Prenatal Cocaine Exposure Differentially Causes Vascular Dysfunction in Adult Offspring

DaLiao Xiao, Xiaohui Huang, Zhice Xu, Shumei Yang, Lubo Zhang

Abstract—Epidemiological studies have shown a clear association of adverse intrauterine environment and an increased risk of cardiovascular diseases and hypertension in adult life. The present study tested the hypothesis that prenatal cocaine exposure causes reprogramming of vascular reactivity, leading to an increased risk of hypertension in adult offspring. Pregnant rats received cocaine (30 mg kg\(^{-1}\) day\(^{-1}\)) or saline from days 15 to 21 of gestational age, and experiments were conducted in 3-month-old offspring. Cocaine had no effect on the baseline blood pressure but significantly increased norepinephrine-stimulated blood pressure and decreased the baroreflex sensitivity in male but not female offspring. The cocaine treatment significantly increased norepinephrine-induced contractions in pressurized resistance–sized mesenteric arteries but not in aortas, which was primarily because of a loss of endothelial NO synthase–mediated inhibition and an enhanced Ca\(^{2+}\) sensitivity in mesenteric arteries. In addition, the cocaine treatment significantly attenuated the endothelium-dependent relaxation in mesenteric arteries in male but not female offspring. Endothelial NO synthase protein levels in aortas but not mesenteric arteries were significantly increased in the cocaine-treated animals. However, cocaine significantly decreased phosphorylation levels of endothelial NO synthase in both aortas and mesenteric arteries. The results suggest that prenatal cocaine exposure programs vascular contractility via changes in endothelial NO synthase–regulated Ca\(^{2+}\) sensitivity of myofilaments in the sex- and tissue-dependent manners in resistance arteries leading to an increased risk of hypertension in male offspring. (Hypertension. 2009;53:937-943.)

Key Words: cocaine ■ fetal programming ■ Ca\(^{2+}\) sensitivity ■ eNOS ■ vascular contractility ■ rat

Epidemiological studies have shown a clear association of adverse intrauterine environment and an increased risk of cardiovascular disease and hypertension in adult life.\(^{1-4}\) In utero exposure to cocaine is a significant public health problem. Offspring born to mothers with a history of cocaine abuse have a high incidence of congenital cardiovascular malformations.\(^{5}\) Recently, we have demonstrated in a rat model that fetal exposure to cocaine during gestation results in an increase in heart susceptibility to ischemia and reperfusion injury in adult male offspring.\(^{6}\) In addition, other studies have demonstrated that prenatal cocaine exposure results in an attenuated vasodilation and enhanced responses to vasoconstrictors in cerebral arteries in the neonate.\(^{7,8}\)

It is unknown whether or to what extent fetal cocaine exposure affects the vascular reactivity of resistance vessels in adult offspring. Given the importance of resistance arteries in the regulation of blood pressure (BP), dysfunction of resistance arteries is likely to contribute to the development of hypertension observed frequently in adult offspring who experienced an adverse intrauterine environment before birth.\(^{9,10}\) To investigate the fetal programming of cardiovascular function in response to fetal cocaine exposure, the present study was designed to test the hypothesis that maternal cocaine administration during pregnancy causes reprogramming of vascular reactivity leading to an increased risk of hypertension in adult offspring. The specific aims of the present study were to determine whether and to what extent prenatal cocaine exposure affects the baseline and norepinephrine-stimulated BP in vivo, KCl- and norepinephrine-induced contractions, the endothelium-dependent relaxations, and endothelial NO synthase (eNOS) activity and protein levels in resistance-sized mesenteric arteries in adult offspring. In addition, we determined the role of Ca\(^{2+}\) signaling in the cocaine-mediated programming of vascular reactivity. To determine the differential effects of prenatal cocaine on large and resistance vessels in the offspring, the studies were also performed in aortas. To investigate the potential sex effects of prenatal cocaine exposure, the studies were performed in both male and female offspring.

Methods

An expanded Methods section is available in the online data supplement at http://hyper.ahajournals.org.
Animals
Pregnant rats were divided into 2 groups: saline control and 15 mg/kg IP of cocaine twice daily from day 15 to 21 of gestational age, as described previously. Offspring were studied at 3 months old. All of the procedures and protocols were approved by the institutional animal care and use committee guidelines.

Measurement of Arterial BP
Baseline and norepinephrine-stimulated BPs were measured in offspring as described previously.

