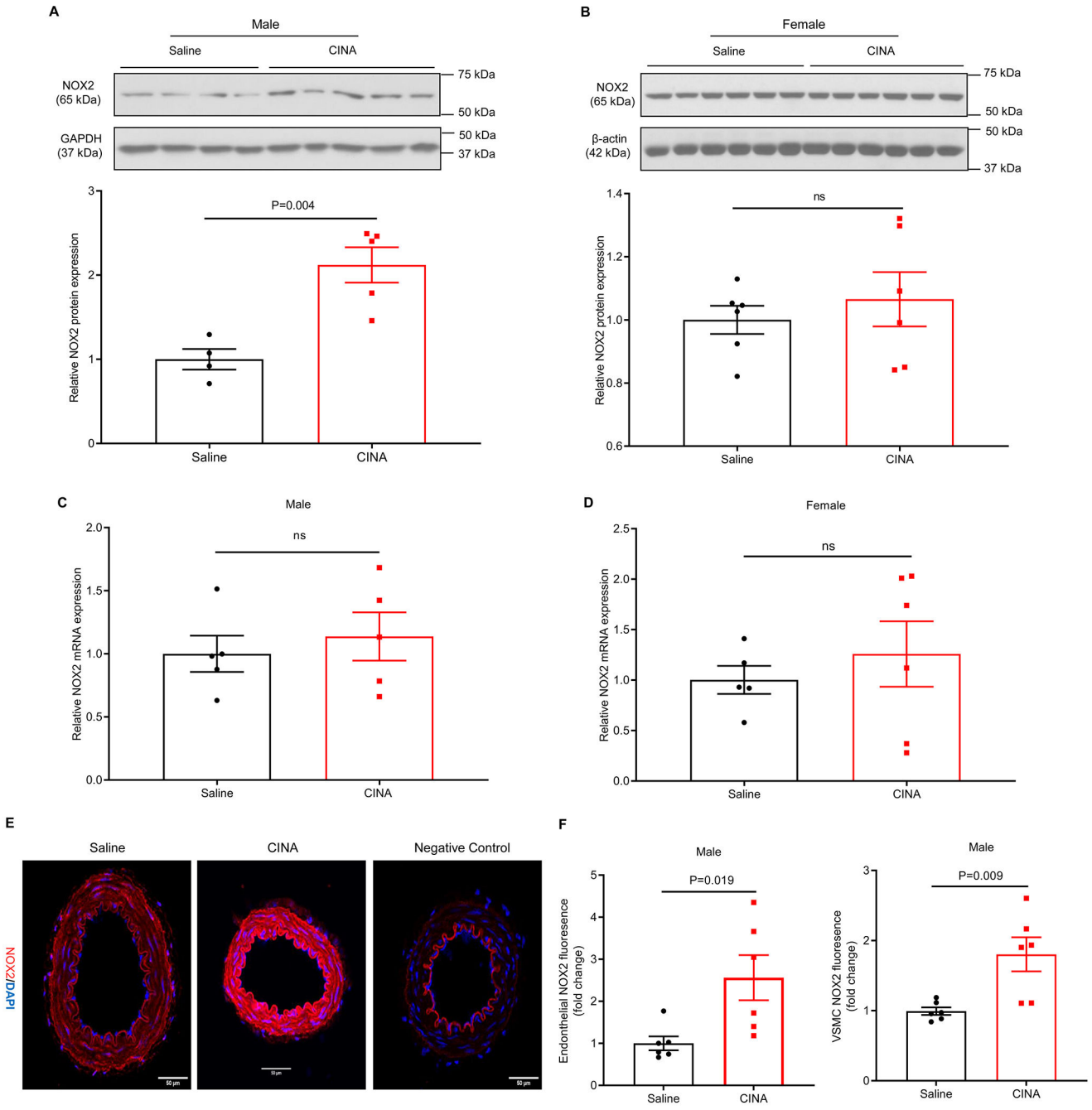

Figure 3. Effect of prenatal CINA on vascular NOX2 gene expression and protein abundances in adult offspring.

Mesenteric arteries (MAs) were isolated from both saline and CINA-exposed adult offspring. **A** and **B**, NOX2 protein abundances were determined by Western blot analysis in both male (**A**) and female (**B**) offspring. **C** and **D**, The mRNA levels of NOX2 were determine by RT-PCR analysis in both male (**C**) and female (**D**) offspring. **E**, Showing representative immunofluorescence images of MA rings in male offspring. NOX2 (red color); Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI) (blue color). Scale

bars: 50 μm. **F**, The fluorescence intensities of NOX2 in both endothelial layer (**F**, **left panel**) and vascular smooth muscle cell (VSMC) layer (**F**, **right panel**) were quantified and expressed as fold change relative to saline control group. Data were presented as means ± SEM and statistical analysis was performed via unpaired student t-tests. ns indicates no significance (P>0.05), saline control vs. CINA, n=4~6 animals.

