Prenatal methamphetamine differentially alters myocardial sensitivity to ischemic injury in male and female adult hearts

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Prenatal methamphetamine differentially alters myocardial sensitivity to ischemic injury in male and female adult hearts. Am J Physiol Heart Circ Physiol 310: H516–H523, 2016. First published December 18, 2015; doi:10.1152/ajpheart.00642.2015.—Methamphetamine is one of the most common illicit drugs abused during pregnancy. The neurological effects of prenatal methamphetamine are well known. However, few studies have investigated the potential effects of prenatal methamphetamine on adult cardiovascular function. Previous work demonstrated that prenatal cocaine exposure increases sensitivity of the adult heart to ischemic injury. Methamphetamine and cocaine have different mechanisms of action, but both drugs exert their effects by increasing dopaminergic and adrenergic receptor stimulation. Thus the goal of this study was to determine whether prenatal methamphetamine also worsens ischemic injury in the adult heart. Pregnant rats were injected with methamphetamine (5 mg·kg⁻¹·day⁻¹) or saline throughout pregnancy. When pups reached 8 wk of age, their hearts were subjected to ischemia and reperfusion by means of a Langendorff isolated heart system. Prenatal methamphetamine had no significant effect on infarct size, preischemic contractile function, or posts ischemic recovery of contractile function in male hearts. However, methamphetamine-treated female hearts exhibited significantly larger infarcts and significantly elevated end-diastolic pressure during recovery from ischemia. Methamphetamine significantly reduced protein kinase Ce expression and Akt phosphorylation in female hearts but had no effect on these cardioprotective proteins in male hearts. These data indicate that prenatal methamphetamine differentially affects male and female sensitivity to myocardial ischemic injury and alters cardioprotective signaling proteins in the adult heart.

NEW & NOTEWORTHY

Prenatal exposure to methamphetamine increases myocardial sensitivity to ischemic injury and alters proteins involved in cardioprotective signaling pathways in the adult female rat heart. These changes were not observed in male hearts. The responses of adult male and female hearts to an ischemic insult are differentially altered by prenatal methamphetamine.

METHAMPHETAMINE is one of the most common illicit drugs abused during pregnancy (2, 29). One study found that 5.2% of pregnant women use methamphetamine at some point during their pregnancy (2). Most studies of prenatal methamphetamine have focused on the impact of this drug on behavior and cognitive effects during early childhood. Prenatal methamphetamine produces a variety of neurological effects, including structural changes in the brain (8, 37), increased prevalence of cognitive and behavioral problems (11, 23), delayed development of gross motor skills (42), and decreased verbal and spatial memory (8). In contrast to these neurological effects, the impact of prenatal methamphetamine exposure on the adult cardiovascular system has not been well studied.

Cocaine is also commonly abused by pregnant women. Like methamphetamine, most studies of prenatal cocaine exposure have focused on neurological and behavioral outcomes. However, animal studies indicate that prenatal cocaine has long-lasting effects on the heart. Bae et al. (3, 4) demonstrated that prenatal cocaine induces apoptosis and hypertrophy in the juvenile heart and increases myocardial sensitivity to ischemic injury in adult male offspring. Prenatal cocaine induces methylation of the promoter region of the protein kinase Cε (PKCε) gene and suppresses the expression of this cardioprotective protein in adult male rat hearts (30). This epigenetic alteration of PKCε expression has been implicated in the enhanced myocardial sensitivity to ischemic injury in male rats prenatally exposed to cocaine (3, 30).

Methamphetamine and cocaine differ in their mechanisms of action, but they produce similar physiological effects. Cocaine inhibits the reuptake of epinephrine, norepinephrine, and dopamine from sympathetic and dopaminergic neurons. In contrast, methamphetamine induces the release of these catecholamines from sympathetic and dopaminergic neurons. Both substances act peripherally as sympathomimetic drugs. Given the fact that cocaine and methamphetamine exert their effects by increasing dopaminergic and adrenergic receptor signaling, we reasoned that prenatal methamphetamine might have an effect on the ischemic adult heart similar to that reported for cocaine (3). The primary goals of this study were to determine whether prenatal methamphetamine alters myocardial sensitivity to ischemic injury in the adult rat heart and whether prenatal methamphetamine differentially alters male and female cardiac responses to an ischemic insult. A secondary goal was to explore the effects of prenatal methamphetamine on the expression and phosphorylation status of cardioprotective proteins in the adult heart.

