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RESEARCH PAPER

Antenatal nicotine induces heightened oxidative stress and vascular dysfunction in rat offspring

Daliao Xiao[1], Xiaohui Huang[1], Shumei Yang[2] and Lubo Zhang[1]

[1]*Center for Perinatal Biology, Division of Pharmacology, Department of Basic Sciences, Loma Linda University School of Medicine, Loma Linda, CA, USA, and* [2]*Department of Chemistry & Biochemistry, California State University, San Bernardino, CA, USA*

Correspondence
Daliao Xiao, Center for Perinatal Biology, Department of Basic Sciences, Loma Linda University, School of Medicine, Loma Linda, CA 92350, USA. E-mail: dxiao@llu.edu

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BACKGROUND AND PURPOSE

Antenatal nicotine exposure causes aberrant vascular reactivity and increased blood pressure in adult male rat offspring in a sex-dependent manner. The present study tested the hypothesis that maternal nicotine administration increases the production of reactive oxygen species resulting in the vascular hypertensive reactivity in male offspring.

EXPERIMENTAL APPROACH

Nicotine was administered to pregnant rats via subcutaneous osmotic minipumps throughout the gestation. The vascular oxidative damage and dysfunction were determined in 5-month-old male offspring. Contraction studies were performed on isolated aortas and their expression of NADPH oxidase (Nox2)/gp91 and nox4 determined by Western blot analysis. In addition, oxidative damage in the vessel wall was determined by measuring malondialdehyde concentrations, vascular superoxide production and SOD activity.

KEY RESULTS

Antenatal nicotine significantly increased angiotensin II-induced arterial contractions in the offspring. The exaggerated vascular contractions were inhibited by both apocynin (a Nox inhibitor) and tempol (a SOD mimetic) in a concentration-dependent manner. In addition, ACh-induced relaxations were impaired in aortas isolated from the nicotine-treated offspring, which were restored by both apocynin and tempol in a concentration-dependent manner. The nicotine treatment significantly decreased the superoxide dismutase activity and increased malondialdehyde, superoxide and nitrotyrosine protein levels in the vascular wall. Consistently, antenatal nicotine exposure significantly enhanced the protein expression of NADPH oxidase Nox2/gp91, but not Nox4 in the aorta.

CONCLUSIONS AND IMPLICATIONS

The present findings suggest that antenatal nicotine exposure results in the programming of heightened oxidative stress and vascular hypertensive reactivity via a Nox2-dependent mechanism, leading to an increased risk of hypertension in adult offspring.

LINKED ARTICLE

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Abbreviations

Ang II, angiotensin II; AT_1R, Ang II type 1 receptor; E_{max}, maximum response; HE, hydroethidium; MDA, malondialdehyde; Nox, NADPH oxidase; pD_2, -log EC_{50}; ROS, reactive oxygen species; SOD, superoxide dismutase

Introduction

Maternal cigarette smoking during gestation is associated with a number of adverse fetal and neonatal outcomes, as well as an increased risk of adverse health consequences in adult offspring. Epidemiological studies have demonstrated that fetal exposure to maternal smoking is associated with elevated BP and/or cardiovascular disease in offspring later in life (Beratis *et al.*, 1996; Blake *et al.*, 2000). As one of the major components in cigarette smoking, nicotine is likely to contribute to the developmental programming of cardiovascular disorders (Slotkin, 1998). Indeed, fetal nicotine exposure results in cardiovascular dysfunction and increased BP during adulthood in different animal models (Pausova *et al.*, 2003; Gao *et al.*, 2008). Recently, we have demonstrated that prenatal nicotine exposure reprogrammes cardiovascular reactivity in adult offspring (Xiao *et al.*, 2007; Lawrence *et al.*, 2008) and causes a gender-specific increase in angiotensin II (Ang II)-induced BP response and vascular contractility in adult male offspring (Xiao *et al.*, 2008). However, the mechanisms underlying the prenatal nicotine-induced hypertensive response to Ang II in offspring are not fully understood.

Ang II plays a key role in the regulation of basal vascular tone and BP, and in pathophysiological changes in vascular function via the activation of NADPH oxidase (Nox) and generation of reactive oxygen species (ROS) (Mehta and Griendling, 2007). Ang II has been shown to be a potent mediator of oxidative stress and oxidant signalling. Recent studies have demonstrated that Ang II-mediated up-regulation of Nox and increased production of ROS play a key role in fetal programming of the heightened vascular contractility and hypertension in offspring (Yzydorczyk *et al.*, 2006). Additionally, it has been demonstrated that maternal smoking is associated with increased levels of ROS and oxidative stress in offspring (Noakes *et al.*, 2007). Furthermore, there is considerable evidence *in vivo* and *in vitro* to suggest that exposure to nicotine results in increased production of ROS in fetal, neonatal and adult tissues (Mayhan and Sharpe, 1999; Bruin *et al.*, 2008). ROS are known to be potent intercellular and intracellular second messengers, and cause vascular dysfunction (Griendling *et al.*, 2000). However, it is not known whether prenatal nicotine exposure causes vascular oxidative damage, and whether the enhanced oxidase activity is mechanistically linked to the developmental programming of vascular dysfunction in response to fetal nicotine exposure in adult offspring.

The present study was designed to test the hypothesis that maternal nicotine administration during gestation causes fetal programming of heightened vascular reactivity via an enhanced ROS production and oxidative stress in the vascular wall, leading to an increased risk of vascular disease in adult offspring. To this end, we investigated whether antenatal nicotine exposure leads to exaggerated Ang II-induced vascular contractions and impaired NO-dependent vasorelaxation in adult offspring, and whether prenatal nicotine increases vascular Nox gene expression, ROS production and target protein nitration. Furthermore, we determine whether the hypertensive response is restored by the inhibition of Nox and a superoxide dismutase (SOD) mimetic, which may provide a mechanistic link between ROS production and vascular hypertensive reactivity in response to antenatal nicotine exposure in adult offspring.