Contraction of Aortic Rings
Norepinephrine-induced contractions were measured in aortic rings in the absence or presence of Nω-nitro-L-arginine (L-NNA; 100 μmol/L, 20 minutes) at 37°C, as described previously. For relaxation studies, the tissues were precontracted with the submaximal concentration (1 μmol/L) of norepinephrine, followed by acetylcholine added in a cumulative manner.

Contraction of Mesenteric Arteries
Resistance-sized mesenteric arteries (~200 μm in diameter) were loaded with fura 2-acetoxyethyl ester and pressurized to 45 mm Hg in an organ chamber, as described previously. Norepinephrine-induced contractions were measured in the absence or presence of L-NNA (100 μmol/L, 20 minutes). For relaxation studies, the arteries were precontracted with 3 μmol/L of norepinephrine, followed by acetylcholine.

Western Immunoblotting
eNOS protein and phospho-eNOSSer1177 levels were determined with Western blot analysis, as described previously.

Real-Time RT-PCR
RNA was extracted from tissue samples with TRIzol, and eNOS mRNA abundance was determined by real-time RT-PCR using gene-specific primers.

Data Analysis
Data are presented as mean±SEM. Experimental number represents offspring from different dams. The differences were evaluated for statistical significance (P<0.05) by 2-way ANOVA or by t test, where appropriate.

Results
Effect of Cocaine on Baseline BP and Body Weight
As shown in Table S1, prenatal cocaine exposure had no significant effects on baseline arterial systolic BP, diastolic BP, mean arterial pressure, heart rate, or body weight in either male or female offspring at 3 months old.

Effect of Cocaine on Norepinephrine-Induced BP Response
Norepinephrine produced time-dependent increases in arterial BP in both control and cocaine-treated offspring. In 3-month-old male offspring, prenatal cocaine caused significant increases in norepinephrine-stimulated systolic BP, diastolic BP, and mean arterial pressure (Figure 1). Heart rate was not affected (Figure S1). The increased BP in response to norepinephrine resulted in a decrease in heart rate via baroreflex. Prenatal cocaine treatment resulted in a significant decrease in the baroreflex sensitivity (control: 0.83±0.11 versus cocaine: 0.45±0.04 ms/mm Hg; P<0.05). In contrast to the findings in males, norepinephrine-induced changes of systolic BP, diastolic BP, and mean arterial pressure in females were not significantly different between the control and cocaine-treated animals (Figure S2). In addition, the baroreflex sensitivity was the same between the 2 groups (0.31±0.07 versus 0.31±0.12 ms/mm Hg).

Effect of Cocaine on Norepinephrine-Induced Contractions
Prenatal cocaine exposure had no significant effects on KCl-induced contractions of aortas (control: 1.9±0.1 versus cocaine: 2.0±0.2 g/mm2; P>0.05) and pressurized mesenteric arteries (control: 64.7±12.5% versus cocaine: 68.6±11.9%, diameter changes at pressure of 45 mm Hg; P>0.05) in 3-month-old male offspring. In aortas, norepinephrine-induced, concentration-dependent contractions were not significantly altered by the cocaine treatment regardless of the presence or absence of the eNOS inhibitor L-NNA (Table). In resistance-sized mesenteric arteries, prenatal cocaine treatment resulted in significant increases in the −log EC50 (pD2) value and maximum response of norepinephrine-induced contractions in the absence of L-NNA (Figure 2A and 2B and Table). In the control animals, inhibition of eNOS with L-NNA significantly potentiated norepinephrine-induced contractions and increased the norepinephrine-induced.
mediated maximal response (Figure 2A and Table). In contrast, in the cocaine-treated animals, norepinephrine-induced contractions were not significantly affected by L-NNA (Figure 2B and Table). In the presence of L-NNA, there were no significant differences in norepinephrine-induced contractions of mesenteric arteries between the control and cocaine-treated animals (Table).

### Table. Effect of Prenatal Cocaine Exposure on Norepinephrine-Mediated Contraction of Aortas and Mesenteric Arteries in Adult Male Offspring at 3 Months of Age in the Absence or Presence of L-NNA

<table>
<thead>
<tr>
<th>Tissues and Treatment</th>
<th>Control</th>
<th>Cocaine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pD2</td>
<td>Emax</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Aorta</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−L-NNA</td>
<td>7.8±0.1</td>
<td>187.5±4.4</td>
</tr>
<tr>
<td>+L-NNA</td>
<td>7.8±0.1</td>
<td>168.7±6.2</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−L-NNA</td>
<td>5.7±0.1</td>
<td>82.0±4.7</td>
</tr>
<tr>
<td>+L-NNA</td>
<td>5.9±0.1</td>
<td>102.4±3.2</td>
</tr>
</tbody>
</table>

n=7 to 8.
*P<0.05, cocaine vs control.
†P<0.05, +L-NNA vs −L-NNA.