METHODS

Animals and prenatal methamphetamine treatment. Female Sprague-Dawley rats were bred at 8 wk of age; gestational day 0 was defined by the presence of a vaginal plug. The rats were housed on a 12:12-h light-dark schedule (lights on at 0700) in standard Plexiglas cages with free access to food and water. Preg nant rats (n = 6 for each treatment group) were weighed every 5 days through the first 16 days of pregnancy and then every 2 days until the pups were born. Pregnant rats were subcutaneously injected once per day (at 1000) with methamphetamine (5 mg·kg⁻¹·day⁻¹) or saline starting at gestational day 1 and continuing throughout the pregnancy. This dose was chosen on the basis of previous work demonstrating that prenatal exposure to methamphetamine (5 mg·kg⁻¹·day⁻¹) alters exploratory behavior, locomotor activity, and memory in adult rats (39, 40, 41). Pups were...
weaned at 21 days of age and housed three to four per cage. Rats were euthanized at 8 wk of age for Langendorff isolated heart experiments. Spontaneous locomotor activity of the animals was measured 2–3 days prior to heart isolation. All procedures were approved by the Institutional Animal Care and Use Committee of Ohio Northern University.

**Spontaneous locomotor activity.** Prenatal methamphetamine alters exploratory behavior and locomotor activity in adult rats (39). Thus, locomotor activity was measured as a “positive control” to verify that our methamphetamine treatments produced a behavioral effect that is consistent with previous work. Spontaneous locomotor activity was measured using a two-channel Opto-M4 Auto-Track System (Columbus Instruments, Columbus, OH). This apparatus (44 x 44 x 21 cm) is equipped with 16 lasers (spaced 2.5 cm apart) on each axis. Each rat was individually placed in the center of the open field, and locomotor activity (determined by the total number of photo beam breaks) was monitored for 20 min. Testing was done between 0900 and 1100 in a dark room illuminated by a red 60-W light bulb. This behavioral test was performed 2–3 days prior to euthanization for Langendorff isolated heart experiments.

**Langendorff isolated heart experiments.** Rats were anesthetized with pentobarbital sodium (75 mg/kg ip) at 8 wk of age. Hearts were excised. The heart was submerged in Krebs solution to maintain constant pressure of 80 mmHg. Contractile function of the left ventricle was measured using an intraventricular balloon connected to a pressure transducer. The balloon was inflated to an end-diastolic pressure of 4 mmHg, and data were continually recorded using a Powerlab 4SP data acquisition system (AD Instruments, Colorado Springs, CO). The heart was submerged in Krebs solution to maintain the temperature of the heart at 37.5°C throughout the experiment, and temperature was continuously monitored using a thermocouple placed on the surface of the heart. Hearts were equilibrated for 25 min prior to the onset of 20 min of ischemia and 2 h of reperfusion. Hearts were excluded after the 25-min equilibration period if developed pressure was <100 mmHg, if coronary flow rate was >20 ml/min, or if there were persistent arrhythmias. Preischemic contractile function was measured immediately prior to ischemia. Postischemic recovery of contractile function was measured following 1 h of reperfusion. Hearts were perfused for an additional 1 h (2 h of total reperfusion) prior to triphenyltetrazolium chloride staining.