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 US National Institutes of Health Guide for the Care and Use of Laboratory Animals. Twenty-five time-dated pregnant Sprague-Dawley rats were randomly divided into two groups: (i) saline control ($n = 13$); and (ii) nicotine ($n = 12$) administration through an osmotic minipump at 4 $\mu g \cdot kg^{-1} \cdot min^{-1}$ from day 4 of pregnancy, as previously described (Lawrence *et al.*, 2008; Xiao *et al.*, 2008). The dose of nicotine resulted in the blood levels closely resembling those occurring in moderate human smokers (Slotkin, 1998). Control rats received saline as the vehicle control. As previously reported (Xiao *et al.*, 2008), the nicotine treatment did not affect the length of gestation, and all of pregnancies reached full term. Newborn pups were kept with their mothers until weaning. At weaning, male pups were separated and housed in groups of two. The offspring were studied at 5 months of age.

Contraction studies

Aortas were isolated from the adult offspring, cut into 4 mm rings and mounted in 10 mL tissue baths containing modified Krebs' solution equilibrated with a mixture of 95% O_2 and 5% CO_2. Isometric tensions were measured at 37°C, as described previously (Xiao *et al.*, 2008). Ang II-induced concentration-dependent contractions were obtained by cumulative additions of the agonist in approximate one-half log increments, and were normalized as % KCl-induced contractions (saline control: 2.6 ± 0.1 g; nicotine-treated: 3.1 ± 0.1 g, $P < 0.05$). In certain experiments, tissues were pre-treated for 20 min with a Nox inhibitor (apocynin) or a SOD mimetic (tempol), followed by the stimulation with increased concentrations of Ang II. For relaxation studies, the tissues were pre-contracted with a submaximal concentration (1 μM) of noradrenaline that produced similar contractions in the saline control (4.3 ± 0.5 g) and nicotine-treated (5.1 ± 0.5 g) animals ($P > 0.05$), followed by ACh stimulation; ACh was added in a cumulative manner.

Immunoblotting

Arteries were homogenized in a lysis buffer. Homogenates were ultrasonicated for 15 s, and then centrifuged at 4°C for 10 min at 10 000× *g*. Supernatants were collected. Samples with equal protein were loaded and separated on SDS-PAGE. The membranes were incubated with mouse anti-gp91[phox] (Nox2) (BD Biosciences), rabbit anti-Nox4 (Abcam Inc), or rabbit antinitrotyrosine (Upstate, Millipore Corporation) antibodies, respectively, followed by a secondary horseradish peroxidase-conjugated antibody. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. Results were quantified with the Kodak electrophoresis documentation and analysis system.

Determination of oxidative damage

Oxidative damage in the vascular tissue was determined by measuring malondialdehyde (MDA) concentrations, using the thiobarbituric acid reactive substances method, as previously described (Draper *et al.*, 1993). Briefly, the samples from aortic homogenates were mixed with 1 mL of 10% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid. They were then heated in a boiling water bath for 30 min. The absorbance of the organic phase containing the pink chromogen was measured spectrophotometrically at 532 nm. The total protein content in each sample was determined using the Bradford method. MDA levels are expressed in OD value μg^{-1} protein.

Determination of the SOD activity

The activity of SOD was determined by a standard kit from Sigma (St Louis, MO, USA), following the manufacturer's instructions. The SOD activity was expressed as the amount of protein causing a 50% inhibition of formazan dye, employing xanthine and xanthine oxidase to generate superoxide radicals.

Detection of vascular superoxide (O_2^-) production

The oxidative fluorescent dye hydroethidium (HE) was used to evaluate O_2^- production *in situ*, as described previously (Miller *et al.*, 1998; Yzydorczyk *et al.*, 2006). Cells are permeable to HE and, in the presence of O_2^-, HE is oxidized to fluorescent ethidium bromide, which is trapped by intercalation with DNA. Briefly, unfixed frozen aorta segments from control and nicotine-treated animals were cut into 20 µm thick sections with a cryostat at –20°C and placed onto a glass slide. HE (2 µM) was applied to each tissue section and coverslipped. The tissue slides were then incubated in a light-protected humidified chamber at 37°C for 30 min. Images were obtained with a fluorescent laser scanning confocal microscope (LSM 710 laser scanning microscope; Zeiss) equipped with a argon laser, using the same imaging settings in each case. The vascular fluorescence was detected with a 514 nm long-pass filter and visualized by excitation at 488 nm and detection at 535 nm. The samples from control and nicotine-treated offspring were processed and imaged in parallel. The quantitative analysis of HE fluorescence intensity was carried out on the confocal images using the Zeiss Zen 2009 software program, and the fluorescence pixel intensities above the background were determined by the software and expressed as per unit area.

Data analysis

Concentration-response curves were analysed by computer-assisted nonlinear regression to fit the data using GraphPad Prism (GraphPad Software, San Diego, CA, USA) to obtain pD_2 (-log EC_{50}) and the maximum response (E_{max}). Data are expressed as means ± SEM. Experimental number represents offspring from different dams. The differences were evaluated for statistical significance ($P < 0.05$) by ANOVA or by *t*-test, where appropriate.

