Pre-eclamptic Fetal Programming Alters Neuroinflammatory and Cardiovascular Consequences of Endotoxemia in Sex-Specific Manners

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ABSTRACT

Pre-eclampsia (PE)-induced fetal programming predisposes offspring to health hazards in adult life. Here, we tested the hypothesis that pre-eclamptic fetal programming elicits sexually dimorphic inflammatory and cardiovascular complications to endotoxemia in adult rat offspring. PE was induced by oral administration of L-NAME (50 mg/kg per day for seven consecutive days) starting from day 14 of conception. Cardiovascular studies were performed in conscious adult male and female offspring preinstrumented with femoral indwelling catheters. Compared with non-PE male counterparts, intravenous administration of lipopolysaccharide (LPS, 5 mg/kg) to PE male offspring caused significantly greater 1) falls in blood pressure, 2) increases in heart rate, 3) rises in arterial dP/dtmax, a correlate of left ventricular contractility, and 4) decreases in time- and frequency-domain indices of heart rate variability (HRV). By contrast, the hypotensive and tachycardic actions of LPS in female offspring were independent of the pre-eclamptic state and no clear changes in HRV or dP/dtmax were noted. Measurement of arterial baroreflex activity by vasoactive method revealed no sex specificity in baroreflex dysfunction induced by LPS. Immunohistochemical studies showed increased protein

expression of toll-like receptor 4 in heart as well as in brainstem neuronal pools of the nucleus of solitary tract and rostral ventrolateral medulla in endotoxic PE male, but not female, offspring. Enhanced myocardial, but not neuronal, expression of monocyte chemoattractant protein-1 was also demonstrated in LPS-treated male offspring. Together, pre-eclamptic fetal programming aggravates endotoxic manifestations of hypotension and autonomic dysfunction in male offspring via exacerbating myocardial and neuromedullary inflammatory pathways.

SIGNIFICANCE STATEMENT

Current molecular and neuroanatomical evidence highlights a key role for pre-eclamptic fetal programming in offspring predisposition to health hazards induced by endotoxemia in adult life. Pre-eclampsia accentuates endotoxic manifestations of hypotension, tachycardia, and cardiac autonomic dysfunction in male offspring via exacerbating myocardial and central inflammatory pathways. The absence of such detrimental effects in female littermates suggests sexual dimorphism in the interaction of pre-eclamptic fetal programming with endotoxemia.

Introduction

Pre-eclampsia (PE) is a new-onset gestational hypertensive status that is commonly associated with aggravated proteinuria, renal insufficiency, and impaired liver function (Brown et al., 2018). PE complicates almost 5% of all pregnancies (Morton, 2016) and negatively affects maternal and fetal health (Fox et al., 2019). Adverse fetal consequences involve antenatal risks of intrauterine growth restriction, preterm birth, and possibly fetal death in utero (Haddad et al., 2004; Madazli et al., 2014; Rezk et al., 2015). Moreover, in utero exposure to hypertensive state during pregnancy can

result in long-term cardiovascular sequelae in offspring, including early onset hypertension, and increased risk of ischemic heart disease and stroke (Davis et al., 2012). The term “fetal programming” describes developmental fetal adaptations during pregnancy with consequent cardiovascular, metabolic, and endocrine disorders in adulthood (Godfrey and Barker, 2001). During PE, the harsh intrauterine environment, induced by angiogenic, inflammatory, and hypoxic insults, causes genomic alterations in mother and fetus that would ultimately modify the expressed phenotype (Stojanovska et al., 2016). Fetal programming in response to prenatal stress alters offspring cardiovascular health. Litters of prenatally stressed rats elicit exaggerated and more sustained elevations in BP and its variability, with female offspring showing more dramatic effects compared with their male counterparts (Igosheva et al., 2004).

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ABBREVIATIONS: BP, blood pressure; BRS, baroreflex sensitivity; FFT, fast-Fourier transform; HF, high frequency; HR, heart rate; HRV, heart rate variability; LF, low frequency; LPS, lipopolysaccharide; LV, left ventricular; MAP, mean arterial pressure; MCP-1, monocyte chemoattractant protein-1; NTS, nucleus tractus solitarius; PE, pre-eclampsia; rMSSD, root mean square of successive beat-to-beat differences in R-R interval durations; RVLM, rostral ventrolateral medulla; SBP, systolic blood pressure; SDNN, SD of beat-to-beat intervals; TLR-4, toll-like receptor 4.

Various animal models of PE are available. An ultra-low-dose of LPS has been used to replicate PE manifestations in rats including hypertension, platelet coagulopathy, and glomerular fibrinogen deposition (Faas et al., 1994). Alternatively, chronic inhibition of nitric oxide synthase with L-NAME (N ω -Nitro-L-arginine methyl ester hydrochloride) in pregnant rats results in hypertension accompanied by thrombocytopenia, reduced glomerular filtration rate, proteinuria, and intrauterine growth restriction (McCarthy et al., 2011; Marshall et al., 2018). Unlike LPS model of PE, serum-free fatty acid, hepatic, and placental fatty deposition are elevated in L-NAME treated dams (Han et al., 2015). This could be related to different pathogenic pathways in the two models, with primarily fatty acid oxidative disorders in L-NAME model and aberrant inflammatory cascade through the activation of the NF- κ B signaling pathway, and damage of endothelial cells in LPS model (Ding et al., 2015). Physiologic and histologic perturbations induced by gestational L-NAME largely resemble clinical manifestations of PE. This includes 1) elevated placental expression of inflammatory, antiangiogenic, and apoptotic factors; 2) reduced antioxidant activity; and 3) increased glomerular area, capillary structure disorder, abnormal protein cast, and reduced nephron numbers (Amaral et al., 2018; Shu et al., 2018; Chen et al., 2019; Zheng et al., 2019).