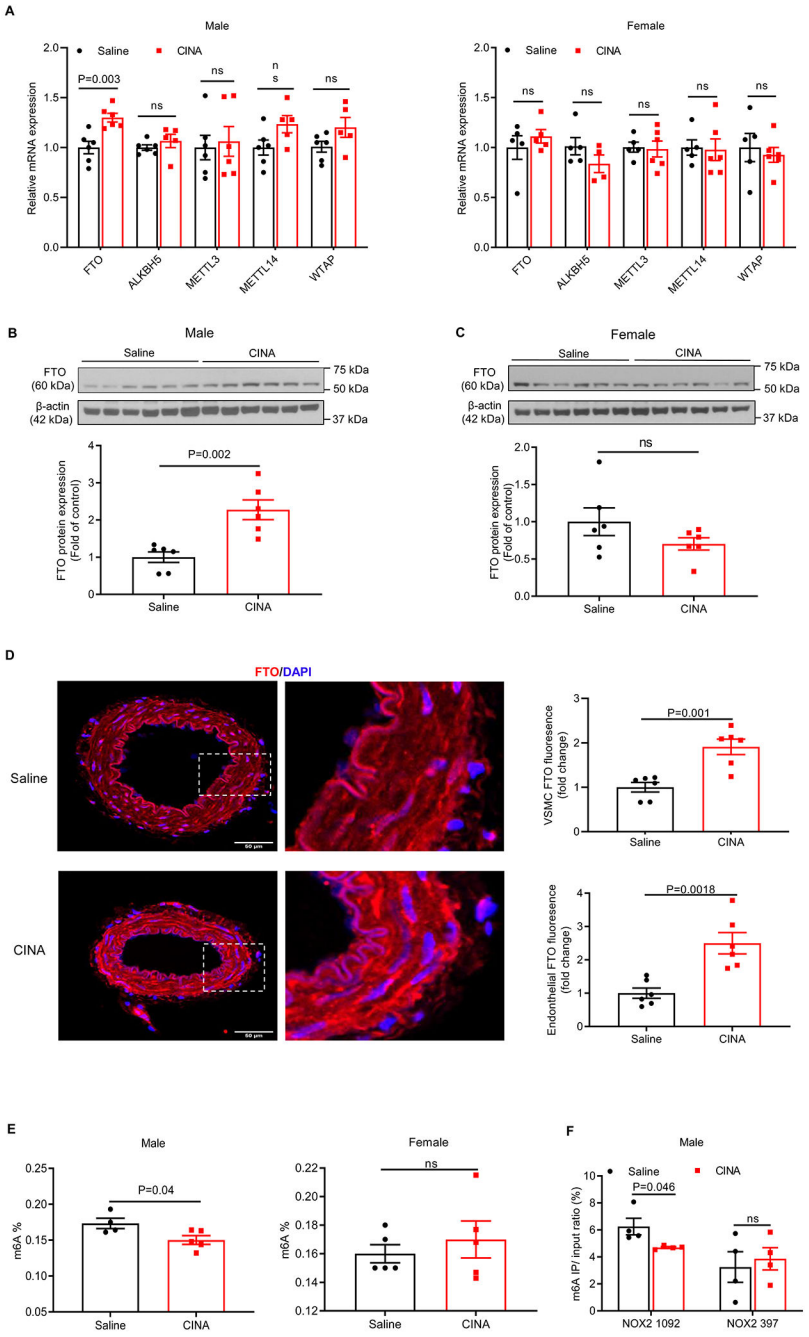

Figure 4. Effect of prenatal CINA on m6A RNA methylation levels in offspring vasculatures.

Mesenteric arteries (MAs) were isolated from both saline and CINA-exposed adult offspring. **A**, The relative mRNA levels of m6A methylation-associated enzymes including FTO, ALKBH5, METTL3, METTL14, and WTAP in MAs were determined by RT-PCR analysis in both male (**left panel**) and female (**right panel**) offspring, n=4~6 animals. **B** and **C**, The protein abundances of FTO in both male (**B**) and female (**C**) offspring MAs were determined by Western blot analysis, n=6 animals. **D**, The localization and expression pattern of FTO in both VSMC and endothelial layers were determined by

Immunofluorescence analysis in saline and CINA-exposed male offspring, FTO (red color); Nuclei were labeled with DAPI (blue color); Scale bars: 50 μm, n=6 animals. **E**, The total m6A RNA methylation levels of MAs in both male (**E**, **left panel**) and female (**E**, **right panel**) offspring were determined by m6A RNA methylation quantification kits as described in the Methods Section, n=6 animals. **F**, The specific m6A methylation levels at NOX2 mRNA of male offspring MAs were determined by MeRIP-qPCR analysis, n=4 pools/group, 3 rats/pool for MeRIP-qPCR experiment. Data were presented as means ± SEM and statistical analysis was performed by unpaired student t-tests. ns indicates no significance (P>0.05).