Effect of Cocaine on Norepinephrine-Induced Ca2+ Responses

[Ca2+]i was measured simultaneously in the same tissues of pressurized mesenteric arteries in which norepinephrine-mediated contractions were determined. Consistent with concentration-dependent vasoconstrictions and decreases in the arterial diameter of pressurized mesenteric arteries (Figure 2A and 2B), norepinephrine produced concentration-dependent increases in [Ca2+]i (Figure 2C and 2D). Prenatal cocaine treatment showed no significant effects on the pD2 values and maximum responses of norepinephrine-induced Ca2+ mobilization in the absence (pD2: 5.93±0.18 versus 6.13±0.10 Rf340/f380; P>0.05; maximal response [Emax]: 1.12±0.03 versus 1.13±0.02 Rf340/f380; P>0.05) or presence of L-NNA (pD2: 6.10±0.18 versus 6.08±0.10 Rf340/f380; P>0.05; Emax: 1.05±0.02 versus 1.08±0.02 Rf340/f380; P>0.05). In addition, L-NNA did not significantly affect norepinephrine-induced Ca2+ mobilization.

The simultaneous measurement of [Ca2+]i and contractions in the same tissue allowed us to determine the norepinephrine-mediated [Ca2+]i, contraction relation in resistance mesenteric arteries. Figure 2E and 2F showed a positive correlation between increased [Ca2+]i, and contractions induced by norepinephrine in mesenteric arteries of both control and cocaine-treated animals. Prenatal cocaine treatment resulted

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Effect of cocaine on norepinephrine-induced contractions in offspring. Norepinephrine-induced contractions (A; control; B; cocaine), [Ca2+]i, (C; control; D; cocaine), and [Ca2+]i-contraction relation (E; control; F; cocaine) were determined in the absence or presence of L-NNA (100 μmol/L, 20 minutes) in pressurized mesenteric arteries from 3-month-old male offspring that had been exposed in utero to saline control or cocaine. The values of pD2 and the maximal response were presented in the Table. n=6 to 7 rats.
but not in aortas (E_max: control, 61.9 ± 2.8% versus cocaine, 67.0 ± 3.1%; P > 0.05; Figure S3). In females, cocaine had no significant effect on acetylcholine-induced relaxations in either aortas or mesenteric arteries (Figure S4).

**Effect of Cocaine on Acetylcholine-Induced Ca^{2+} Responses**

Acetylcholine-mediated relaxation and reduction in vascular wall [Ca^{2+}]_i in mesenteric arteries were measured simultaneously in the same tissues. Consistent with acetylcholine-induced relaxations, it produced concentration-dependent reductions in [Ca^{2+}]_i in mesenteric arteries precontracted with norepinephrine, which were not significantly different in the arteries between control and cocaine-treated animals (Figure 3B). Analysis of the relations between the decrease in vascular wall [Ca^{2+}]_i and relaxation depicted from the results of simultaneous measurements of [Ca^{2+}]_i and diameter changes in pressurized resistance mesenteric arteries indicated that there was a significant difference in the acetylcholine-induced Δ[Ca^{2+}]_i relaxation relation in mesenteric arteries between control and cocaine-treated animals, with a significantly decreased relaxation at the same level of the [Ca^{2+}]_i reduction in the arteries from cocaine-treated animals (Figure 3C).

**Effect of Cocaine on eNOS and Phospho-eNOS^{ser1179} Levels**

eNOS mRNA and protein abundance and phospho-eNOS^{ser1179} levels in aortas and mesenteric arteries in male offspring were determined by real-time RT-PCR and immunoblotting. As shown in Figure 4A, prenatal cocaine treatment had no significant effect on eNOS mRNA abundance in either aortas or mesenteric arteries. However, eNOS protein levels were significantly increased in the aorta, but not in the mesenteric arteries, from cocaine-treated animals as compared with those from the control animals (Figure 4B). In contrast, the ratio of phospho-eNOS^{ser1179}/total eNOS protein was significantly decreased in both aortas and mesenteric arteries from cocaine-treated animals (Figure 4C).