Results

Effect of antenatal nicotine on Ang II-induced contractions and ACh-induced relaxations of aortas in offspring

Figure 1A shows Ang II-induced concentration-dependent contractions of aortas. The maximal responses to Ang II were significantly increased in aortas of the nicotine-treated animals, as compared with the control (29.0 ± 2.1% vs. 16.3 ± 1.3% KCl response, $P < 0.05$). Figure 1B shows that the antenatal nicotine treatment significantly decreased ACh-induced relaxations, both the maximal response (51.4 ± 2.8% vs. 77.2 ± 2.8%, $P < 0.05$) and pD_2 values (6.3 ± 0.1 vs. 6.7 ± 0.1, $P < 0.05$).

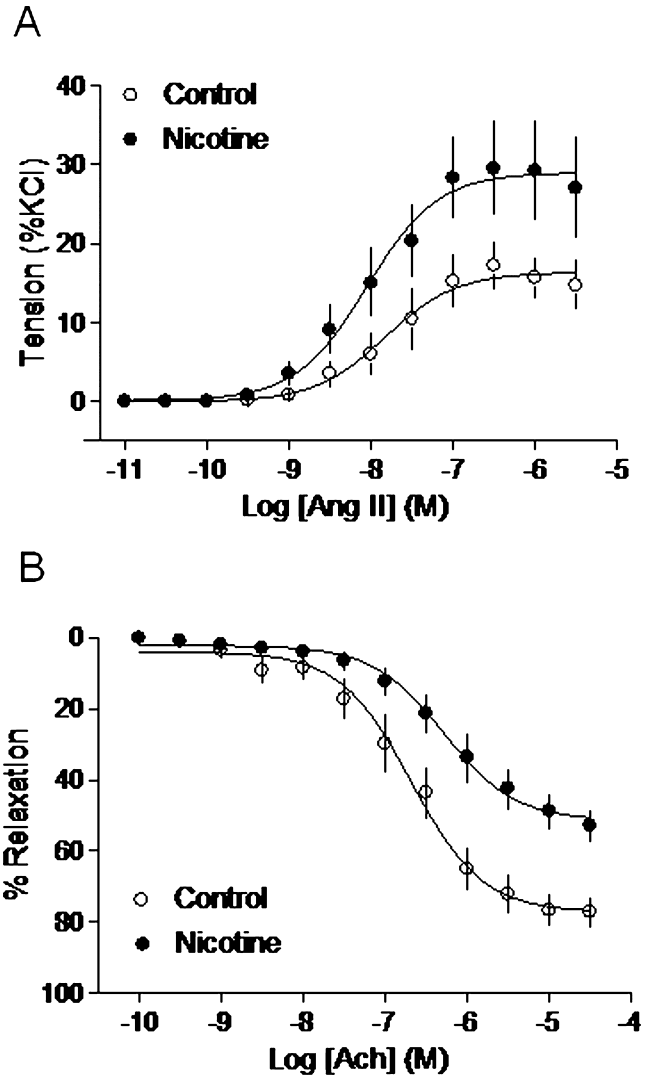

Figure 1

Effect of antenatal nicotine on Ang II-induced contractions and ACh-induced relaxations. Pregnant rats were treated with saline (control) or nicotine, and Ang II-induced contractions (A) and ACh-induced relaxations (B) were determined in aortas isolated from 5 months old male offspring. Data are means ± SEM of tissues from nine to ten animals.

Effect of apocynin and tempol on Ang II-induced contractions

To determine whether ROS play a role in nicotine-mediated heightened vascular contractions, arterial rings were pre-treated with apocynin or tempol, respectively. As shown in Figure 2A, apocynin had no significant effect on Ang II-induced contractions of arteries from saline control offspring (E_{max} of 0, 30, 100 and 300 μM apocynin-treated groups: 16.3 ± 1.3%, 14.9 ± 0.8%, 13.6 ± 1.6% and 11.3 ± 1.2% KCl response, $P > 0.05$, one-way ANOVA). However, apocynin produced a concentration-dependent inhibition of the maximal responses to Ang II induced in the nicotine-treated groups (E_{max} of 0, 30, 100 and 300 μM apocynin-treated groups: 29.0 ± 2.1%, 16.6 ± 1.3%, 11.2 ± 1.0% and 5.5 ± 0.9% KCl response, $P < 0.05$, one-way ANOVA) (Figure 2B). Similar to apocynin, tempol had no significant effect on Ang II-induced contractions in saline control animals (E_{max} of 0, 30, 100 and 300 μM tempol-treated groups: 16.3 ± 1.3%, 17.9 ± 1.4%, 17.3 ± 1.3% and 16.5 ± 1.2% KCl response, $P > 0.05$, one-way ANOVA) (Figure 2C), but produced a concentration-dependent inhibition of the maximal responses to Ang II induced in the nicotine-treated groups (E_{max} of 0, 30, 100 and 300 μM tempol-treated groups: 29.0 ± 2.1%, 19.5 ± 1.6%, 14.7 ± 2.1% and 13.8 ± 1.2% KCl response, $P < 0.05$, one-way ANOVA) (Figure 2D). Tempol (100 μM) abolished the difference in Ang II-induced contractions between the control and nicotine-treated animals (Figure 2C and D).