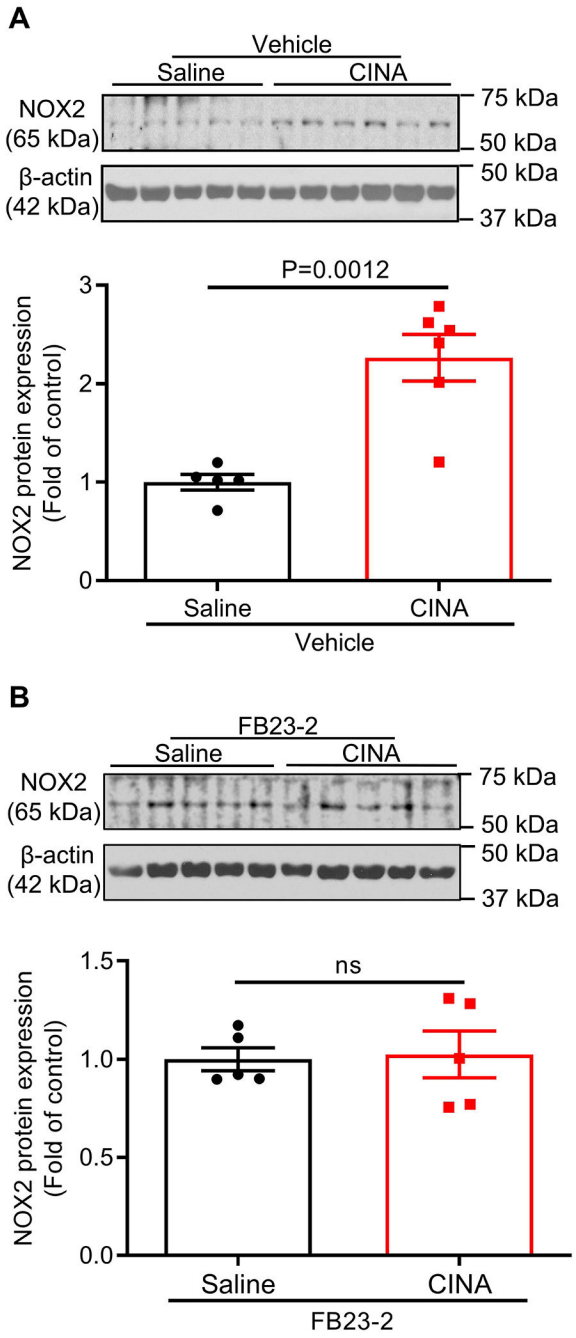

Figure 5. Effect of *ex vivo* treatment of FTO inhibitor on CINA exposure-mediated NOX2 protein expression in offspring vasculatures.

Mesenteric arteries (MAs) were freshly isolated from both saline and CINA-exposed adult male offspring. The MA rings were ex vivo cultured in DMEM F-12 culture medium in a culture incubator at 5% CO_2 and 37°C in the presence of FTO inhibitor FB23-2 (10 μmol/L) or vehicle. **A** and **B**, After 48 hours treatment, NOX2 protein levels without (**A**) and with (**B**) FB23-2 treatment were then determined by Western blot analysis. Data were presented as means ± SEM and statistical analysis was performed by unpaired student t-tests. ns indicates no significance (P>0.05), n=5~6 animals.

