Perinatal Nicotine Exposure Increases Angiotensin II Receptor-Mediated Vascular Contractility in Adult Offspring

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Abstract

Previous studies have reported that perinatal nicotine exposure causes development of hypertensive phenotype in adult offspring.

Aims: The present study was to determine whether perinatal nicotine exposure causes an epigenetic programming of vascular Angiotensin II receptors (ATRs) and their-mediated signaling pathway leading to heightened vascular contraction in adult offspring.

Main methods: Nicotine was administered to pregnant rats via subcutaneous osmotic minipumps from day 4 of gestation to day 10 after birth. The experiments were conducted at 5 months of age of male offspring.

Key Findings: Nicotine treatment enhanced Angitension II (Ang II)-induced vasoconstriction and 20-kDa myosin light chain phosphorylation (MLC20-P) levels. In addition, the ratio of Ang II-induced tension/MLC-P was also significantly increased in nicotine-treated group compared with the saline group. Nicotine-mediated enhanced constrictions were not directly dependent on the changes of [Ca2+]i concentrations but dependent on Ca2+ sensitivity. Perinatal nicotine treatment significantly enhanced vascular ATR type 1a (AT1aR) but not AT1bR mRNA levels in adult rat offspring, which was associated with selective decreases in DNA methylation at AT1aR promoter. Contrast to the effect on AT1aR, nicotine decreased the mRNA levels of vascular AT2R gene, which was associated with selective increases in DNA methylation at AT2R promoter.

Significance: Our results indicated that perinatal nicotine exposure caused an epigenetic programming of vascular ATRs and their-mediated signaling pathways, and suggested that differential regulation of AT1R/AT2R gene expression through DNA methylation mechanism may be involved in nicotine-induced heightened vasoconstriction and development of hypertensive phenotype in adulthood.

Introduction

Maternal cigarette smoking is the single most widespread perinatal insult in the world [1]. Epidemiological studies have shown that maternal smoking is associated with increased risk of cardiovascular disease in offspring later in life [1–3]. As one of the major active components in cigarette smoking, nicotine readily crosses the placenta and produces higher nicotine concentrations in the fetal circulation than that experienced by the mother [4]. The heightened nicotine concentration may contribute to the maternal cigarette smoking-induced developmental programming of cardiovascular dysfunction in offspring. Indeed, nicotine use during pregnancy can cause cardiovascular disorders and hypertension of offspring in different animal models [5–7]. Our recent studies have demonstrated that perinatal nicotine exposure reprograms cardiovascular functions and causes an exaggerated vascular reactivity and heightened blood pressure (BP) response in adult offspring [8–13]. However, the epigenetic and molecular mechanisms underlying the perinatal nicotine-induced heightened vascular reactivity in offspring are not fully understood.

It has been well documented that Angiotensin II (Ang II) has been implicated in pathophysiology of hypertension and other cardiovascular dysfunction induced by adverse in utero environment during the fetal development [14–16]. In an animal model of dietary restriction during pregnancy, elevated BP in adult offspring was associated with an increased sensitivity to Ang II [16]. Inhibition of Ang II type 1 receptor (AT1R) in early postnatal life following maternal dietary restriction prevented development of hypertension in adult offspring [16,17]. In addition, a differential increase in the AT1R and decrease in AT2R expression has been
reported at an early age of spontaneously hypertensive rats [18].
Our recent studies have demonstrated that perinatal nicotine exposure enhances AT1R but decreases AT2R protein levels in the vasculatures, resulting in a significant increased vascular reactivity and elevated BP response to Ang II in adult offspring [9]. Furthermore, previous studies have demonstrated that fetal nicotine exposure impairs kidney development and reprograms renal ATR expression which may contribute to fetal programming of hypertension [19,20]. These studies suggest that the program-
ing of Ang II receptor-mediated signaling pathways is a mechanistic link between programmed cardiovascular dysfunction and intrauterine adverse factors during early development. However, the mechanisms underlying perinatal nicotine-mediated Ang II receptors expressions and transduction signaling in the developing vasculatures remain elusive.