**Discussion**

The present study has demonstrated in a rat model that prenatal cocaine exposure causes a hypertensive reactivity of resistance-sized mesenteric arteries in adult male offspring. The major findings of the present study are as follows: (1) prenatal cocaine exposure caused a sex-dependent increase in arterial BP response to norepinephrine and a decrease in the baroreflex sensitivity in male offspring; (2) α_{1}-adrenoceptor agonist noradrenaline-induced vascular contractions are significantly enhanced in resistance mesenteric arteries but not in aortas in response to prenatal cocaine exposure; (3) this functional alteration of vascular reactivity is independent of changes in Ca^{2+} mobilization but mainly depends on enhanced Ca^{2+} sensitivity in mesenteric arteries; (4) the basal eNOS activity inhibits norepinephrine-mediated contractions of resistance mesenteric arteries but not aortas, which are abolished by the cocaine treatment; (5) endothelium-dependent relaxation is significantly attenuated in mesenteric arteries but not in aortas in male offspring in a sex-dependent
manner, which is mediated by increased Ca\(^{2+}\) sensitivity in mesenteric arteries; and (6) fetal cocaine exposure significantly increased eNOS protein levels in aortas but decreased the ratio of phosphorylated eNOS in both aortas and mesenteric arteries of adult offspring.

The finding that fetal cocaine exposure increased the susceptibility of elevated BP in adult offspring in the present study is consistent with recent studies in humans and animal models showing a link between adverse intrauterine environments and fetal programming, resulting in an increased risk of hypertension and ischemic heart disease in adulthood.\(^{1,16–18}\) Previous studies suggested that intrauterine cocaine exposure increased a risk of persistently elevated BP during early and later childhood.\(^{19–22}\) The present finding that prenatal cocaine affected male offspring predominantly is in agreement with previous studies showing that female offspring were less sensitive in the manifestation of hypertension caused by adverse prenatal insults.\(^{3}\) In addition, our recent study has demonstrated that fetal cocaine exposure results in increased heart susceptibility to ischemia and reperfusion injury in adult male but not female offspring.\(^{6}\) These studies suggest sex-dependent fetal programming of cardiovascular dysfunction induced by prenatal cocaine exposure. It is likely that multiple mechanisms are involved in fetal programming of cardiovascular response. In the present study, we found that prenatal cocaine treatment caused a significant decrease in the baroreflex sensitivity in male offspring. Consistent with this finding, previous studies have demonstrated that cocaine suppresses baroreflex control of BP.\(^{23,24}\) Studies in other models of fetal programming also showed a decrease in baroreflex function in offspring.\(^{11,25,26}\) Studies in other models of fetal programming of cardiovascular response in offspring.

In the present study, KCl-induced contractions of both aortas and resistance mesenteric arteries were not significantly different between the control and cocaine-treated animals, suggesting that the electromechanically-mediated contractile signal pathway in arteries is not altered by prenatal cocaine exposure. This is in agreement with the previous finding that glucocorticoid exposure during early gestation had no significant effect on KCl-induced contractions in newborn lamb coronary arteries.\(^{27}\) In contrast, our recent study showed that KCl-induced vascular contractility was enhanced in adult rats that were exposed to nicotine before birth.\(^{12}\) These findings suggest a stimuli specificity in fetal programming of electromechanical coupling in the vascular smooth muscle in adult offspring.

Unlike the finding with KCl-induced contractions, prenatal cocaine exposure significantly increased norepinephrine-induced contractions of resistance mesenteric arteries in adult offspring. In contrast, norepinephrine-mediated contractions of aortas were not affected by the cocaine treatment, suggesting that fetal cocaine exposure causes a tissue-selective programming of resistance vascular reactivity to the sympathetic neurotransmitter in adult offspring. Consistent with the present finding of altered agonist-mediated vascular contractions, Yakubu et al.\(^{7}\) have reported that prenatal cocaine exposure significantly enhances 5-hydroxytryptamine-, endothelin-1-, and clonidine-induced contractions of cerebral arterioles in neonatal piglets.