Effect of apocynin and tempol on ACh-induced relaxations

In control offspring, apocynin at the concentration of 300 μM, but not 100 μM, significantly shifted the curve of ACh-induced relaxations to the left (pD_2: 7.4 ± 0.1 vs. 6.7 ± 0.1, $P < 0.05$) and increased ACh-induced maximal relaxations (E_{max}: 83.0 ± 1.3% vs. 77.2 ± 1.1%, $P < 0.05$) (Figure 3A). As shown in Figure 3B, in the nicotine-treated offspring, apocynin significantly shifted the curve of ACh-induced relaxations to the left at both concentrations of 100 μM (pD_2: 6.9 ± 0.1) and 300 μM (pD_2: 7.5 ± 0.1) as compared with the absence of apocynin (pD_2: 6.3 ± 0.1) ($P < 0.05$, one-way ANOVA), and significantly increased ACh-elicited maximal relaxations from 51.4 ± 2.8% (absence of apocynin) to 72.2 ± 2.9% (100 μM apocynin) and 83.2 ± 3.3% (300 μM apocynin) ($P < 0.05$, one-way ANOVA). In the presence of apocynin (both at 100 μM and 300 μM), there were no significant differences in ACh-induced relaxations between the control and nicotine-treated animals (Figure 3A and B). Similarly, the pretreatment with 300 μM, but not 100 μM, tempol significantly shifted the curve of ACh-induced relaxations to the left (pD_2: 7.1 ± 0.1 vs. 6.7 ± 0.1, $P < 0.05$) and decreased ACh-elicited maximal relaxations (E_{max}: 69.0 ± 2.3% vs. 77.2 ± 1.1%, $P < 0.05$) in saline control offspring (Figure 3C). However, the pretreatment with tempol at both 100 μM and 300 μM shifted the curve of ACh-induced relaxations to the left (pD_2: from 6.3 ± 0.1 to 6.7 ± 0.2 to 7.0

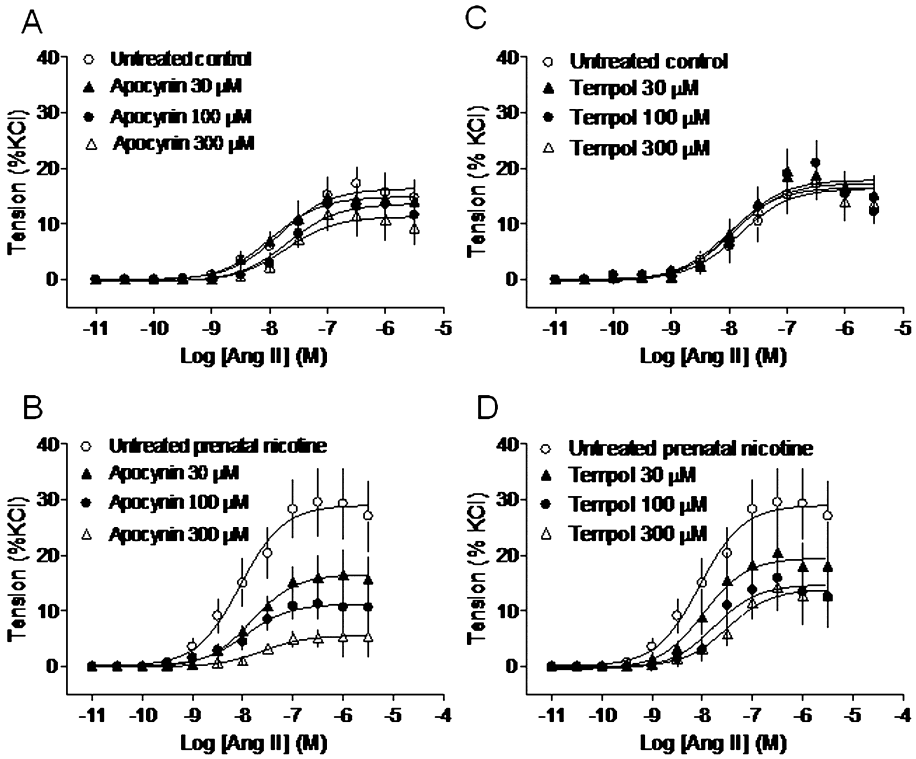

Figure 2

Effect of apocynin and tempol on Ang II-induced contractions. Pregnant rats were treated with saline (control) or nicotine, and Ang II-induced contractions in the absence or presence of apocynin or tempol were determined in aortas isolated from 5 months old male offspring of saline control (A, C) or nicotine-treated (B, D) groups. Data are means ± SEM of tissues from five to ten animals.