**Measurement of infarct size.** Hearts were perfused with 1% triphenyltetrazolium chloride at 7.5 ml/min for 8 min and then submerged in 1% triphenyltetrazolium chloride and incubated at 37°C for 15 min. Hearts were subsequently frozen at −80°C, sliced into ~1-mm sections, soaked in 10% neutral buffered formalin, and then photographed with a Nikon SMZ 800 microscope equipped with a Nikon DS-Fi1 digital camera. The infarcted surface area and total surface area of each slice were measured using ImageJ software (National Institutes of Health). Infarct size is expressed as a percentage of the area at risk. The area at risk was defined as the entire ventricular myocardium, since hearts were exposed to global ischemia.

**Western blots.** Hearts were perfused for 5 min on the Langendorff isolated heart system to flush blood from the tissue. The left ventricle was immediately flash-frozen in liquid nitrogen and stored at −80°C. Ventricular tissue was homogenized with a Polytron in homogenization buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 1% sodium dodecyl sulfate, phosphatase inhibitor cocktail 2 (catalog no. P5726, Sigma), phosphatase inhibitor cocktail 3 (catalog no. P0044, Sigma), and protease inhibitor cocktail (catalog no. P8340, Sigma)) and immediately boiled for 5 min. Homogenates were centrifuged at 4°C for 10 min at 14,000 rpm. Supernatant protein (30 μg) was separated on a 10% polyacrylamide electrophoresis gel and subsequently transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk and then blotted overnight for PKCe, phosphorylated ERK, and phosphorylated Akt. Membranes were stripped and reblotted for total ERK, total Akt, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All antibodies (catalog nos. 4376, 9271, 9102, and 9272) were obtained from Cell Signaling Technology (Danvers, MA), except for the PKCe antibody (catalog no. sc-214), which was obtained from Santa Cruz Biotechnology (Dallas, TX). Western blots were quantified by measurement of band densities with ImageJ software. Band intensities of phosphorylated ERK were divided by band intensities of total ERK. Band intensities of phosphorylated Akt were divided by band intensities of total Akt. PKCe expression was normalized to GAPDH.

**Statistical analysis.** Most data (cardiac contractile function, infarct size, PKCe expression, ERK phosphorylation, Akt phosphorylation, and spontaneous locomotor activity) were compared by two-way ANOVA and Tukey’s post hoc analysis, with sex and drug treatment (prenatal saline or prenatal methamphetamine) as factors. Body weights were compared by three-way ANOVA, with sex, drug treatment (prenatal saline or prenatal methamphetamine), and age (repeated measures at 21 days and 8 wk of age) as factors. Litter sizes were compared by independent t-test, with drug treatment (prenatal saline or prenatal methamphetamine) as the independent factor. P ≤ 0.05 was considered statistically significant.

**RESULTS**

**Effects of methamphetamine on maternal body weight, litter size, and pup body weight.** The rate of body weight gain in pregnant rats injected with methamphetamine was similar to that in rats injected with saline (Fig. 1). All females delivered pups on gestational day 21 or 22. Methamphetamine treatment had no effect on litter size. Litter size was 12 ± 1.2 pups in methamphetamine-treated females (n = 6 pregnant rats) and 14 ± 0.4 pups in saline-injected females (n = 6 pregnant rats). Methamphetamine caused no noticeable physical abnormalities, and all pups survived until 8 wk of age, when they were euthanized for experiments.

Body weights of pups were measured at 21 days and 8 wk of age (Fig. 2). Three-way ANOVA (with sex, prenatal metham-

![Fig. 1. Effect of methamphetamine on maternal body weight.](http://ajpheart.physiology.org/)}
Prenatal methamphetamine treatment and repeated measures of age as factors) revealed a significant overall effect of time, sex, and prenatal methamphetamine treatment \( [F = 5.82 (112,3), P = 0.001] \). As expected, body weights of male and female pups were significantly greater at 8 wk than 21 days of age \( [F = 132 (63,1), P < 0.0001] \), and body weights of 8-wk-old males were significantly greater than body weights of 8-wk-old females \( [F = 450 (67,1), P < 0.001] \), irrespective of prenatal exposure to methamphetamine. Prenatal methamphetamine treatment had no effect on male or female body weight at 21 days (Fig. 2A) or 8 wk (Fig. 2B) of age.