Sepsis is a fatal organ dysfunction caused by disrupted host response to infection. Endotoxemia is employed to model the hyperinflammatory state characteristic of early sepsis (Dickson and Lehmann, 2019). LPS from Gram negative pathogens is classically used to fire the immune responses, leading to hyperinflammation and microcirculatory perturbations (Dickson and Lehmann, 2019). Experimental data from our laboratory (Sallam et al., 2016) and others (Zila et al., 2015) demonstrated that endotoxemia elicits hypotensive, tachycardic, and cardiac autonomic depressant consequences. These harmful derangements could be related to the excessive generation of inflammatory cytokines, which induce mitochondrial damage, cripple calcium homeostasis, and perturb the neuroautonomic tone (Flierl et al., 2008). We and others have previously reported sexual dimorphism in immune response to endotoxemia, with females experiencing less adverse clinical outcomes than age-matched males (Saia et al., 2015; Klein and Flanagan, 2016; El-Lakany et al., 2018).

Although sex differences in cardiovascular consequences of endotoxemia have been demonstrated, the influence of PE on cardiovascular sequelae of endotoxemia in adult offspring remains largely obscure. This prompted us to test the novel hypothesis that PE fetal programming elicits sexually dimorphic inflammatory, cardiovascular, and autonomic complications in response to endotoxemia in adult rat offspring. Endotoxemia was induced by intravenous administration of a single dose of LPS (5 mg/kg) (Lv et al., 2006) in conscious age-matched PE and non-PE male and female offspring. Cardiac autonomic activity was assessed by: 1) HRV analysis, which determines global autonomic control and cardiac sympathovagal balance (Stein et al., 1994; El-Mas and Abdel-Rahman, 2013), and 2) arterial baroreceptor testing, which symbolizes reflex control of cardiac autonomic function (Smyth et al., 1969). Molecular studies were undertaken to assess inflammatory state in myocardial tissues as well as in brainstem neuronal pools of the nucleus tractus solitarius (NTS) and rostral ventrolateral medulla (RVLM). The latter are

brainstem nuclei that play critical roles in cardiovascular and cardiac autonomic control (Saha, 2005) as well as in central processing of central neuroinflammatory signals including those triggered by the endotoxic insult (Sirivelu et al., 2012; Sallam et al., 2019).

Materials and Methods

Adult Wistar rats (170–240 g; Faculty of Pharmacy Animal Facility, Alexandria University, Alexandria, Egypt) were used in this study. All experiments were approved by the Institutional Animal Care and Use Committee and carried out in accordance with the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) as adopted and promulgated by the National Institutes of Health. This protocol was approved by the Institutional Animal Care and Use Committee, Alexandria University, Egypt (Approval No. AU0620191113262).

Induction of Pre-eclampsia. Pregnancy was induced by introducing adult nulliparous female rats to larger males (ratio 1:1) to allow for overnight mating. The day of conception was determined by checking the spermatozoa in the vaginal lavage or detection of a vaginal plug. PE was induced by daily oral administration of 50 mg/kg of L-NAME for 7 days starting from day 14 of conception (Pandhi et al., 2001). The development of PE was assessed by measurement of systolic blood pressure (SBP) by the tail cuff technique as well as by assessment of urinary protein level. Ten weeks after spontaneous delivery, adult male and female offspring were processed for measurement of SBP and proteinuria. Rats were then subjected to intravascular cannulation for undertaking cardiovascular studies as detailed below.

Tail-Cuff Plethysmography. Tail-cuff measurements of SBP were conducted in conscious pregnant rats and adult offspring using a computerized data acquisition system with LabChart-7 pro software (Power Laboratory 4/30, model ML866/P; AD Instruments, Bella Vista, Australia) as described in our previous studies (El-Mas et al., 2015). Heart rate (HR) was computed from BP waveforms and displayed on another trace of the recording system. A specialized tail cuff and pulse transducer (Pan Laboratory, Spain) were used for SBP measurement depending on the periodic obstruction of tail arterial blood flow. SBP was measured three or four times and values were averaged to the mean.

Urine Collection and Protein Analysis. For 24-hour urine collection, pregnant dams (gestational day 20) and adult offspring (10 weeks old) were housed in metabolic cages with stainless steel mesh wire bottom. Rats were allowed ad libitum access to standard rat chow and water. Urine samples were collected under light mineral oil and kept at -80°C until processed (El-Mas and Abdel-Rahman, 2007). Urinary protein levels were assessed using pyrogallol red method (Zhang et al., 2009) using Cromatest standard kit (LiNEAR Chemicals, Spain) according to the manufacturer's guidelines.