The present study was designed to test the hypothesis that maternal nicotine administration during pregnancy causes a differential epigenetic regulation of AT1R and AT2R gene expression via DNA methylation mechanism, leading to increase in down-stream signaling transduction pathway and enhance vascular reactivity in adult offspring. The specific aims of the present study were to determine in adult offspring whether and to what extent perinatal nicotine exposure affects 1) arterial mRNA levels of AT1R and AT2R, 2) DNA methylation levels in specific CpG sites at AT1R and AT2R promoter regions, 3) Ang II-induced intracellular Ca2+ ([Ca2+]i) signaling, 4) 20-kDa myosin light chain phosphorylation (MLC20-P) levels, and 5) vascular contractile function.

Material and Methods

Experimental animals

All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University, and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Time-dated pregnant Sprague-Dawley rats were randomly divided into two groups: 1) saline control; and 2) nicotine administration through an osmotic minipump at 4 µg/kg/min from day 4 of pregnancy to day 10 after birth, as previously described [8–13]. In brief, on the fourth day of pregnancy, rats were lightly anesthetized with ketamine and xylazine, and an incision was made on their back to insert osmotic minipumps (Type 2ML4). The incision was closed with four sutures. Twelve pregnant rats were implanted with minipumps containing nicotine solution, and other eleven pregnant rats were implanted with minipumps containing only saline which served as the vehicle control. A total of 134 pups from the control and 141 pups from nicotine-treated pregnant rats were delivered. Our previous studies [8,9,12] and current studies did not show any significant differences in litter size following the nicotine exposure. Therefore, the litter size was intact as nature in each dam and all of the newborn pups were kept with their mothers until weaning. At weaning (3-weeks age), the male and female offspring were separated. Because our previous studies have demonstrated that fetal nicotine exposure causes a hypertensive response in male but not female offspring [9], the male offspring were kept and used for present studies at 5 months of age. Male offspring were anesthetized with ketamine and xylazine and sacrificed by removing the heart, and aortas or mesenteric arteries were isolated for functional and molecular biological studies at 5 months of age. In each experiment, the sample sizes represented the number of animal used and each animal was from a different litter.

Contractions of aortic rings

At 5 months of age, aortas were isolated and cut into 4-mm rings and mounted in 10-ml tissue baths containing modified Krebs solution equilibrated with a mixture of 95%O2 and 5%CO2. Isometric tensions were measured at 37°C, as described previously [9]. After 60 min of equilibration, each ring was stretched to the optimal resting tension as determined by the tension developed in response to 120 mmol/L KCl added at each stretch level. Tissue were then stimulated with different doses of Angiotensin II (Ang II, 10−9−10−6 M) (Sigma; St, Louis, MO), and contractile tensions and 20-kDa myosin light chain phosphorylation (MLC20-P) levels were measured simultaneously in the same tissues. Tensions developed were continuously recorded with an online computer. To measure phosphorylated MLC20 simultaneously in the same tissue, arterial rings were snap frozen with liquid N2-cooled clamps at the indicated times and were rapidly immersed in a dry ice-acetone slurry that contained a 10% trichloroacetic acid (TCA) and 10 mM DTT mixture. Tissues were then stored at −80°C until analysis of MLC20 phosphorylation.

Measurement of MLC20 phosphorylation

Tissue MLC20 phosphorylation levels were measured as described previously [21]. Briefly, tissues were brought to room temperature in a dry ice-acetone-TCA-DTT mixture and then washed three times with ether to remove the TCA. Tissues were then extracted in 100 µL of sample buffer containing 20 mM Tris base and 23 mM glycine (pH 8.6), 3.0 M urea, 10 mM DTT, 10% glycerol and 0.04% bromophenol blue, as previously described [19]. Samples (20 µL) were electrophoresed at 12 mA for 2.5 h after a 30 min pre-run in 1.0 mm mini-polyacrylamide gels containing 10% acrylamide, 0.27% bisacrylamide, 40% glycerol and 20 mM Tris Base (pH 8.8). Proteins were transferred to nitrocellulose membranes and subjected to immunoblot with a specific MLC20 antibody (1:500, Sigma, St. Louis, MO). Goat anti-mouse IgG conjugated with horseradish peroxidase was used as a secondary antibody (1:2000, Calbiochem). Bands were detected with enhanced chemiluminescence (ECL), visualized on films and analyzed with the Kodak ID image analysis software. Moles of phosphate per mole of MLC20 (fraction of MLC20 phosphorylated) were calculated by dividing the density of the phosphorylated band by the sum of the densities of the phosphorylated plus the unphosphorylated bands.