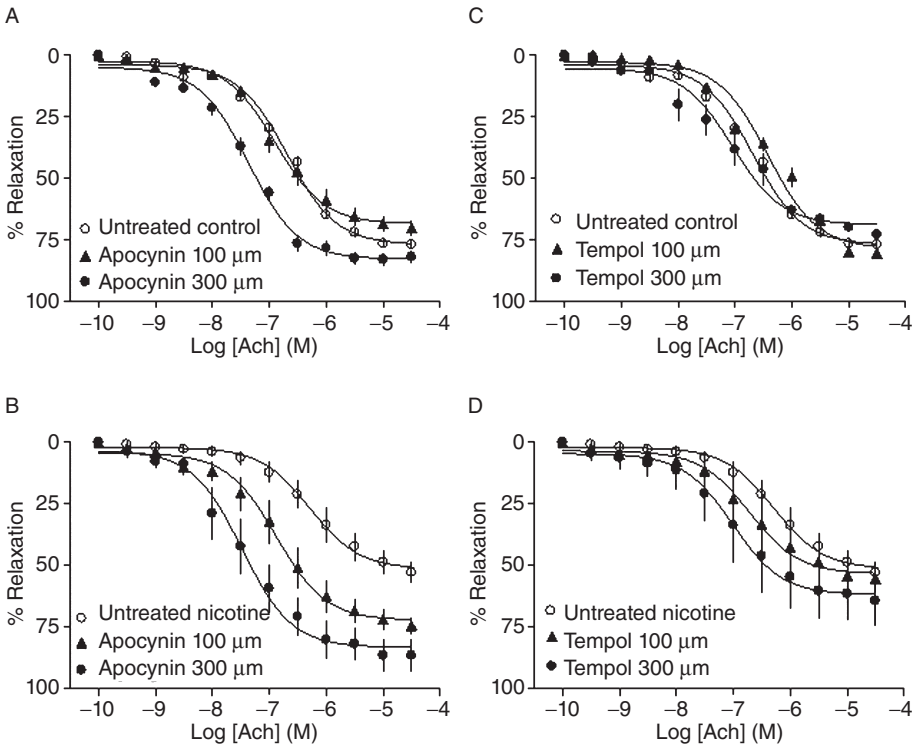

Figure 3

Effect of apocynin and tempol on ACh-induced relaxations. Pregnant rats were treated with saline (control) or nicotine, and ACh-induced relaxations in the absence or presence of apocynin or tempol were determined in aortas isolated from 5 months old male offspring of saline control (A, C) or nicotine-treated (B, D) groups. Data are means ± SEM of tissues from four to nine animals.

± 0.2, $P < 0.05$, one-way ANOVA) and increased ACh-elicited maximal relaxations (E_{max}: from 51.4 ± 2.8% to 53.4 ± 3.1% to 61.8 ± 5.0%, $P < 0.05$, one-way ANOVA) in the nicotine-treated animals (Figure 3D).

Effect of antenatal nicotine on vascular oxidative damage, antioxidant enzyme activity and superoxide levels in offspring

Oxidative damage, as assessed by MDA levels in the vascular wall, was significantly increased in the nicotine-treated offspring than that in saline control animals (0.017 ± 0.002 vs. 0.011 ± 0.001 OD values μg^{-1} protein, $P < 0.05$) (Figure 4A). It is well known that SOD is the most important antioxidant enzyme responsible for the oxidative balance in the vessels, which is regulated by oxidative stress. As shown in Figure 4B, the SOD activity was significantly decreased in the arteries of nicotine-treated offspring as compared with the control group (2.3 ± 0.2% vs. 5.8 ± 0.1% inhibition rate μg^{-1} protein, $P < 0.05$). Consistently, as shown in Figure 5, the production of superoxide anion (O_2^-) was significantly increased in the arterial wall in the nicotine-treated animals compared to that in saline controls. In addition, O_2^- was detected in the endothelium, media and adventitial layers of the aorta, and predominately located in the smooth muscle cells.

Effect of nicotine on Nox and nitrotyrosine protein levels

As shown in Figure 6, the Nox catalytic subunit gp91phox (Nox2) (saline control: 0.015 ± 0.0077 vs. nicotine: 0.051 ± 0.0097, $P < 0.05$), but not Nox4 (saline control: 0.040 ± 0.0035 vs. nicotine: 0.058 ± 0.013, $P > 0.05$), protein abundance determined by the Western blotting was significantly greater in the nicotine-treated offspring compared with that in the saline control groups. This suggests that increased oxidative stress observed in the nicotine-treated offspring is probably mediated in part by Nox2-dependent ROS overproduction. At the same time, we determined the nitrotyrosine protein levels in the arterial rings. As shown in Figure 7, arterial wall nitrotyrosine protein levels were significantly higher in the nicotine-treated group (0.276 ± 0.078) than those in the saline control group (0.090 ± 0.011) ($P < 0.05$).

Discussion

The present study demonstrates that antenatal nicotine-induced vascular hypertensive responses to Ang II and endothelium-dependent relaxations in male offspring are restored by a Nox inhibitor or a SOD mimetic. This is associated with a reduction in the SOD activity and an increase in MDA levels and superoxide production in the vasculature. These findings suggest that nicotine-mediated fetal programming of the heightened vascular reactivity is at least partly regulated through the increased production of ROS. Previous studies have shown that maternal smoking and/or nicotine exposure are associated with increased levels of oxidative stress markers, ROS in fetal, neonatal and adult tissues (Mayhan and Sharpe, 1999; Noakes *et al.*, 2007; Bruin *et al.*,