Prenatal methamphetamine decreases locomotor activity in adult female rats. The effect of prenatal methamphetamine on adult cardiovascular function has not been previously investigated. However, the behavioral effects of prenatal methamphetamine are well established. In light of the possibility that prenatal methamphetamine would have no effect on the ischemic heart, we used locomotor activity as a “positive control” to verify that our methamphetamine treatments had a behavioral effect that is consistent with previous work and to confirm the efficacy of prenatal methamphetamine treatment in our own hands. Locomotor activity was measured 2–3 days prior to euthanization of the rats for isolated heart experiments. Consistent with the work of Schutova et al. (39), prenatal methamphetamine had a sex-dependent effect on locomotor activity. Prenatal methamphetamine significantly decreased locomotor activity of adult females but had only a small (statistically insignificant) effect in males (Fig. 3). These data confirm the previous report that prenatal methamphetamine has sex-dependent effects on locomotor activity (39). More importantly, these data affirm that prenatal methamphetamine treatment in our own laboratory has an effect that is consistent with the findings of other investigators.

Prenatal methamphetamine exerts sex-dependent effects on the ischemic myocardium during adulthood. The impact of prenatal methamphetamine on the adult heart was examined in hearts from 8-wk-old male and female rats subjected to 20 min

![Graph](http://ajpheart.physiology.org/Downloadedfrom)
of global ischemia followed by reperfusion. Infarct sizes were measured by triphenyltetrazolium chloride staining. Infarcts were significantly smaller in saline-treated female than salineltreated male hearts (Fig. 4). Prenatal methamphetamine had no effect on infarct size in male hearts (Fig. 4). In contrast, methamphetamine significantly increased infarct size in female hearts.

Preischemic parameters of contractile function in male and female hearts were similar, irrespective of treatment with prenatal methamphetamine (Table 1). Parameters of postsischemic recovery of contractile function were independently analyzed as absolute values (e.g., mmHg, mmHg/s, and beats/min) and as percentages of their preischemic contractile function. Two-way ANOVA (with sex and methamphetamine as factors) and Tukey’s post hoc analysis indicated that postsischemic recovery of $-\frac{dP}{dt}$ was significantly greater in female than male hearts, irrespective of exposure to prenatal methamphetamine [$F = 119.7$ (1, 23), $P < 0.001$; Table 1]. Recovery of $+\frac{dP}{dt}$ was significantly greater in hearts from saline-treated female than saline-treated male rats when the data were expressed as a percentage of their preischemic values [$F = 7.2$ (23, 1), $P = 0.05$]. Postsischemic recovery of pressure was also greater in female than male rats [$F = 5.4$ (1, 23), $P < 0.005$], but Tukey’s post hoc analysis identified no significant interaction between sex and methamphetamine treatment.
significant differences in developed pressure among individual experimental groups. Methamphetamine had no significant effect on any parameters of postischemic recovery of contractile function in male or female hearts (Table 1).

Prenatal methamphetamine differentially alters the expression and phosphorylation status of cardioprotective proteins in adult male and female hearts. Prenatal cocaine worsens ischemic injury in adult male hearts, and this effect has been attributed to decreased expression of PKCε (3, 30, 31, 47, 48). Thus we examined the impact of prenatal methamphetamine on PKCε expression in 8-wk-old male and female hearts. Prenatal methamphetamine had no effect on PKCε expression in male hearts (Fig. 5A). However, expression of this cardioprotective protein was significantly decreased in female hearts prenatally exposed to methamphetamine (Fig. 5).

We also examined the impact of prenatal methamphetamine on basal levels of Akt and ERK phosphorylation. Methamphetamine had no effect on Akt phosphorylation in male hearts (Fig. 5B). Female hearts exhibited a small, but statistically significant, decrease in Akt phosphorylation (Fig. 5B). Methamphetamine had no effect on ERK phosphorylation in male or female hearts (Fig. 5C).