It is likely that multiple mechanisms are involved in fetal programming of vascular hypertension. Previous studies demonstrated that in utero cocaine exposure altered α-adrenoceptor mRNA levels and binding sites in the brain,\(^{28–30}\) as well as the myocardial β-adrenoceptor signaling pathway in neonatal rats.\(^{31}\) In the present study, inhibition of eNOS by L-NNA significantly increased norepinephrine-induced contractions of mesenteric arteries in adult offspring, suggesting a significant component of basal eNOS activity in suppressing α-adrenoceptor-mediated contractions. The cocaine treatment abolished the effect of L-NNA, and there were no significant differences in norepinephrine-mediated contractions of mesenteric arteries between control and cocaine-treated animals in the presence of L-NNA. These results suggest that cocaine-mediated enhancements of norepinephrine-induced contractions in resistance mesenteric arteries are primarily attributed to the loss of the eNOS-mediated relax-
ation component rather than increased norepinephrine-induced contractions, per se, in the resistance arteries. This is supported by the finding of a significant decrease in acetylcholine-induced relaxation of mesenteric arteries, but not aortas, in cocaine-treated offspring. Consistent with the sex-dependent BP response observed, the decreased endothelium-dependent relaxation of mesenteric arteries was found only in male offspring. The involvement of endothelium/NO in fetal programming of vascular function has been studied, and the results are controversial. Our recent study demonstrated that enhanced α1-adrenoceptor–mediated contractions of the aorta in male adult offspring after prenatal nicotine exposure were primarily attributed to the loss of the eNOS-mediated relaxation component in α1-adrenoceptor–mediated contractions. In contrast, fetal nicotine exposure enhanced arterial sensitivity to angiotensin II primarily because of increased angiotensin II–induced contractions, per se, rather than the loss of the eNOS-mediated relaxation component. Taken together, these findings suggest tissue-specific and stimuli-dependent fetal programming of endothelial function and vascular reactivity.

To evaluate endothelium-dependent mechanisms, eNOS protein abundance and phosphorylation levels were examined in both aortas and mesenteric arteries. In aortas, the cocaine treatment significantly enhanced eNOS protein, but not mRNA, abundance, suggesting that cocaine-mediated programming of eNOS expression in the aorta is at the translational level. The finding of attenuated eNOS phosphorylation levels suggests a counterbalance of eNOS protein expression and its activity, which may lead to the minimal effect on endothelium-dependent relaxation observed in the aorta of cocaine-treated animals. Unlike aortas, the finding of no difference in eNOS protein abundance but a significant decrease in eNOS phosphorylation levels in mesenteric arteries of cocaine-treated animals suggests that decreased eNOS activity contributes to the decreased endothelium-dependent relaxation and the increased vascular contractility of resistance arteries in adult offspring that were exposed to cocaine before birth. This is in agreement with the previous studies showing that intrauterine malnutrition decreased eNOS activity and NO production without affecting eNOS gene expression in rat mesenteric arteries. In addition, our recent study in a rat model of prenatal nicotine exposure demonstrated that alteration of eNOS activity without changes in eNOS protein abundance contributed to the vascular dysfunction. Although the present study provides evidence of a correlation between decreased phospho-eNOSSer1179 levels and impaired endothelium-dependent relaxation, it should be noted that Ser1179 is not the only site that can be phosphorylated, and changes in Ser1179 phosphorylation and eNOS activity are not seen in all endothelial studies. Whether and to what extent fetal cocaine exposure may cause programming of other phosphorylation events of eNOS remain intriguing areas for future investigation.