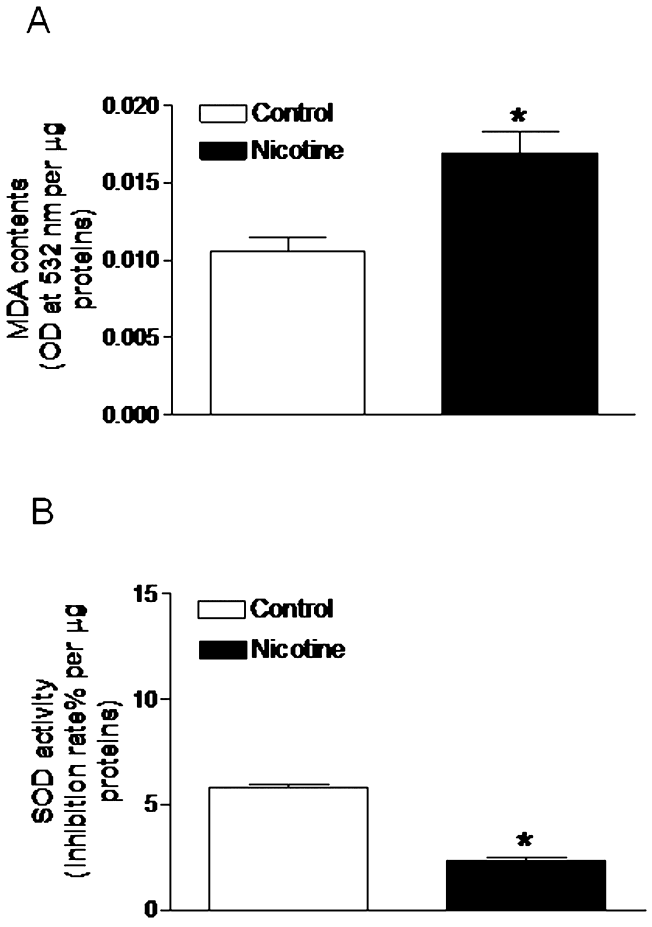

Figure 4

Effect of antenatal nicotine on vascular malondialdehyde (MDA) and superoxide dismutase (SOD) activities. Pregnant rats were treated with saline (control) or nicotine, MDA (A) and SOD activity (B) were determined in aortas isolated from 5 months old male offspring. Data are means ± SEM of tissues from five animals. *$P < 0.05$ versus control.

2008). In addition, numerous studies have demonstrated that an increased ROS production in vessels contributes to vascular dysfunction (Shinozaki *et al.*, 2004; Viswanad *et al.*, 2006; Lassegue and Griendling, 2010). To our knowledge, the present study is the first to demonstrate in a rat model that heightened oxidative stress directly contributes to nicotine-induced fetal programming of vascular hypertensive reactivity in adult offspring. This is consistent with the previous studies showing that the increased vascular ROS contributes significantly to fetal programming of vascular dysfunction in offspring of low protein-fed pregnant rats (Yzydorczyk *et al.*, 2006; Cambonie *et al.*, 2007).

Whereas the increased KCl-induced contractions in nicotine-treated offspring may be mediated in part by the increased arterial media thickness, as demonstrated previously (Xiao *et al.*, 2008), the finding that Ang II-induced arterial contractions, normalized to the KCl response, were significantly increased in the nicotine-treated animals indicates a specific increase in the Ang II-mediated response. This is consistent with the previous studies showing that prenatal nicotine exposure enhanced the vascular contractility and BP response to Ang II in adult offspring (Xiao *et al.*, 2008). It has been demonstrated that the prenatal nicotine-mediated hypertensive response is associated with an up-regulation of Ang II type 1 receptor (AT_1R) protein expression in vessels, and the heightened vascular response is normalized by an AT_1R blocker losartan (Xiao *et al.*, 2008). These findings suggest a pathogenic role for the Ang II/AT_1R signalling pathway in the development of hypertensive phenotypes in the offspring of prenatal nicotine exposed. An increasing body of evidence suggests that Ang II/AT_1R directly regulates the Nox/ROS signalling pathway, leading to enhanced vasoconstrictions in pathophysiological conditions (Touyz and Schiffrin, 2000; Touyz, 2004; Yzydorczyk *et al.*, 2006). The present finding that the enhanced Ang II-induced vasoconstrictions in the nicotine-treated offspring were restored by apocynin suggests an involvement of heightened Nox activity in the enhanced vascular reactivity, although apocynin is not limited to the inhibition of Nox (Pietersma *et al.*, 1998). Nox have emerged as key sources of ROS in the vasculature, which are essential to normal vascular function and participate in the development of vascular disease (Lassegue and Griendling, 2010). Most Nox subunits are found in vascular cells, including the catalytic Nox2, which is the earliest member of the newly discovered Nox family (Lassegue and Clempus, 2003). In the present study, we found that the expression of Nox2, but not Nox4, was increased in the nicotine-treated offspring as compared with the saline control animals. This was associated with an increase in O_2^- production and oxidative damage of the vasculature as indicated by an increase in MDA levels. These findings suggest that antenatal nicotine-mediated hypertensive response to Ang II in offspring results from an up-regulation of AT_1R-mediated Nox2 gene expression in the vasculature. Similar observations have been reported indicating that aortic Nox2 is elevated in spontaneously hypertensive rats, in rats exposed to aldosterone plus salt, and in Ang II-infused mice (Lassegue and Clempus, 2003; Park *et al.*, 2008). Additionally, the previous studies have demonstrated that hypertension is enhanced by the Ang II treatment in Nox2 overexpressing mice (Bendall *et al.*, 2007). On the other hand, hypertension can be improved by the Nox2 deletion (Jung *et al.*, 2004). Although these collective findings suggest a causal role of Nox2 in antenatal nicotine-mediated programming of vascular dysfunction in offspring, the direct involvement of vascular Ang II concentrations and/or AT_1R in the increased vascular ROS remains to be determined.

The decreased vascular SOD activity in nicotine-treated offspring observed in the present study suggests a reduction in antioxidant defence. In particular, the present finding that the pretreatment with tempol (membrane permeable SOD mimetic) restored the enhanced Ang II-induced contractions and ACh-induced relaxations in nicotine-treated offspring, suggests that the reduction in SOD activity plays a key role in the programming of the vascular dysfunction observed in the nicotine-treated animals. Tempol has been reported to regulate the vascular contractility and to improve vascular dysfunction in several different animal models (Feng *et al.*, 2001; Shastri *et al.*, 2002; Viswanad *et al.*, 2006; Costa *et al.*, 2009). It has been demonstrated that tempol restores the Ang II-mediated hypertensive response and reduces BP by scav-