Intravascular Cannulation. Rats were anesthetized with thiopental (50 mg/kg, i.p.) and femoral artery and vein cannulation was performed as described previously (el-Mas et al., 1997; El-Mas and Abdel-Rahman, 1999; El-Mas et al., 2006, 2009). As detailed below, full cardiovascular and autonomic investigations were conducted 2 days later in conscious, freely moving rats.

Time-Domain Analysis of HRV. Two time-domain measures of the cardiac autonomic activity were used, the SD of beat-to-beat intervals (SDNN) and the root mean square of successive beat-to-beat differences in R-R interval durations (rMSSD) (Stein et al., 1994; Omar and El-Mas, 2004). The R-R intervals were computed from the HR (i.e., the reciprocal of the HR in milliseconds). The SDNN is comparable to the total power of the spectrum of R-R variability, which measures the overall autonomic balance of the heart. The rMSSD is largely validated as a measure of the parasympathetic input to the heart and, therefore, correlates with the high-frequency power of the spectrum (Stein et al., 1994; Sgoifo et al., 1997). SDNN and rMSSD

were measured before (baseline) and at 15-minute intervals after drug treatments. For each time point, the 5-minute values of each variable were averaged.

Frequency-Domain Analysis of HRV. Spectral hemodynamic fluctuations, quantitative indices of cardiac autonomic control (Stein et al., 1994; El-Mas and Abdel-Rahman, 2007; Sallam et al., 2016), were used to reflect changes in sympathetic and vagal outflows. Hemodynamic variability was analyzed in the frequency domain using fast-Fourier transform (FFT) algorithms of R-R data series. The FFT algorithm for direct transformation of data points into power spectral density graphs was used. Data were interpolated to obtain equally spaced samples with an effective sampling frequency of 10 Hz (0.1 seconds). A second-order interpolation was used to fit a smooth curve to the existing data points and to produce smoother visual representation of data. The evenly spaced (equidistant) sampling allowed direct spectral analysis using the FFT algorithm. Spectra were integrated into two specific frequency bands, low-frequency (LF) (0.25–0.75 Hz) and high-frequency (HF) (0.75–3 Hz) bands and expressed in normalized units (LF_{nu} and HF_{nu}). Spectral data were estimated before (baseline) and at 15-minute intervals after drug treatments. For each time point, the 5-minute values of each variable were averaged.

Baroreflex Testing. Baroreflex sensitivity (BRS) was evaluated using the vasoactive method (Smyth et al., 1969; El-Mas et al., 2012). This method is based on the measurement of bradycardic and tachycardic responses to peripherally induced increases and decreases in BP evoked by bolus intravenous injections of randomized doses of phenylephrine (BRS_{phenylephrine}) and sodium nitroprusside (BRS_{nitroprusside}) (1–16 µg/kg each), respectively, at 5-minute intervals. The relationship between changes in MAP and associated reciprocal changes in HR were assessed by regression analysis for individual animals. The slope of the dynamic part of the curve expressed as beats per minute per millimeters of Hg was considered as an index of BRS.

Immunohistochemistry. The technique described in our previous studies (El-Mas et al., 2006; Helmy et al., 2015; Sallam et al., 2019) was employed for immunohistochemical measurement of protein expression of TLR-4 and MCP-1 in rat heart (anterior portion of the apex) and brainstem pools of NTS and RVLM. Cardiac and brain

tissues were fixed in 10% formaldehyde solution and embedded in paraffin blocks. Sections (5 µm) of heart or brainstem (–12.0 to –12.48 mm relative to bregma, see Fig. 1) were cut and put on positively charged adhesive glass slides (Thermo Scientific, Berlin, Germany), then deparaffinized in xylene and rehydrated in a series of declining ethanol concentration (100%, 95%, and 70%). Heat-induced epitope retrieval was performed by immersing slides in coplin jars containing 10 mM citrate buffer solution and incubated in a microwave at power 100 for 1 minute then power 30 for 9 minutes. Endogenous peroxidases were blocked by 3% hydrogen peroxide for 10 minutes. The primary polyclonal antibodies, rabbit anti-TLR-4 (PA5-23124), rabbit anti-MCP-1 (PA5-34505) (ThermoFisher Scientific), were diluted (1:300) as instructed by the manufacturer, applied to the slides and then sections were incubated at 4°C overnight. The secondary antibody (horseradish peroxide conjugate) was applied for 30 minutes. The chromogen 3,3'-diaminobenzidine was prepared and applied as instructed by the manufacturer for protein visualization. Slides were counterstained with hematoxylin and dipped in ascending concentrations of alcohol and then xylene. Images were taken by OptikamB9 digital camera (Optika microscopes, Italy) and Fiji Image J software version 1.51n (National Institutes of Health, Bethesda, MD) was employed to quantitate the area fraction of chromogen 3,3'-diaminobenzidine-positive staining in cardiac and brainstem areas of NTS and RVLM.

Measurement of Serum MCP-1. Blood samples were withdrawn at the end of each experiment through the arterial catheter. The collected blood was centrifuged at 1118g for 10 minutes and serum was stored at –80°C for subsequent analyses. Serum MCP-1 was measured using the MCP-1 Rat Instant ELISA Kit (cat no: BMS631INS; ThermoFisher Scientific) according to the manufacturer's protocol.