In addition, we determined the downstream mechanisms of intracellular Ca2+ mobilization and Ca2+ sensitivity in cocaine-mediated fetal programming of the resistance arterial contractility and found that the enhanced norepinephrine-induced contractions of resistance arteries in cocaine-treated animals were not associated with increased [Ca2+]i. In the present study, the simultaneous measurement of [Ca2+]i, with tension development in the same intact tissue allowed us to directly determine the precise relationship between [Ca2+]i and tension in arteries and, thus, to determine the Ca2+ sensitivity of myofilaments with unimpaired excitation–contraction coupling processes and retained regulatory targets for second messenger pathways. The finding of enhanced Δ[Ca2+]i, induced by norepinephrine in resistance mesenteric arteries of cocaine-treated animals indicates an increase in the Ca2+ sensitivity of myofilaments in the resistance arteries. Indeed, the increased Ca2+ sensitivity has been demonstrated to be critical in the fetal programming of vascular dysfunction and hypertension. It is likely that inhibition of the eNOS activity plays a key role in fetal programming of the Ca2+ sensitivity in resistance arteries in cocaine-treated animals (see Figure S5 for a diagram). This is supported by the following evidence obtained in the present study. L-NNA–mediated increases in norepinephrine-induced contractions of resistance mesenteric arteries in control animals were not associated with increased [Ca2+]i, but rather with an increase in Δ[Ca2+]i, indicating that basal eNOS activity inhibits the Ca2+ sensitivity in resistance arteries. In the presence of L-NNA, there were no significant differences in norepinephrine-induced Ca2+ sensitivity in resistance mesenteric arteries between control and cocaine-treated animals. The role of eNOS in programming of the Ca2+ sensitivity in resistance arteries was further supported by the finding of a significant decrease in acetylcholine-induced relaxation at the same level of [Ca2+]i, reduction in mesenteric arteries from cocaine-treated animals. It has been demonstrated in permeabilized vascular smooth muscle preparations that the addition of cGMP-precavitated, cGMP-dependent protein kinase reduces myosin phosphorylation and contractile tension proportionally despite a constant [Ca2+]i, indicating that cGMP inhibits the Ca2+ sensitivity of contractile proteins.

Perspectives
The present study has demonstrated that prenatal cocaine exposure results in reprogramming of eNOS activity and enhanced Ca2+ sensitivity in resistance arteries leading to an increased resistance arterial contractile sensitivity and BP response to norepinephrine in a sex-dependent manner. These findings indicate that fetal cocaine exposure not only causes increased perinatal morbidity and mortality, as recognized previously, but also has long-lasting effects and increases the susceptibility of elevated arterial BP later in adult life. The present finding is consistent with recent studies in humans and animal models and re-enforces the notion that multiple in utero adverse factors during pregnancy cause fetal programming and increase the risk of hypertension in the adult. Given that cocaine abuse in pregnant women is a significant problem, the present finding suggests that intrauterine cocaine exposure may be a significant risk factor for cardiovascular disease in later life in male offspring. As is often the case with novel findings, the present study may raise more questions than it answers. For instance, are the effects mediated by a direct effect of cocaine on the fetus or
indirectly through its effect on the mother? What are the epigenetic mechanisms involved in reprogramming of the eNOS activity in resistance arteries, which persist into adulthood? In addition, whether or to what extent do sex steroid hormones contribute to the sex differences in cocaine-mediated fetal programming of resistance vascular function? Undoubtedly, these questions warrant further investigation.

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**Disclosures**

None.

**References**


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Prenatal cocaine exposure differentially causes vascular dysfunction in adult offspring

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Running title: Prenatal cocaine and hypertension in offspring

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Materials and Methods

Experimental animals
Time-dated pregnant Sprague–Dawley rats were purchased from Charles River Laboratories (Portage, MI) and were randomly divided into two groups: 1) saline control; and 2) cocaine 15 mg/kg i.p. twice daily at 10:00 a.m. and 4:00 p.m. from day 15 to 21 of gestational age, as described previously. The intraperitoneal route and the dose used closely resembles intranasal administration in human in the kinetics and plasma cocaine levels attained. The animals were allowed to give birth naturally, and pups were weaned and separated by sex 21 days after birth. The offspring were given food and water ad Librum and were subjected to no further treatment prior to sacrifice. Mesenteric arteries and aortas were isolated from 3-month-old male offspring from seven litters in each group. 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.

Measurement of arterial BP
BP was measured in 3-month old male and female offspring, as described previously. Animals were implanted with catheters in femoral arteries for recording of arterial BP and heart rate (HR). A catheter was subcutaneously implanted in the back of animals for drug administration. Two days after recovery from surgery, BP and HR were measured continuously in conscious animals. After the baseline recording for about 60 min, animals were administered norepinephrine (500 µg/kg of body weight) with subcutaneous injection via the implanted catheter, and BP and HR were recorded for 60 min. Arterial BP responses at this dose of norepinephrine reached a submaximal level, as determined in preliminary studies. Arterial systolic BP (SBP) and diastolic BP (DBP), mean arterial BP (MAP), and HR data were recorded continuously throughout each study with data acquisition software (Powerlab 16/SP and Chart version 4, ADInstruments, Colorado Springs, CO). The baroreflex sensitivity was calculated as the slope of Δ pulse interval/Δ SBP (msec/mmHg).