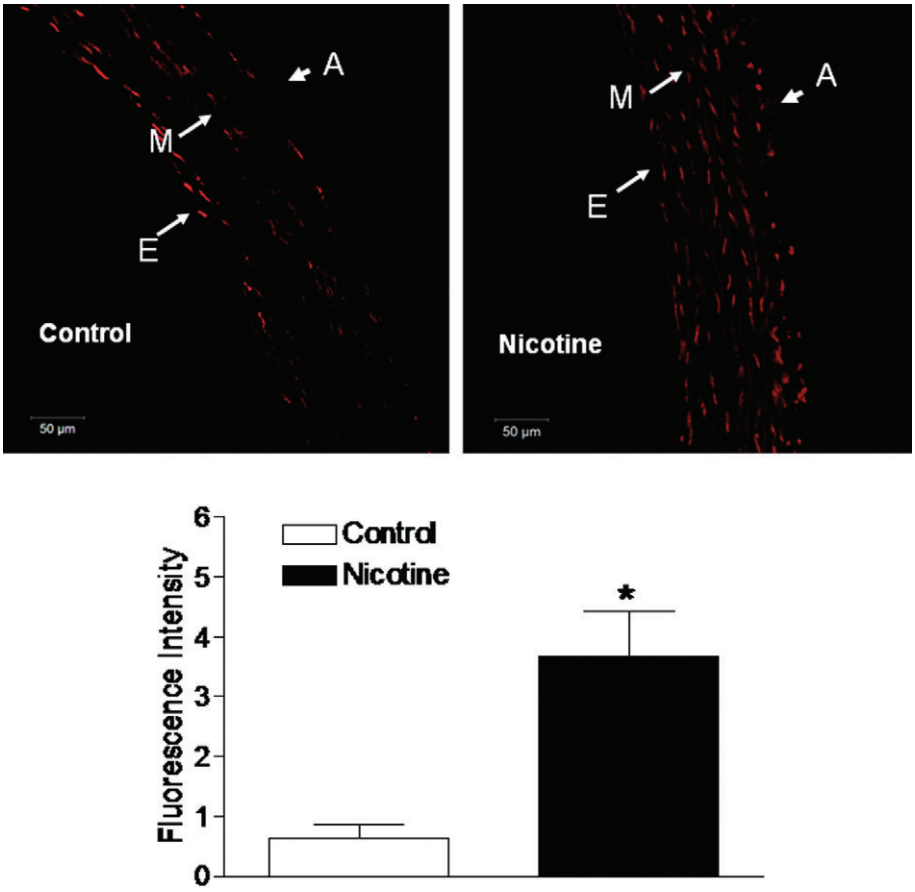

Figure 5

Effect of antenatal nicotine on vascular superoxide production. Pregnant rats were treated with saline (control) or nicotine, and aortas were isolated from 5 months old male offspring. Vessels were labelled with the oxidative dye dihydroethidium that produces red fluorescence when oxidized to ethidium bromide by superoxide anion. Images were captured and quantified with a fluorescence confocal microscope (20 × objective). E, endothelial layer; M, media layer; A, adventitial layer. Data are means ± SEM of tissues from 5 animals. *$P < 0.05$ versus control.

enging superoxide in spontaneously hypertensive rats and streptozotocin diabetic rats, indicating an important role for superoxide in the development of cardiovascular disease (Shastri *et al.*, 2002; Viswanad *et al.*, 2006). While it may be relatively difficult to directly demonstrate that tempol-mediated improvement of the endothelium-dependent relaxation is due to increased bioavailability of NO, the studies in spontaneously hypertensive rats showing that the i.v. infusion of tempol decreased mean arterial pressure and this response was blocked by a NOS inhibitor L-NAME (Schnackenberg *et al.*, 1998), suggest that this may be the case.

The present finding that ACh-induced relaxations were significantly decreased in nicotine-treated offspring suggests a reduction of NO-mediated signalling. In a previous study we demonstrated that the prenatal nicotine treatment does not alter eNOS protein expression in arteries of offspring, suggesting that NO bioavailability but not NO synthesis is altered by the nicotine exposure (Xiao *et al.*, 2007). Whether antenatal nicotine alters phosphorylated eNOS or iNOS expression in the blood vessels remains to be determined. Nonetheless, it has been demonstrated that smoking causes dysfunctional eNOS due to the reduced bioactivity of tetrahydrobiopterin (Heitzer *et al.*, 2000; Ueda *et al.*, 2000), which may lead to uncoupling of eNOS and an increased production of superoxide. It has been known that excess superoxide reacts with NO and disrupts its physiological signalling by generating peroxynitrite ($ONOO^-$) (Grunfeld *et al.*, 1995; Myatt, 2010). Indeed, recent studies have shown that treatment with SOD reverses the decreased NO levels in spontaneously hypertensive rats (Grunfeld *et al.*, 1995; Tschudi *et al.*, 1996). Thus, the finding that both apocynin and tempol restored ACh-induced relaxations in nicotine-treated offspring provides evidence that increased O_2^- contributes to the scavenging of NO and the reduction of relaxation induced by ACh.

Peroxynitrite is a potent oxidant and a potent vasoconstrictor (Belik *et al.*, 2004). It has been demonstrated that *in vivo* administration of peroxynitrite increases vascular resistance and BP, suggesting that the *in vivo* production of peroxynitrite is likely to contribute to the development of hypertension (Kooy and Lewis, 1996). Peroxynitrite oxidizes arachidonic acid to form isoprostanes that exert a potent vasoconstrictor effect (Romero and Reckelhoff, 1999, 2000). Additionally, peroxynitrite may nitrate tyrosine residues in