DISCUSSION

The primary finding of this study is that prenatal exposure to methamphetamine worsens cardiac ischemic injury in the adult heart and that this occurs in a sex-dependent manner. Prenatal methamphetamine also induced sex-dependent decreases in the expression of PKCε and phosphorylation of Akt, proteins that are known to play important roles in cardioprotective signaling pathways. These findings are consistent with prior work demonstrating that prenatal cocaine, another illicit sympathomimetic drug, also worsens ischemic injury in the adult heart (3, 4). This is the first study, to our knowledge, to demonstrate that prenatal exposure to methamphetamine alters cardiovascular function in the adult rat.

PKCε and Akt have well-established roles in protecting the heart from ischemic injury. Phosphorylation of these proteins is a critical step in the activation of cardioprotective signaling pathways (6, 13, 17). PKCε expression and Akt phosphorylation were decreased in female rats following prenatal exposure to methamphetamine. These data do not establish a cause-effect relationship between these cardioprotective proteins and myocardial sensitivity to ischemic injury in methamphetamine-treated rats, but they do correlate with the observation that prenatal methamphetamine increased myocardial sensitivity to ischemic injury in female hearts, but not male hearts, where we found no effect of prenatal methamphetamine on Akt phosphorylation or PKCε expression. These findings are also consistent with previous work suggesting that prenatal cocaine worsens ischemic injury by causing epigenetic changes in PKCε expression (3, 30, 31, 47, 48).
In addition to prenatal methamphetamine and cocaine, prenatal nicotine also suppresses PKCε expression in the adult heart (24, 30, 34, 45) and increases the susceptibility of adult offspring to myocardial ischemic injury (3, 25, 26, 46). Suppression of PKCε expression results from cocaine- or nicotine-induced methylation of CpG dinucleotide sequences in the promoter region of DNA encoding this cardioprotective protein (24, 30, 47). A common link between cocaine, nicotine, and methamphetamine is that prenatal use of each of these substances decreases uterine blood flow (9, 27, 32, 33, 40, 43) and decreases the partial pressure of oxygen in fetal circulation (1, 16, 42). Fetal hypoxia also decreases cardiac PKCε expression (34) and increases myocardial sensitivity to ischemic injury (26, 46) in adult offspring. Collectively, these data suggest that the detrimental effects of prenatal methamphetamine, cocaine, and nicotine on the ischemic adult heart may be a consequence of fetal hypoxia (1, 16, 42), rather than a direct pharmacological effect of these drugs on the heart. However, other potential mechanisms may contribute to worsening of ischemic injury. Like cocaine, maternal methamphetamine administration increases fetal blood catecholamine concentrations (12) and fetal blood pressure (43, 44). Chronic β-adrenergic receptor stimulation (19, 20) and hypertension (36) increase myocardial sensitivity to ischemic injury. Thus, chronic elevation of blood pressure or chronic β-adrenergic receptor stimulation in utero could potentially induce changes in the heart that contribute to the worsening of ischemic injury.

Despite increased infarct sizes in female hearts, postischemic recovery of contractile function was unaffected by methamphetamine. Many investigators have observed a correlation between increased infarct size and decreased postischemic recovery of contractile function following an ischemic insult. However, others have reported that postischemic recovery of contractile function does not always correlate with infarct size (10, 35, 38, 44). Some investigators have attributed this disparity to the phenomenon of myocardial stunning in which myocytes are viable but do not contract properly following reperfusion (8). Contractile function measurements may also be confounded by the low coronary flow rate during reperfusion. Postischemic coronary flow rates in hearts from saline- and methamphetamine-exposed rats were 31–38% lower than their respective preischemic flow rates (Table 1). This decrease in coronary perfusion and subsequent decrease in the delivery of oxygen may suppress contractile function and mask differences in the contractile capacity of myocytes. In light of these factors, infarct size is generally regarded as more reliable than postischemic recovery of contractile function as an indicator of ischemic injury (10, 28, 38).