Contractions of pressurized small mesenteric arteries

Also at 5 months of age, the mesenteric arcade was excised and small mesenteric arteries (~200 µm in diameter) were dissected out under a dissecting microscope. The arterial segments were mounted and pressurized in an organ chamber (Living Systems, Burlington, VT), as previously described [9]. Vascular intracellular Ca2+ ([Ca2+]i) were measured in the same tissues loaded with the Ca2+ indicator Fura 2-AM, as previously described [9]. The vessels were pressurized to 45 mmHg that was considered the optimum pressure as shown in previous studies [9]. The pressurized arteries were stimulated with KCl (120 mmol/L) or single Ang II concentrations (10−7 M) until the maximal decrease in arterial diameter was obtained. Arterial diameter and Ca2+ signal were recorded using the SoftEdge Data Acquisition Subsystem (IonOptix, Milton, MA), as described previously [9].
Real-Time Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

RNA was extracted from aortic rings and abundance of AT1aR, AT1bR, and AT2R mRNA was determined by real-time reverse transcription–polymerase chain reaction using an iCycler Thermal cycler (Bio-Rad, Hercules, CA), as described previously [22,23]. The primers used were: AT1aR, 5’-ggagagatggggctactg-3’ (forward) and 5’-ctttctggaggggttgtgtgat-3’ (reverse); AT1bR, 5’-atgtcctgctctccta-3’ (forward) and 5’-tgacctccatcccttcttg-3’ (reverse); and AT2R, 5’-caatctggctgtggctgactt-3’ (forward) and 5’-tgcatatcagagttgccaaga-3’ (reverse). Real-time reverse transcription–polymerase chain reaction was performed in a final volume of 25 μL. Each polymerase chain reaction mixture consisted of 600 nmol/L of primers, 33 U of M-MLV reverse transcriptase (Promega, Madison, WI), and iQ SYBR Green Supermix (Bio-Rad) containing 0.625 U Taq polymerase, 400 μmol/L each of dATP, dCTP, dGTP, and dTTP, 100 mmol/L KCl, 16.6 mmol/L ammonium sulfate, 40 μmol/L Tris-HCl, 6 μmol/L MgSO4, SYBR Green I, 1 μmol/L fluorescein, and stabilizers. The following reverse transcription–polymerase chain reaction protocol was used: 42°C for 30 minutes, 95°C for 15 minutes followed by 40 cycles of 95°C for 20 seconds, 55°C for 1 minute, 72°C for 20 seconds. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal reference and serial dilutions of the positive control was performed on each plate to create a standard curve. Polymerase chain reaction was performed in triplicate, and threshold cycle numbers were averaged.

Quantitative Methylation-Specific Polymerase Chain Reaction (qMS-PCR)

CpG methylation at rat AT1aR and AT2R gene promoter was determined as previously described [22,23]. Briefly, genomic DNA were isolated from aortic rings from control and nicotine-treated 5 month-old offspring using a GenElute Mammalian Genomic DNA mini-Prep kit (Sigma), denatured with 2 N NaOH at 42°C for 15 min, and treated with sodium bisulfite at 55°C for 16h, as previously described [13,22,23]. Bisulfite treatment of DNA converted cytosines to uracils. However, methylated cytosines at CpG were not converted. DNA was purified with a Wizard DNA clean up system (Promega). The bisulfite-modified DNA was used as a template for PCR. Specific primers were designed to amplify the target regions of interest with unmethylated CpG by detecting uracils and those with methylated CpG by detecting cytosines. GADPH was used as an internal reference gene. Real-time methylation-specific PCR was performed using iCycler real-time PCR system (BioRad). Data were presented as the percentage of methylation of the regions of interest (methylated CpG unmethylated CpG X 100%). The significant differences in the level of methylation of individual CpG between control and nicotine treated offspring were tested by Student’s unpaired t-test.

Statistical analysis

Data were presented as the mean ± SEM. The differences were evaluated for statistical significance (P<0.05) by ANOVA or by t test, where appropriate.

Results

Effects of nicotine on Ang II-induced contraction and MLC20 phosphorylation

Ang II produced dose-dependent increases in contractions of aortic rings from both saline control and nicotine-treated animals.
dose-dependent increases in phosphorylation levels of MLC$_{20}$ were also significantly enhanced in aortic rings of nicotine-treated rats compared with saline control rats (Fig. 2). To further determine whether nicotine affected the force-MLC$_{20}$ phosphorylation relation, Ang II-induced tensions (Fig. 1) were plotted against their corresponding MLC$_{20}$P levels of both control and nicotine-treated groups (Fig. 2). The slope value obtained from the tension-MLC$_{20}$P relation curve in nicotine-treated group was significantly higher than that in control group (11.02 ± 1.1 vs. 7.04 ± 0.96, P < 0.05).