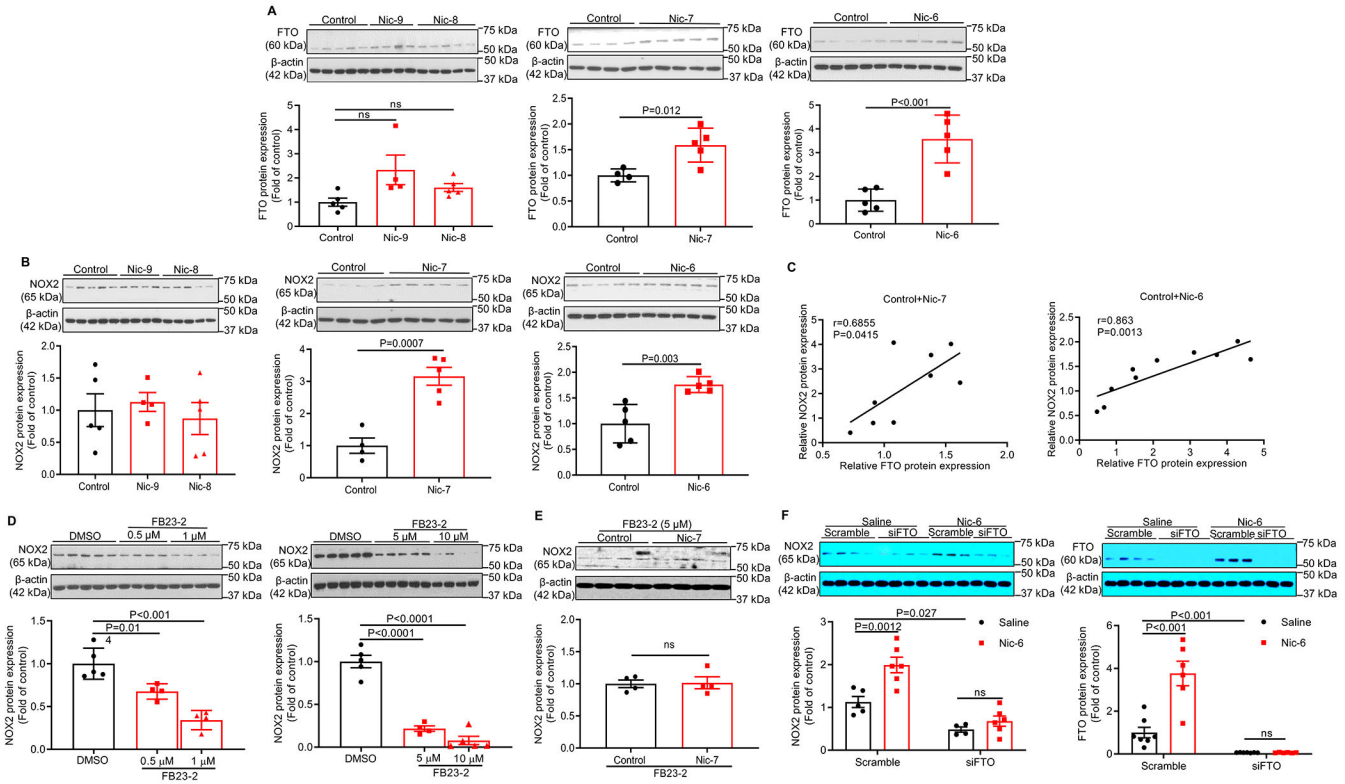

Figure 6. The direct effect of nicotine on FTO and NOX2 expression and the role of FTO in nicotine-mediated NOX2 over-expression in vitro cultured HUVECs.

Commercial Human umbilical vein endothelial cells (HUVECs) were treated with different concentrations of nicotine (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} μM). After 48 hours treatment, the protein levels of FTO and NOX2 in HUVECs were determined by Western blot analysis. **A** and **B**, Nicotine concentration-dependently increases FTO protein levels (**A**) and NOX2 protein levels (**B**) in the cultured HUVECs. **C**, At the concentration of 10^{-7} (**C, left panel**) and 10^{-6} (**C, right panel**) μM, nicotine-mediated changes of NOX2 expression were positively correlated with the changes of FTO in the cultured HUVECs. **D**, Treatment with FTO inhibitor, FB23-2 (0.5, 1, 5, 10 μM) for 24 hours produced concentration-dependent decreases in NOX2 expression in the cultured HUVECs. **E**, Pretreatment with FB23-2 (5 μM) for 24 hours, there was no differences of NOX2 protein abundance between control and nicotine (10^{-7} M)-exposed groups in the cultured HUVECs. **F**, Pretreatment with FTO siRNA for 24 hours attenuated the protein abundances of NOX2 (**F**, **left panel**) and FTO (**F**, **right panel**) as compared to the scramble controls. In addition, knock-down of FTO with the siRNA eliminated the differences of NOX2 (**F**, **left panel**) and FTO (**F**, **right panel**) protein expression between the control and nicotine (10^{-6} M)-exposed groups in HUVECs. All data were presented as means ± SEM and statistical analysis was performed by unpaired student t-test (**A, B** and **C**) and pearson's correlation coefficient (**C**) or one-way ANOWA followed by Bonferroni multiple comparison test (**D** and **F**). ns indicates no significance (P>0.05), n=4~6.