Protocols and Experimental Groups. Eight groups of rats (*n* = 8 each) preinstrumented with femoral indwelling catheters were used to test the effect of PE on cardiovascular, autonomic, and inflammatory manifestations of endotoxemia in male and female offspring. For each sex, four groups of rats were employed and categorized into: 1) saline-treated non-PE offspring, 2) LPS-treated non-PE offspring, 3) saline-treated PE offspring, and 4) LPS-treated PE offspring. On the experiment day, 2 days after femoral catheterization, the arterial

Fig. 1. Anatomic localizations of the NTS and RVLM of rat brainstem.

catheter was connected to a BP transducer (model P23XL; Astro-Med, West Warwick, RI) that was connected through MLAC11 Grass adapter cable to a computerized data acquisition system with LabChart7 pro software (Power Laboratory 4/35, model ML866/P; AD Instruments Pty Ltd., Castle Hill, Australia). The HR was computed from BP waveforms and displayed on another trace of the recording system.

After an initial stabilization period of at least 30 minutes, rats were allocated to receive intravenous dose of LPS (5 mg/kg) (Lv et al., 2006) or equal volume of saline over a period of 10 minutes. Hemodynamic monitoring continued for 2 hours post-treatment. Changes in MAP, HR, time (SDNN, rMSSD) and spectral (total power and LF:HF ratio) measures of HRV, maximum rate of rise in left ventricular pressure (dp/dt max, a correlate of systolic contractility), were computed at 15-minute intervals. The cumulative hypotensive effect of LPS over the entire 2-hour observation period of the study was computed by measuring the area under the curve of the hypotensive response for individual experiments using trapezoidal integration with zero line taken as the baseline (GraphPad Prism, version 3.02). This was computed by summing incremental areas of each trapezoid below the effect-time curve. After the 2-hour hemodynamic monitoring period, baroreflex curves were constructed by the vasoactive method as described above. Afterward, rats were euthanized using an overdose of thiopental (100 mg/kg), and hearts and brainstems were quickly removed, fixed in 10% formaldehyde solution, and embedded in a paraffin blocks within 24 hours to be used for immunohistochemical assessment of the protein expression of TLR-4 and MCP-1.

Drugs. Thiopental (Thiopental; Biochemie GmbH, Vienna, Austria), povidone-iodine solution (Betadine; Nile Pharmaceutical Co., Cairo, Egypt), Pencitard (NCPC North best Co., Hebei, China), Heparin (5000 IU/ml; Nile pharmaceutical Co.), LPS (from *Escherichia coli* 0111: B4), L-NAME, phenylephrine hydrochloride, and sodium nitroprusside (Sigma Aldrich, St. Louis, MO) were purchased from commercial vendors. All drugs were dissolved in saline.

Statistical Analysis. Values are expressed as means $\pm$ S.E.M. The unpaired Student *t* test or the one-way or repeated measures ANOVA followed by the Tukey's post hoc test was used to test for statistical significance. These analyses were performed by GraphPad InStat, software release 3.05. Probability levels less than 0.05 were considered significant.

Results

L-NAME Induces Pre-eclamptic Manifestations in Pregnant Female Rats. The daily treatment of pregnant rats with L-NAME (50 mg/kg per day) for seven consecutive days resulted in significant rises in SBP (137.2 ± 5.2 vs. 109.9 ± 1.7 mm Hg) and falls in HR (336.1 ± 7.7 vs. 376.5 ± 8.3 beats/min) compared with saline-treated (non-PE) values. In 24-hour urine samples collected during the 20th day of gestation, significantly higher quantities of protein were detected in urine of PE (333.3 ± 51.1 mg/dl) compared with non-PE females (72.3 ± 6.8 mg/dl). This corresponds to approximately a fivefold increase in urine protein in PE females.

Effects of PE on Baseline Cardiovascular and Autonomic Functions in Male and Female Offspring. Table 1 summarizes baseline values of cardiovascular and autonomic functions in 10-week-old male and female offspring of PE and non-PE rats measured prior to intravenous administration of LPS or saline. The body weights of PE offspring (male or female) were significantly less than those of non-PE offspring of the same sex. Moreover, compared with their respective non-PE counterparts, male offspring of PE rats exhibited significantly 1) higher levels of proteinuria and SBP, and 2) lower baseline values of time (SDNN and rMSSD) and frequency (total power) indices of HRV. Unlike male offspring, none of these

parameters was different between PE and non-PE female offspring (Table 1).

Pre-eclampsia Exacerbates Endotoxic Cardiovascular Manifestations in Male, but Not, Female Offspring.

The effects of PE on cardiovascular and autonomic responses to endotoxemia in adult offspring are shown in Figs. 2–5. In all rat groups, intravenous infusion of LPS (5 mg/kg) resulted in significant falls in BP compared with values of saline-treated rats during the 2-hour duration of the experiment (Fig. 2, A and C). The hypotensive action of LPS peaked at approximately 60 minutes and declined thereafter and was associated with synchronized rises in HR (Fig. 2, B and D). Both the hypotensive and tachycardic effects of LPS were significantly potentiated in PE male offspring compared with their non-PE counterparts. Such PE effects were not evident in female offspring, as similar decreases and increases in MAP and HR, respectively, were observed in PE and non-PE female offspring.