Effects of nicotine on Ang II-induced contraction and \([\text{Ca}^{2+}]_i\):

As shown in Figure 3A (trace), Ang II (100 nM) produced vasoconstrictions and decreases in the arterial diameter of pressurized small mesenteric arteries, which were associated with increases in \([\text{Ca}^{2+}]_i\). As shown in Figure 3 (bar graph), nicotine treatment significantly enhanced Ang II-induced contractile responses compared with the control group (Fig. 3B). Ang II-induced increases in \([\text{Ca}^{2+}]_i\), were not significant differences between nicotine-treated and control groups (Fig. 3C). However, the ratio of diameter change/\([\text{Ca}^{2+}]_i\)-induced by Ang II was significantly higher in nicotine-treated group that in control group (Fig. 3D) (974.4 ± 125.3 vs. 479.2 ± 81.8 \(\mu\)m/R$_{340/380}$, P < 0.05).

Effect of nicotine on vascular expression patterns of AT$_1$R and AT$_2$R in adult offspring

Our previous studies have demonstrated that perinatal nicotine exposure significantly increases AT$_1$R but decreases AT$_2$R protein levels, resulting in a significant increase in the ratio of AT$_1$R/AT$_2$R protein abundances in the aorta of adult male offspring [9]. To determine whether the protein expressions were regulated through transcriptional mechanism, we measured the mRNA abundance of ATR in the aorta of adult offspring. As shown in Figure 4, nicotine treatment significantly increased AT$_1a$R mRNA but not AT$_1b$R mRNA abundance in aorta as compared with the control. However, vascular AT$_2$R mRNA abundance was significantly decreased in nicotine-treated group as compared with the control group.

Effect of nicotine on DNA methylation of CpG locus at AT$_1$R and AT$_2$R promoter

Recent studies indicated that alteration of CpG methylation in sequence-specific transcription factor binding sites played an important role in epigenetic modification of gene expression

Figure 3. Effect of nicotine on Ang II-induced contractions and Ca$^{2+}$ mobilization in mesenteric arteries in adult offspring. Ang II-induced contractions of pressurized mesenteric arteries were determined in male adult offspring that had been exposed in utero to saline control or nicotine. A, Typical recordings of Ang II (100 nM)-induced decreases in the arterial diameter and increments in [Ca$^{2+}$], in the same tissue from saline control and nicotine-treated groups. Ang II-induced increases in [Ca$^{2+}$], were not significant differences between nicotine-treated and control groups (Fig. 3C). However, the ratio of diameter change/[Ca$^{2+}$]-induced by Ang II was significantly higher in nicotine-treated group that in control group (Fig. 3D) (974.4 ± 125.3 vs. 479.2 ± 81.8 μm/R$_{340/380}$, P < 0.05).

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patterns in the developing fetus in response to perinatal stress [13,24–26]. From rat AT1aR gene bank, we have identified, at least five transcription factors binding sites located in CpG locus at rat AT1aR gene promoter region (Figure 5A). As shown in Figure 5B, the methylation levels of CpG locus at ERα, β binding site (−484) and Sp1 transcription factor binding site (−96) of AT1aR promoter region were significantly decreased in the aorta of adult offspring with maternal nicotine administration, when compared with the control. However, nicotine did not significantly alter the methylation status of CpG sites at −2809, −2725 and −2150. In contrast to the effect of nicotine on AT1aR, nicotine selectively increased methylation levels of CpG locus at CREB binding site (−444) and GRE binding site (+11) of AT2R promoter region as compared with the control (Figure 6). However, the methylation levels at the CpG site (−52) near TATA box were not significant difference between the nicotine-treated and control animals.