Contractions of aortic rings
Aortas were 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. After 60 min of equilibration, each ring was stretched to the optimal resting tension as determined by the tension developed in response to 120 mM KCl added at each stretch level. Norepinephrine-induced concentration-dependent contraction curves were obtained by cumulative addition of the agonist in approximate one-half log increments. In certain experiments, tissues were pretreated with a nitric oxide synthase inhibitor N⁶-nitro-L-arginine (L-NNA) (100 µM, 20 min) as described in our previous study, and then stimulated with increased concentrations of norepinephrine. For relaxation studies, the tissues were precontracted with the sub-maximal concentration (1 µM) of norepinephrine, followed by acetylcholine, added in a cumulative manner.

Contractions of pressurized resistance-sized mesenteric arteries
The mesenteric arcade was excised and resistance-sized mesenteric arteries (~200 µm in diameter) were dissected out under a dissecting microscope. The arterial segments were canulated and pressurized in an organ chamber (Living Systems, Burlington, VT), as previously...
described. Vascular intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)) were measured in the same tissues loaded with the Ca\(^{2+}\) indicator fura 2-AM, as previously described. The vessels were pressurized to 45 mmHg that was considered the optimum pressure as shown in previous studies. The pressurized arteries were stimulated with KCl (120 mM) or increased concentrations of norepinephrine. In certain experiments, tissues were pretreated with L-NNA (100 µM, 20 min) as described in our previous study, and then stimulated with increased concentrations of norepinephrine. For relaxation studies, the pressurized arteries were pre- contracted with the sub-maximal concentration of norepinephrine (3 µM), followed by increasing concentrations of acetylcholine. Arterial diameter and Ca\(^{2+}\) signal were recorded using the SoftEdge Data Acquisition Subsystem (IonOptix, Milton, MA), as described previously.

**Western immunoblotting**

eNOS protein and phosphorylation levels were determined with Western blot analysis, as described previously. Arteries were homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris.HCl, 10 mM EDTA, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 µg/ml leupeptin, and 5 µg/ml aprotinin, pH 7.4. Homogenates were centrifuged at 4°C for 10 min at 10,000g, and supernatants were collected. Protein was quantified in the supernatant using the protein assay kit from Bio-Rad. Samples with equal protein were loaded on 7.5% polyacrylamide gel with 0.1% sodium dodecyl sulfate (SDS) and separated by electrophoresis at 100 V for 2 h. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding sites in the membranes were blocked by overnight incubation at 4°C in a Tris-buffered saline solution containing 5% dry milk. The membranes were then incubated with mouse eNOS monoclonal antibody, and the antibody for eNOS phosphorylation at S1179 (p-eNOS\(^\text{Ser}1179\)), followed by a secondary horseradish peroxidase-conjugated goat anti-mouse antibody. Proteins were visualized with enhanced chemiluminescence reagents, and the blots were exposed to Hyperfilm. Results were quantified with the Kodak electrophoresis documentation and analysis system and Kodak ID image analysis software.

**Real-time RT-PCR**

RNA was extracted from tissue samples using TRIzol reagents (Invitrogen, Carsbad, CA). eNOS mRNA levels were determined by real-time RT-PCR using the iCycler Thermal cycler (BioRad, Hercules, CA). Specific eNOS primers were 5’-TTCTGGCAAGACCGATTACACGACAT-3’ (forward) and 5’-AAAGGCGGAGAGGACTTGTCCAAA-3’ (reverse). Real-time RT-PCR was performed in a 25 µl-reaction mixture according to the instruction of iScript one-step RT-PCR kit (BioRad). RT-PCR was carried out under the following conditions: 42 °C for 45 min and then 95 °C for 15 min, followed by 45 cycles of 95 °C for 15 sec, 56 °C for 20 sec and 72 °C for 10 sec. GAPDH was used as an internal reference. The relative amount of gene expression was calculated by comparison of cycle thresholds with the housekeeping gene of GAPDH.

**Materials**

Norepinephrine, N\(^\text{G}\)-nitro-L-arginine (L-NNA), acetylcholine, cocaine and other chemicals were obtained from Sigma (St. Louis, MO). Fura-2 AM was purchased from Molecular Probes (Eugene, OR). Mouse eNOS monoclonal antibody was from Transduction Laboratory (Lexington, KY). Phospho-eNOS\(^\text{Ser}1179\) antibody was from Cell Signaling Technology (Danvers, MA). Electrophoresis and immunoblotting reagents were from Bio-Rad (Hercules, CA).
**Data analysis**
Concentration-response curves were analyzed by computer-assisted nonlinear regression to fit the data using GraphPad Prism (GraphPad Software, San Diego, CA) to obtain pD2 (-log EC50) and the maximum response (E_max). Results were expressed as means ± SEM. Experimental number (n) represents offspring from different dams. The differences were evaluated for statistical significance (P < 0.05) by two-way ANOVA or by t-test, where appropriate.