In addition to the effect of methamphetamine, we also observed that infarcts in hearts from saline-treated male rats were significantly larger than those from saline-treated female rats (Fig. 4). This sex-dependent effect has been reported by others (7, 22). Some have attributed this finding to increased activity of sarcolemmal ATP-sensitive potassium channels in the female heart (7, 22). Others have reported that reperfusion of female, but not male, hearts is accompanied by increased signaling through PKCε and Akt (5). This is consistent with our finding that PKCε expression is increased in hearts from saline-treated female rats compared with saline-treated male rats. We also observed a modest (but statistically insignificant) increase in Akt phosphorylation in saline-treated female hearts compared with saline-treated male hearts. These data support the work of previous investigators who reported that female hearts are more resistant to ischemic injury than male hearts as a result of enhanced signaling through these cardioprotective pathways (5).

Prenatal methamphetamine increased myocardial sensitivity to ischemic injury, suppressed cardiac PKCε expression, decreased Akt phosphorylation, and decreased locomotor activity in female rats. However, none of these effects were observed in their male siblings. Consistent with our data, Schutova et al. (39) reported that prenatal methamphetamine decreased locomotor activity in adult female rats but had no significant effect on locomotor activity in male rats. Prenatal methamphetamine also alters tyrosine hydroxylase expression in dopaminergic neurons of different brain regions in a sex-dependent manner (14, 15). Other sex-dependent effects of prenatal methamphetamine, some of which occur exclusively in males, have been reported (14, 18, 40). It is unclear why the effects of prenatal methamphetamine on the adult heart occurred exclusively in females. It is possible that the sex-dependent effects of prenatal methamphetamine reported here and in previous studies result from sex differences in the expression of different types of adrenergic or dopaminergic receptors or their downstream signaling proteins in different regions of the brain and peripheral tissues in the male and female fetus. However, this is speculative in the absence of data to directly compare the expression and function of different adrenergic or dopaminergic receptor subtypes in male and female embryonic tissues. Further work is needed to better understand the mechanism by which prenatal methamphetamine induces sex-dependent effects in male and female adult offspring.

Few studies have addressed the potential effects of prenatal methamphetamine on the adult cardiovascular system. Inoue et al. (21) reported an increased number of mRNA transcripts encoding α- myosin heavy chain and disarrangement of myofibers in newborn rat pups, but these effects were reversed by postnatal days 9 and 14, respectively. We are unaware of any other animal studies investigating the effects of prenatal methamphetamine on the adult heart. Human studies investigating the potential impact of prenatal methamphetamine use on adult cardiovascular function are also absent from the published literature. This may reflect the fact that methamphetamine abuse has not been a widespread problem for a long enough period of time to study the effects of prenatal exposure of this drug on the outcome of middle-aged or elderly individuals who have a higher prevalence of cardiovascular disorders.

In conclusion, our work provides the first evidence that prenatal methamphetamine worsens ischemic injury in the adult heart and that this occurs in a sex-dependent manner. An important difference between this study and human use of methamphetamine is that humans tend to use methamphetamine at irregular intervals characterized by acute binges separated by time periods without the drug. In contrast, the rats in this study received methamphetamine at regular daily time intervals that may not mimic the typical human pattern of methamphetamine abuse. In addition, we induced ischemia in hearts from young adult rats. In contrast, most human myocardial infarctions occur in individuals of advanced age. It is unknown how prenatal methamphetamine might influence myocardial sensitivity to ischemic injury in older rats or in the human geriatric population. These factors are limitations of
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

B.R.R. and M.S.D. developed the concept and designed the research; B.R.R., S.L.S., A.D.B., L.S., and M.S.D. performed the experiments; B.R.R. and M.S.D. analyzed the data; B.R.R. and M.S.D. interpreted the results of the experiments; B.R.R. prepared the figures; B.R.R. drafted the manuscript; B.R.R. and M.S.D. edited and revised the manuscript; B.R.R. and M.S.D. approved the final version of the manuscript.

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