Discussion

Our previous studies have demonstrated that perinatal nicotine exposure causes a sex-dependent increase in blood pressure response in adult male rat offspring [9]. The present study provides new evidence that epigenetic programming of vascular Ang II receptors gene expressions and their-mediated signaling pathway plays a key role in nicotine-mediated developmental programming of hypertensive phenotype in adult offspring. The major findings in present study are the following: (1) perinatal nicotine exposure enhanced contractile responses to Ang II in both aortic and mesenteric arteries in adult offspring; (2) the enhanced vasoconstriction was not associated with changes of intracellular Ca2+ concentration ([Ca2+]i) but dependent on Ca2+ sensitivity of myofilaments; (3) nicotine enhanced Ang II-induced MLC20 phosphorylation level and the ratio of tension/MLC20-P; (4) nicotine exposure differentially increased AT1aR mRNA but decreased AT2R mRNA abundance in vasculatures; (5) the enhanced AT1aR gene expression was associated with decreased
methylation levels of specific CpG sites at AT1aR promoter, and the decreased AT1aR expression was associated with selectively increased methylation levels of specific CpG sites at AT1aR promoter.

In the present study, we found that Ang II-induced vasoconstrictions were significantly increased in both aortic and mesenteric arteries of adult male offspring in perinatal nicotine treated group compared with the control group, which is consistent with previous findings that perinatal nicotine exposure enhances vasoconstriction and blood pressure response in male offspring in the similar animal model [8–10]. Heightened vascular contractility and programmed elevation of blood pressure has been reported in several different animal models, including in utero and neonatal exposure to secondhand smoke [27], antenatal glucocorticoid exposure [15] and maternal food restriction [28]. However, little is known about the programming of contractile signaling of vascular smooth muscle in response to in utero adverse environmental stress. Smooth muscle contraction is regulated through both thick and thin-filament regulatory pathways [29–31]. Thick-filament regulation is mediated through MLC20 phosphorylation-dependent pathway including both Ca2+-dependent and –independent mechanisms. Increased intracellular Ca2+ concentration leads to activation of myosin light chain kinase (MLCK) and phosphorylation of MLC20, but Ca2+-independent mechanisms mainly involve inactivation of myosin light chain phosphatase (MLCP) and decreased MLC20 dephosphorylation. Present findings that nicotine enhanced Ang II-induced vasoconstriction without significant change of intracellular Ca2+ concentration, suggesting that nicotine-mediated enhanced vasoconstriction may predominately regulated through Ca2+-independent mechanism, i.e. Ca2+ sensitivity of myofilaments. Because alterations in the activities of MLCK and/or MLCP at fixed [Ca2+]i, can alter the Ca2+ sensitivity of MLC20 phosphorylation, the present results that nicotine enhanced Ang II-induced MLC20 phosphorylation levels, suggest that nicotine exposure may regulate MLCK or/and MLCP activities independent of changes in [Ca2+]i. Previous studies have reported that MLCP is the primarily involved in agonist-induced Ca2+ sensitization, but MLCK is mainly regulated through agonist-induced changes in [Ca2+]i, [31]. These findings suggest that alteration of MLCP activity may play a key role in the regulation of the Ca2+ sensitivity of MLC20 phosphorylation in adaptation of vasculatures to nicotine exposure. Ang II-induced vascular contraction is regulated through G-protein coupled receptor-mediated signaling pathways [32]. Typically, Ang II binding with its receptor activates phospholipase C, leading to generate inositol trisphosphate (IP3) which increases in [Ca2+]i, and increase diacylglycerol (DAG) production which results in activation of protein kinas C (PKC). It has been demonstrated that PKC is able to modulate the Ca2+ sensitivity via phosphorylation of MLCP subunit, which leads to inhibition of MLCP activity [33]. Therefore, changes of Ang II/ ATR-mediated PKC may play a key role in the adaptation of vascular thick-filament function to perinatal nicotine exposure.

In addition to the thick filament regulation, thin filament regulatory pathway also plays a key role in regulation of Ca2+ sensitivity. Smooth muscle thin filament-associated proteins, such as caldesmon, can inhibit myosin ATPase activity and generate vascular force without changes of [Ca2+]i, and MLC20 phosphorylation level [34]. In support this concept, our present results by experiments of simultaneous measurement of tension and MLC20 phosphorylation in the same tissue indicated that the ratio of Ang II-induced tension to MLC20 phosphorylation level was higher in nicotine-treated group than in saline control group, which suggest that thin filament regulatory pathway, i.e. increased tension at fixed MLC20-P, occurs in Ang II-mediated contraction and it is enhanced by nicotine exposure. In present study we can simultaneously measure the changes of pressurized arterial diameter and Ca2+ signal only in the small mesenteric artery (100–200 μm in diameter) but not in the large aortic ring by using the IonOptix instrument system. However, we can simultaneously measure the changes of vascular tension and phosphorylation level of 20-kDa myosin light chain only in the aortic ring but not in the mesenteric artery in tissue bath. Therefore, we have to measure Ang II-induced changes of Ca2+ signal in mesenteric artery and but myosin light chain phosphorylation level in aortic ring. Although the technique limitation may affect our data interpretation, our previous studies have demonstrated that the effect of nicotine on Ang II-induced vascular contractile response is the same in both mesenteric artery and aortic ring [9]. This suggests that Ang II-induced signal response to nicotine exposure may not be changed by vessel type.