**References**
Table S1. Effect of prenatal cocaine exposure on basal arterial blood pressure (BP) of adult rat offspring at 3-month old

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>MAP (mmHg)</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>HR (bpm)</th>
<th>BW (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control M</td>
<td>113.1 ± 3.3</td>
<td>126.0 ± 3.3</td>
<td>108.7 ± 3.8</td>
<td>371.9 ± 14.2</td>
<td>528 ± 7.8</td>
</tr>
<tr>
<td>Cocaine M</td>
<td>118.3 ± 2.7</td>
<td>126.9 ± 2.9</td>
<td>115.5 ± 2.9</td>
<td>359.0 ± 7.4</td>
<td>516 ± 10.5</td>
</tr>
<tr>
<td>Control F</td>
<td>118.1 ± 5.5</td>
<td>149.0 ± 6.0</td>
<td>103.8 ± 6.0</td>
<td>422.0 ± 18</td>
<td>296 ± 4.0</td>
</tr>
<tr>
<td>Cocaine F</td>
<td>120.9 ± 2.9</td>
<td>148.4 ± 4.8</td>
<td>107.7 ± 2.6</td>
<td>408.0 ± 9.8</td>
<td>293 ± 4.8</td>
</tr>
</tbody>
</table>

MAP, mean arterial BP; SBP, systolic BP; DBP, diastolic BP; HR, heart rate; BW, body weight; M, male; F, female; n = 8-9
Figure S1. Effect of cocaine on norepinephrine-induced heart rate responses in offspring. Heart rate responses to norepinephrine (500 µg/kg, s.i.) were measured in 3-month old male and female offspring that had been exposed in utero to saline control or cocaine. n=8-9 rats.
Figure S2. Effect of cocaine on norepinephrine-induced blood pressure (BP) responses in female offspring. Systolic BP (SBP), diastolic BP (DBP), mean arterial BP (MBP) responses to norepinephrine (500 µg/kg, s.i.) were measured in 3-month old female offspring that had been exposed in utero to saline control or cocaine. n=8-9 rats.
**Figure S3. Effect of cocaine on acetylcholine-induced relaxations of aortas in male offspring.** Acetylcholine-induced relaxations were measured in aortas in 3-month old male offspring that had been exposed *in utero* to saline control or cocaine. Aorta segments were pre-contracted with norepinephrine (1 μM) followed by a cumulative addition of acetylcholine. n=6-8 rats.


Figure S4. Effect of cocaine on acetylcholine-induced relaxations of aortas and mesenteric arteries in female offspring. Acetylcholine-induced relaxations were measured in aortas and mesenteric arteries in 3-month old female offspring that had been exposed in utero to saline control or cocaine. Aorta segments were pre-contracted with norepinephrine (1 μM) followed by a cumulative addition of acetylcholine. Pressurized mesenteric artery segments were pre-contracted with norepinephrine (3 μM) at basal pressure of 45 mmHg followed by a cumulative addition of acetylcholine. n=6-8 rats.
Figure S5. eNOS-mediated inhibition of α1-adrenoceptor-mediated pharmacomechanical coupling in the mesenteric artery. Activation of α1-adrenoceptors (α1AR) stimulates inositol 1,4,5-trisphosphate (IP3) synthesis. IP3 binds to the IP3 receptor (IP3R) and releases Ca2+ from intracellular Ca2+ stores. Ca2+ activates myosin light chain kinase (MLCK) and increases phosphorylation of the 20-kDa regulatory light chain of myosin (MLC20) and arterial contractions. Dephosphorylation of MLC20 is mediated by myosin light chain phosphotase (MLCP) resulting in relaxation. Phosphorylation of eNOS increases eNOS activity and synthesis/release of nitric oxide (NO). NO increases cGMP that inhibits the Ca2+ sensitivity of contractile myofilaments possibly by activating MLCP activity. Cocaine-mediated fetal programming leads to decreased eNOS activity and attenuated cGMP-mediated inhibition of the Ca2+ sensitivity.