The areas under the LPS hypotensive curves, which represent cumulative falls in blood pressure over the 2-hour observation period, were computed to determine the intersex differences in the LPS response. Significantly greater areas under the curves of LPS hypotension were noted in female compared with male offspring of non-PE rats (60 ± 4 vs. 48 ± 3 mm Hg.min, $P < 0.05$). In PE rats, by contrast, the accentuated LPS hypotension in male offspring resulted in significantly greater areas under the curves than those of female offspring (96 ± 16 vs. 64 ± 8 mm Hg.min, $P < 0.05$).

Changes in time and frequency measures of HRV were determined as indices of cardiac autonomic activity. The data revealed that the time-domain parameter SDNN (Fig. 3A) as well as total power of the spectral profile (Fig. 4A), measures of the overall cardiac autonomic control, were significantly reduced at some time points after LPS injection in non-PE male offspring. These effects were more intensified in LPS-treated male offspring of PE mothers and remained so throughout the 2-hour observation period of the study (Figs. 3A and 4A). Similar patterns of enhanced LPS-induced reductions in the time (rMSSD, Fig. 3B) and frequency domain (HF_{nu}, Fig. 4B) indices of cardiac vagal activity in male offspring of PE compared with respective non-PE offspring. By contrast, spectral measures of cardiac sympathetic activity (LF_{nu}, Fig. 4C) or sympathovagal balance (LF:HF ratio, Fig. 4D) were not altered by LPS in all male offspring preparations. In female offspring of PE or non-PE mothers, the time-domain parameters of SDNN (Fig. 3C) and rMSSD (Fig. 3D) showed slight, inconsistent, and similar reductions in response to LPS. None of the spectral indices of HRV was altered by LPS in female offspring (data not shown).

LPS did not affect the maximum rate of rise of BP waves (dp/dtmax, correlate of LV contractility) in non-PE male (Fig. 5A) or female (Fig. 5B) offspring. The changes caused by LPS in these parameters were not statistically different from those seen in saline-treated rats. However, a significant increase in LV contractility was observed in male offspring of PE endotoxic rats (Fig. 5A).

Modulation of Baroreflex Dysfunction in Endotoxic Rats by Pre-eclampsia is Not Sex Specific.

Slopes of the baroreflex curves established by the vasoactive method are shown in Fig. 6. In male offspring, LPS caused significant decreases in reflex bradycardic (BRS_{phenylephrine}; Fig. 6A) and tachycardic responses (BRS_{nitroprusside}; Fig. 6B), and these effects were independent of the pre-eclamptic state. In female

TABLE 1

Effect of PE on body weight, proteinuria, and baseline (prior to LPS administration) cardiovascular parameters in adult offspring

Values are means $\pm$ S.E.M.

Parameter	Males		Females	
	Non-PE	PE	Non-PE	PE
Body weight (g)	213.0 $\pm$ 6.8	194.1 $\pm$ 4.5*	168.2 $\pm$ 3.6	144.3 $\pm$ 3.9*
Proteinuria (mg/dl)	221.5 $\pm$ 13.4	249.2 $\pm$ 7.6*	94.2 $\pm$ 9.3	89.5 $\pm$ 11.0
SBP (mm Hg)	121.0 $\pm$ 3.3	133.3 $\pm$ 3.9*	106.6 $\pm$ 1.9	112.3 $\pm$ 2.1
HR (beats/min)	379.6 $\pm$ 9.6	382.9 $\pm$ 11.8	382.3 $\pm$ 7.9	402.2 $\pm$ 11.0
SDNN (ms)	5.2 $\pm$ 0.1	3.7 $\pm$ 0.3*	3.4 $\pm$ 0.3	4.0 $\pm$ 0.3
rMSSD (ms)	5.9 $\pm$ 0.7	4.3 $\pm$ 0.4*	3.7 $\pm$ 0.3	4.2 $\pm$ 0.3
Total power (ms ²)	25.2 $\pm$ 5.1	13.6 $\pm$ 2.4*	12.4 $\pm$ 2.1	14.9 $\pm$ 2.1
LF _{nu}	10.7 $\pm$ 1.9	7.8 $\pm$ 1.1	10.8 $\pm$ 1.6	10.9 $\pm$ 1.7
HF _{nu}	84.7 $\pm$ 2.7	83.1 $\pm$ 2.6	83.3 $\pm$ 2.3	83.8 $\pm$ 2.2
LF/HF	0.16 $\pm$ 0.03	0.1 $\pm$ 0.01	0.13 $\pm$ 0.02	0.14 $\pm$ 0.02
dP/dt _{max} (mm Hg/s)	2195 $\pm$ 250	2498 $\pm$ 299	2481 $\pm$ 233	2410 $\pm$ 313

dP/dt_{max}, maximum rate of rise in left ventricular pressure.