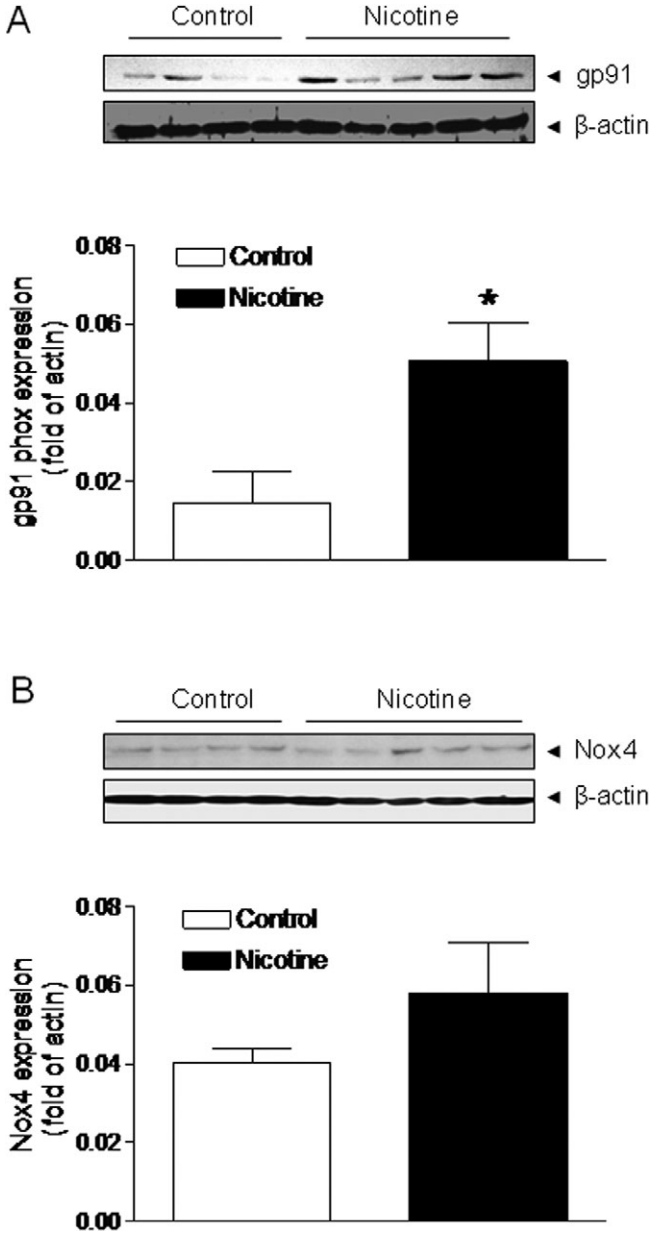

Figure 6

Effect of antenatal nicotine on vascular NADPH oxidase subunits. Pregnant rats were treated with saline (control) or nicotine, and protein abundance of Nox2/gp91 (A) and Nox4 (B) was determined in aortas isolated from 5 months old male offspring. Data are means ± SEM of tissues from four to five animals. *$P < 0.05$ versus control.

target proteins to produce nitrotyrosine, a marker of the endogenous formation of peroxynitrite (Beckman and Koppenol, 1996). Peroxynitrite-induced tyrosine nitration of PA700 has been shown to be one of the key mechanisms in Ang II-induced hypertension (Xu *et al.*, 2009). In the present study, we found that vascular nitrotyrosine protein levels were significantly higher in nicotine-treated offspring than those in the control groups, suggesting that the increased superoxide may react with NO to produce the longer-lived and more potent pro-oxidant peroxynitrite in arteries. The

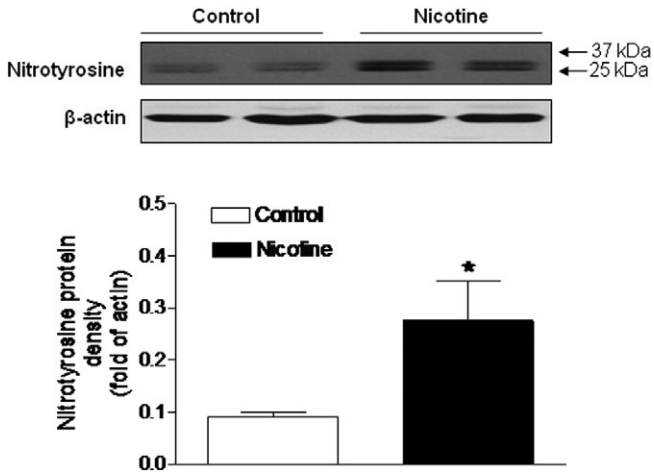

Figure 7

Effect of antenatal nicotine on vascular nitrotyrosine levels. Pregnant rats were treated with saline (control) or nicotine, and protein abundance of nitrotyrosine was determined in aortas isolated from 5 months old male offspring. Data are means ± SEM of tissues from four animals. *$P < 0.05$ versus control.

role of peroxynitrite/nitrotyrosine signalling in nicotine-mediated fetal programming of vascular dysfunction remains to be determined.

Together with the previous studies, the present investigation provides evidence in a rat model that prenatal nicotine exposure increases vascular superoxide production via an up-regulation of Ang II/AT_1R-mediated Nox2 signalling in adult offspring. The increased superoxide production and peroxynitrite formation play an important role in the developmental programming of vascular hypertensive reactivity, supporting the concept of heightened oxidative stress in fetal programming of cardiovascular disease. Although it may be difficult to translate the present findings directly to humans, the possibility that fetal nicotine exposure may result in the programming of heightened oxidative stress and vascular dysfunction in offspring provides a mechanistic understanding worthy of investigation in humans. This is because maternal cigarette smoking and use of nicotine gum and patches are a major stress to the developing fetuses, and large epidemiological studies indicate a link between *in utero* adverse stimuli during gestation and an increased risk of hypertension in adulthood.

Conflicts of interest

The authors declare no conflicts of interest.

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