Our previous studies have demonstrated a significant increase in AT1R but decrease in AT2R protein abundances, resulting in a significant increase in the ratio of AT1R/AT2R of vasculatures in adult offspring in response to fetal nicotine exposure [9]. It suggests that epigenetic mechanisms are involved in reprogramming of the gene expression pattern of Ang II receptors in arteries that persist into adulthood. In the present studies, our data indicated that nicotine treatment significantly increased AT1aR mRNA but decreased AT1aR mRNA abundance in vasculatures of adult offspring as compared with the control group, suggesting that nicotine-mediated alteration of ATR protein level is mainly regulated at transcriptional level. Change of transcriptional level of gene in fetal programming suggests epigenetic mechanisms involved. One of the major epigenetic mechanisms is DNA methylation. Recent studies have reported that DNA methylation is a key mechanistic link between cigarette smoking and cancer, as well as prenatal cigarette-smoke exposure and the development of adult chronic diseases [35]. DNA methylation is one of the most important mechanisms for epigenetic modification of gene expression patterns and occurs at cytosine in the CpG dinucleotide sequence [36,37]. Methylation of CpG islands in gene promoter region can alter chromatin structure and transcription, leading to a long-term shutdown of the gene expression. In addition, methylation of a single CpG dinucleotide at sequence-specific transcription factor binding sites may inhibit gene expression through changes in the binding affinity of transcription factors [38]. Recent studies have demonstrated that CpG methylation in sequence-specific transcriotion factor binding sites play an important role in epigenetic modification of gene expression patterns in the developing fetus in response to different perinatal stresses [13,24–26]. In the present study, we have identified several CpG islands located in the transcription factor binding sites in rat AT1aR promoter. Of these CpG sites, nicotine selectively decreased the methylation levels at -484 and -96 CpG locus, suggesting that hypomethylation of the selective CpG locus may be an important epigenetic mechanism in up-regulation of AT1R gene expression of vasculatures in response to perinatal nicotine exposure. Since CpG at the sites of -449 and -63 have been identified respectively as ER2,β and Sp1 transcription factor binding sites in rat AT1aR promoter, we might speculate that nicotine-mediated decrease in sequence-specific CpG methylation at ER2,β or Sp1 binding site may be a novel mechanism of altering those transcription factors binding affinity to AT1aR promoter and altering AT1aR promoter activity and gene expression. Indeed, previous studies have demonstrated that fetal stress-mediated increased sequence-specific CpG methylation at Sp1 and Egr1 binding sites at protein kinas Ce promoter results in

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Figure 7. Diagram of potential mechanisms underlying fetal nicotine exposure-induced hypertensive response in adult offspring. Perinatal nicotine exposure enhances AT1R and attenuate AT2R gene expression through DNA methylation mechanism. Nicotine-mediated enhanced AT1R/AT2R ratio predominately heightens Ca2+ sensitivity of myofilaments, leading to exaggerated vasoconstriction through both MLC20 phosphorylation-dependent (thick filament) and -independent (thin filament) signaling pathways and development of hypertensive phenotype in adult offspring.

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pregnancy is a major stress to the developing fetus and newborn, which may cause an increased risk of hypertension and other cardiovascular disease in adulthood, further studies on the epigenetic regulation of AT1R and AT2R gene expression patterns in the developing cardio-vasculature should provide more insights into molecular mechanisms in maternal smoking-related cardiovascular disease, and may suggest new insights of therapeutic strategies in the treatment of cardiovascular dysfunction in adulthood.

Author Contributions
Conceived and designed the experiments: DX CD YL XH LZ. Performed the experiments: DX CD YL XH. Analyzed the data: DX CD YL XH LZ. Contributed reagents/materials/analysis tools: DX LZ. Wrote the paper: DX LZ.

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