* $P < 0.05$ vs. corresponding non-PE values in the same rat sex.

offspring, reflex bradycardic responses were significantly attenuated by LPS in PE rats only (Fig. 6C), but a depressant action of LPS on tachycardic reflexes was observed in both PE and non-PE female offspring (Fig. 6D). Additionally, comparisons of intersex values showed that BRS in LPS-treated non-PE male offspring was significantly lower than respective values in female offspring for phenylephrine (1.2 ± 0.05 vs. 1.66 ± 0.17 beats/min per millimeters of Hg, $P < 0.05$) as well as sodium nitroprusside (0.92 ± 0.13 vs. 1.52 ± 0.18 beats/min per millimeters of Hg, $P < 0.05$). By contrast, no sex differences were observed in BRS values of LPS-treated male and female offspring of PE mothers (Fig. 6).

Pre-eclampsia Sensitizes Male Offspring to Endotoxic Myocardial and Brainstem Inflammation. The effects of LPS on immunohistochemical protein expressions of TLR-4 and MCP-1 in cardiac and brainstem tissues of the offspring of PE or control (non-PE) mothers are depicted in Figs. 7–9. No sex differences were observed in the expression of either protein in all tissues of control offspring. A clear evidence

of upregulated TLR-4 expression in cardiac and neuronal pools of NTS and RVLM was noted in LPS-treated offspring of non-PE rats of the two sexes compared with respective control values in non-PE offspring. These inflammatory responses to LPS in cardiac and neuronal tissues were exacerbated in male, but not female, PE offspring rats (Figs. 7A, 8A, and 9A).

Alternatively, immunohistochemical analysis of MCP-1 expression revealed site- and sex-specific effects. Compared with saline treatment of non-PE males, LPS caused significant elevations in MCP-1 expression in cardiac and neuronal tissues of (Figs. 7B, 8B, and 9B). In this circumstance, the treatment of PE male offspring with LPS resulted in significantly higher expression levels of MCP-1 in cardiac, but not neuronal, tissues. In LPS-treated female offspring of non-PE mothers, elevated MCP-1 expression was observed in cardiac, but not NTS or RVLM, tissues, and these effects of LPS remained unaltered in PE female offspring (Figs. 7B, 8B, and 9B). ELISA determinations showed that serum MCP-1 was

Fig. 2. Effect of pre-eclampsia on changes in MAP (panels A and C) and HR (panels B and D) evoked by intravenous LPS (5 mg/kg) or equal volume of saline in adult male and female offspring rats. Values are means $\pm$ S.E.M. of eight observations. The repeated measures ANOVA followed by the Tukey's post hoc test was employed to measure statistical significance. * $P < 0.05$ vs. respective "control, saline" values, + $P < 0.05$ vs. respective "control, LPS" values in the same rat sex.

Fig. 3. Effect of pre-eclampsia on changes in time-domain indices of heart rate variability (SDNN, panels A and C; rMSSD, panels B and D) evoked by intravenous LPS (5 mg/kg) or equal volume of saline in adult male and female offspring rats. Values are means $\pm$ S.E.M. of eight observations. The repeated measures ANOVA followed by the Tukey's post hoc test was employed to measure statistical significance. * $P < 0.05$ vs. respective "control, saline" values, * $P < 0.05$ vs. respective "control, LPS" values in the same rat sex.

increased after LPS challenge in non-PE offspring, and this effect was potentiated in the two sexes of PE offspring (Fig. 7C). Generally, rises in serum MCP-1 by LPS were more noted in male compared with female offspring of non-PE (99 ± 3 vs. 78 ± 3 pg/ml, $P < 0.05$) or PE mothers (160 ± 6 vs. 137 ± 5 pg/ml, $P < 0.05$) (Fig. 7C).

Discussion

This study is the first to report on sex specificity of PE fetal programming on inflammatory and cardiovascular effects of

endotoxemia in adult rat offspring. Compared with non-PE counterparts, LPS treatment of male offspring of PE mothers exhibited greater 1) falls in BP and time and frequency indices of cardiac autonomic function, 2) rises in HR and cardiac contractility, and 3) increases in abundance of inflammatory cytokines in cardiac brainstem tissues. Because none of these exaggerated effects were noted in female offspring, it is concluded that fetal programming to PE-mediated intrauterine adaptations primes male offspring toward endotoxic sequels in adulthood.

The development of PE in current study was verified by rises in SBP and proteinuria. Notably, the adverse implications of PE

Fig. 4. Effect of pre-eclampsia on changes in frequency-domain indices of heart rate variability (total power, panel A; HF_{nu}, panel B; LF_{nu}, panel C; and LF:HF, panel D) evoked by intravenous LPS (5 mg/kg) or equal volume of saline in adult male and female offspring rats. Values are means $\pm$ S.E.M. of eight observations. The repeated measures ANOVA followed by the Tukey's post hoc test was employed to measure statistical significance. * $P < 0.05$ vs. respective "control, saline" values, * $P < 0.05$ vs. respective "control, LPS" values in the same rat sex.

Fig. 5. Effect of pre-eclampsia on changes in the maximum rate of rise of blood pressure waves (dP/dt_{max} , correlate of left ventricular contractility) evoked by intravenous LPS (5 mg/kg) or equal volume of saline in adult male (panel A) and female (panel B) offspring rats. Values are means $\pm$ S.E.M. of eight observations. The repeated measures ANOVA followed by the Tukey's post hoc test was employed to measure statistical significance. * $P < 0.05$ vs. respective "control, saline" values, * $P < 0.05$ vs. respective "control, LPS" values in the same rat sex.

go beyond the gestational period and are likely to impose long-term and possibly sexually related health influences in offspring. For instance, whereas sex-independent metabolic derangements were observed in mouse offspring born to PE mothers treated with soluble fms-like tyrosine kinase-1, hypertension appeared in male offspring only (Lu et al., 2007; Bytautiene et al., 2011). Fetal undernutrition induced by placental insufficiency during late gestation causes hypertension and mesenteric artery dysfunction in two consecutive generations of male and female offspring (Anderson et al., 2006). Our results demonstrated higher SBP and proteinuria in PE male, but not female, offspring suggesting preferential male vulnerability to fetal programming in L-NAME PE model. Together, PE insult type and gestational intervention time play key roles in identifying fetal programming profile and after-effects in offspring of the two sexes.

The present study reveals three important observations that reinforce the advantageous cardiovascular state of female offspring of PE mothers against endotoxemia. First, although LPS caused hypotension and tachycardia in the two sexes of

non-PE rat offspring, HRV signs of cardiac autonomic dysfunction such as reduced overall cardiac autonomic balance (SDNN and total power) and parasympathetic (rMSSD and HF), but not sympathetic (LF), activity were evident only in male offspring. These findings are consistent with greater disturbances in respiratory function observed in male sex such as pulmonary inflammation and hyperventilation, which are believed to be modulated by cardiac vagal function (Tang et al., 1998; Kosyrev et al., 2012; Perez-Quilis et al., 2017). In this context, the reason for the unaltered spectral index of cardiac sympathovagal balance (LF:HF) is not clear. This may relate to the view that in addition to sympathetic drive, other factors including vagal control, sex, and age could contribute to LF component of HRV (Stein et al., 1994; Sgoifo et al., 1997; Parati et al., 2006). Likewise, vagal activity is not entirely responsible for HF oscillations of HRV. Residual HF oscillations observed after autonomic blockade or cardiac transplantation are caused by mechanical respiratory modulation of sinus node (Bernardi et al., 1989).

Second, whereas hypotensive and HRV depressant actions of LPS were remarkably exaggerated in male offspring of PE

Fig. 6. Effect of pre-eclampsia on $BRS_{phenylephrine}$ (panels A and C) and $BRS_{nitroprusside}$ (panels B and D) in adult male and female offspring rats treated with intravenous LPS (5 mg/kg) or equal volume of saline. Values are means $\pm$ S.E.M. of eight observations. The repeated measures ANOVA followed by the Tukey's post hoc test was employed to measure statistical significance. * $P < 0.05$ vs. respective non-pre-eclamptic "control, saline" values.

Fig. 7. Effect of PE on changes in serum MCP-1 (panel C) and cardiac immunohistochemical protein expression of TLR-4 (panel A) and MCP-1 (panel B) evoked by intravenous LPS (5 mg/kg) in adult male and female offspring rats. Representative images for immunostained sections from the heart are shown. Values are means $\pm$ S.E.M. of five observations. The one-way ANOVA followed by the Tukey's post hoc test was employed to measure statistical significance. * $P < 0.05$ vs. respective "control" values, * $P < 0.05$ vs. respective "PE" values, # $P < 0.05$ vs. respective "LPS" values in the same rat sex.

rats, the privileged female status remained a consistent feature in LPS-treated female offspring of PE mothers because no intensification of LPS hypotension was observed and little or no changes in HRV indices occurred. Considering the significantly greater BP falls caused by LPS in female compared with male offspring of non-PE rats, the lack of potentiated LPS hypotension in female offspring of PE rats might be explained, in part, by the pre-existing dampening of compensatory mechanisms. The latter are functional and molecular ramifications that are activated to offset undesirable effects of endotoxemia (Yamaguchi et al., 2006). Third, male, but not female, endotoxic offspring exhibited dramatic rises in myocardial contractility correlate (dP/dtmax), which might serve an adaptive response to weakened cardiomotor vagal input in males. Considering that altered cardiac autonomic and contractility functions often predispose to cardiovascular disturbances and accelerated mortality in endotoxemia (Klückner et al., 2011; Mazzeo et al., 2011; Sallam et al., 2018), the preservation of such traits in female offspring infers their resistance to harsh intrauterine environment featured during PE.

Contradictory information is available regarding whether baroreflex changes are causally related to other hemodynamic actions of endotoxemia (Bigger et al., 1989; Vayssettes-

Courchay et al., 2005). In this study, baroreceptor assessment by vasoactive method unraveled data that were not exactly analogous to those of hemodynamic or HRV investigations. The attenuated reflex bradycardic ($BRS_{phenylephrine}$) and tachycardic ($BRS_{nitroprusside}$) responses in endotoxic male offspring of non-PE mothers was not accentuated in male counterparts from PE mothers. Alternatively, reflex bradycardia was reduced by LPS in female offspring of PE, but not non-PE, mothers. Because reflex bradycardia and tachycardia are predominantly mediated through vagal and sympathetic activity, respectively (El-Mas et al., 2002; La Rovere and Christensen, 2015), current data indicate that unlike HRV, PE unveiled LPS attenuation of reflex cardiomotor vagal activity in female offspring. More studies are warranted to define the precise role of cardiac neural control in PE fetal programming of endotoxemia.

Inflammatory signals in peripheral and central tissues participate in endotoxic organ damage (Dantzer, 2009; Cazar-eth et al., 2014). Peripheral inflammatory signals enter the brain through NTS, which arbitrates, along with its polysynaptic medullary and hypothalamic connections, cardiovascular irregularities of endotoxemia (Lin et al., 1999; Pavlov et al., 2003; Sirivelu et al., 2012). Recently, we reported that upregulation of brainstem neuroinflammatory pathways of

Fig. 8. Effect of PE on changes in immunohistochemical protein expression of TLR-4 (panel A) and MCP-1 (panel B) in NTS evoked by intravenous LPS (5 mg/kg) in adult male and female offspring rats. Representative images for immunostained sections from the NTS are shown. Values are means $\pm$ S.E.M. of five observations. The one-way ANOVA followed by the Tukey's post hoc test was employed to measure statistical significance. * $P < 0.05$ vs. respective "control" values, + $P < 0.05$ vs. respective "PE" values, # $P < 0.05$ vs. respective "LPS" values in the same rat sex.

NF κ B mediates endotoxic hypotension and autonomic depression (Sallam et al., 2019). The current study implicates brainstem neuroanatomical pools of TLR-4 in PE-related fetal programming that worsened endotoxic cardiovascular manifestations in adult male, but not female, offspring. This is supported by the findings that exacerbated reductions in BP and HRV by endotoxemia in male progeny of PE mothers were paralleled with potentiated TLR-4 expression in heart and brainstem pools of NTS and RVLm. Sex specificity of this interaction was corroborated by the lack of such intensified effects in female offspring of same litters. Increased placental TLR4 expression is also seen in PE induced by the single administration of a very low dose of LPS early during pregnancy (Xue et al., 2015). TLR-4 is a key recognition receptor that serves as principal driver of host inflammatory response to bacterial infections (Iwasaki and Medzhitov, 2004; Fink, 2014). TLR4 upregulation by LPS triggers downstream activation of I κ B kinase and NF- κ B and subsequent generation of a wide array of cytokines, chemokines (Li et al., 2017; Lee et al., 2019). These molecules mediate LPS-induced cardiac mitochondrial dysfunction and apoptosis (Tien et al., 2010) and positively correlate with worsened clinical outcomes and mortality (Akira and Takeda, 2004; Liu and Malik, 2006).

MCP-1, a potent chemotactic factor for monocytes, is generated after LPS binding to TLR-4 and provokes systemic inflammation and organ damage (Zhu et al., 2017). Our data showed that MCP-1 expression profile mimicked that of TLR-4 in cardiac, but not neuronal, tissues. In heart, the increased abundance of MCP-1 in response to LPS challenge was exaggerated in male, but not female, offspring of PE mothers. By contrast, brainstem nuclei of male offspring of PE rats exhibited upregulated MCP-1 expression, but no further increments were observed in same rats after LPS treatment. These results suggest preferential involvement of cardiac, but not neuronal, MCP-1 in heightened endotoxic hypotension and cardiac autonomic depression in male offspring.

One potential limitation of current study is the use of dP/dt_{max} as a measure of cardiac contractility. The LabChart-7 pro software adopted here allowed computation of maximum rate of pressure rise across BP wave form (dP/dt_{max}), which serves as an indirect measure of cardiac contractile force (Mehta et al., 1998). The validity of arterial dP/dt_{max} as an index of left ventricular contractility remains questionable because it is derived from arterial pressure measured with a fluid-filled catheter and is influenced by preload and vascular filling conditions. Cardiac function is typically

Fig. 9. Effect of PE on immunohistochemical protein expression of TLR-4 (panel A) and MCP-1 (panel B) in the RVLM evoked by intravenous LPS in conscious offspring. Representative images for immunostained sections from the RVLM are shown. Values are means $\pm$ S.E.M. of five observations. The one-way ANOVA followed by the Tukey's post hoc test was employed to measure statistical significance. * $P < 0.05$ vs. respective "control" values, + $P < 0.05$ vs. respective "PE" values, # $P < 0.05$ vs. respective "LPS" values in the same rat sex.

measured by electrocardiographic or echocardiographic techniques (Carroll et al., 2006; Adeyemi et al., 2009). Nevertheless, some studies have established significant correlation between arterial dP/dtmax and end-systolic elastance, the gold standard measure of LV contractility, during hemodynamic perturbations including endotoxemia (Morimont et al., 2012).

Taken together, current molecular and neuroanatomical evidence suggests possible roles for PE-induced fetal programming in offspring predisposition to health hazards induced by endotoxemia in adult life. PE amplifies endotoxic manifestations of hypotension, tachycardia, and cardiac autonomic dysfunction in male offspring via exacerbating myocardial and neuroinflammatory pathways of the NTS and RVLM. The lack of such injurious states in female littermates suggests sexual dimorphism in the capacity of PE fetal programming to shape endotoxic outcomes.

Authorship Contributions

Participated in research design: El-Gowilly, Helmy, El-Mas.

Conducted experiments: Abuissa, Wedn.

Performed data analysis: Abuissa, Wedn, El-Gowilly, Helmy, El-Mas.

Wrote or contributed to the writing of the manuscript: Abuissa, Wedn, El-Gowilly, Helmy, El-Mas.

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