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Cardiovascular risk and the effect of nitric oxide synthase inhibition in female rats: The role of estrogen

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ABSTRACT

It is known that autonomic modulation is responsive to ovarian hormone levels and that estrogen increases nitric oxide (NO) bioavailability. However, little is known about the interaction of nitric oxide synthase (NOS) isoforms with autonomic modulation, oxidative stress and cardiovascular risk in females. This study aimed to investigate cardiovascular, autonomic and oxidative parameters after selective NOS inhibition. A spectral analysis of systolic arterial pressure (SAP) and heart rate variability (HRV) was performed. NO levels, total antioxidant capacity (TRAP), lipid hydroperoxides (LOOH) and paraoxonase 1 (PON1) activity were measured in the plasma of rats treated with L-NG-nitroarginine methyl ester (L-NAME), S-methylisothiourea (SMT) or saline. Wistar rats, ovariectomized (OVX) with or without estradiol treatment (1 mg/kg/day) or with a false ovariectomy (SHAM), were submitted to artery and vein catheterization. Cardiovascular parameters were evaluated before and after the administration of saline or NOS inhibitors. After 2 h, plasma samples were collected for biochemical measurement. At baseline, cardiovascular and autonomic parameters were not different among the groups. L-NAME, the constitutive NOS isoform (cNOS) inhibitor, promoted an increase in mean arterial pressure (MAP) and a reduction in the low frequency band (LF) of SAP of SHAM rats, but this increase was smaller in OVX animals, which also showed a reduction in PON1 activity. The decreased activity of PON1 caused by L-NAME was prevented in the OVX + E group. SMT, an inducible NOS isoform (iNOS) inhibitor, promoted an increase in MAP and in the LF of SAP, in interbeat interval (IBI) parameters at LFnu and in LF/HF ratio of HRV in all groups, but the OVX + E had lower levels of NO when compared with the OVX group. Our data suggest that while cNOS contributes to maintaining the activity of PON1 in OVX rats, iNOS activity maintains the levels of NO in OVX + E rats.

1. Introduction

It is recognized that gender differences exist in cardiovascular disease and in cardiovascular function (Hayward et al., 2000). Men are generally at a greater risk for cardiovascular and renal disease than age-matched premenopausal women, and physiologically, men's blood pressure tends to be higher (Reckelhoff, 2001). Premenopausal women also have greater endothelium-dependent vasodilation compared to similarly aged men, a difference that disappears after menopause (Sarabi et al., 1999).

Cardiac autonomic modulation also changes with ovarian hormone levels: post-menopausal women have weaker vagal modulation and stronger cardiac sympathetic modulation compared with non-age-matched premenopausal women (Liu et al., 2003). Autonomic functions are altered after an oophorectomy in premenopausal women (Mercuro et al., 2000), and estrogen replacement therapy restores these alterations (Liu et al., 2003).

A noninvasive method to assess sympathovagal balance is the power spectral analysis of heart rate variability (HRV) (Petrofsky et al., 2009). Alterations in HRV, which primarily reflect the tonic autonomic modulation, may have substantial clinical implications (Campos et al., 2014). In the spectral analysis, female rats had the most prominent high frequency (HF) component, which represents the parasympathetic drive, during estrus compared with the OVX and diestrus groups; the

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estrous cyclicity, as well as the cycle-related HF changes, disappeared with ovariectomy (Kuo et al., 2010), corroborating estradiol's participation in autonomic modulation. It was also demonstrated that HRV are significantly higher in both high-estradiol-dose and low-estradiol-dose groups compared to the OVX group (Campos et al., 2014).

Estrogen is an important hormone for the cardiovascular system. This importance comes to light with aging and consequential menopause when estrogen is lost and there are some inflammatory alterations (Knowlton and Lee, 2012). Alterations such as increased TNF, IL-6 (Chung et al., 2009; Donato et al., 2008) and oxidative stress (Jackson and McArdle, 2011; Stice et al., 2011) are factors that contribute to cardiovascular dysfunction. Estrogen also promotes the indirect upregulation of antioxidant gene expression and increases endothelial nitric oxide synthase (eNOS) activity while decreasing superoxide production (reviewed by Knowlton and Lee, 2012).

Paraoxonase-1 enzyme (PON1) may be involved in the relation between the estrogen and the antioxidant systems. Synthesized in the liver, this hydrolase is associated with HDL lipoproteins, functions as a "bioscavenger" (Bojic et al., 2014; Li et al., 2013) and is considered a cardiovascular risk marker. It has been observed in humans that surgical menopause reduces the enzyme activity of PON1 (Kumru et al., 2005), while estradiol stimulates PON1 activity (Ahmad and Scott, 2010). These estrogenic effects are promising for the treatment of disorders related to oxidative stress.

Estrogen is among the many hormones that increase NO production (Duckles and Miller, 2010). Estrogen receptors, which are alpha, beta and G-protein coupled (ERalpha, ERbeta and GPER, respectively), have identified as having NO-linked mechanisms in adult animals. 17Beta-estradiol stimulates the expression of eNOS and iNOS in cardiac myocytes (Nuedling et al., 1999). ERalpha is co-localized to endothelial cells where it activates eNOS (Knowlton and Lee, 2012). Additionally, estradiol increases eNOS expression and NO in cultured human coronary artery endothelial cells (Duckles and Miller, 2010). Production of NO in the endothelium is also the mechanism of GPER vasodilation.

Aging results in the downregulation of GPER in rats and is associated with reduced vascular relaxation responsiveness to estradiol or ER agonists (Prossnitz and Barton, 2014). Additionally, it was established that the majority of estrogen-stimulated NO production in coronary artery smooth muscle is from neural nitric oxide synthase (nNOS) (White et al., 2010). Additionally, estrogen attenuates vasoconstriction by an ERbeta-mediated increase in iNOS expression in wild-type mouse blood vessels (Zhu et al., 2002).

However, the systemic effects of NOS isoforms and their interaction with autonomic modulation, oxidative stress and cardiovascular risk in females have not yet been addressed. This study aimed to investigate cardiovascular, autonomic and oxidative parameters after selective NOS inhibition.

2. Methods

2.1. Animals

Adult female Wistar rats were maintained in ventilated and controlled temperature chambers (22 ± 1 °C) on a 12-hour light-dark cycle with food (Nuvilab CR-1; Nuvital®, Colombo, Paraná, Brazil) and tap water available ad libitum. The experiments were conducted during the light phase. All experimental protocols were approved by the Institutional Animal Ethics Committee of the State University of Londrina, Paraná, Brazil (CEUA-UEL - Comissão de Ética no Uso de Animais, process number 276.2013.81) and performed in accordance with the standards established by the National Council for Animal Testing Control (CONCEA), Brazil.

2.2. Surgical proceedings and experimental groups

Rats were subjected to bilateral ovariectomy or false surgery under ketamine and xylazine anesthesia (100 and 6.7 mg/kg, i.p.; Ceva Santé Animale, São Paulo, Brazil) and 24 h after were divided into experimental groups (n = 4–8): an ovariectomy control group (OVX) that received 0.5 mL/kg estradiol vehicle per day, p.o. (almond oil, Generophlora drugs, Londrina, Paraná, Brazil); an OVX plus estradiol treatment (OVX + E) group that received estradiol valerate 1 mg/kg per day, p.o. (Hangzhou Hetd Industry Co., Zhejiang, China) for 8 weeks (Ceylan-Isik et al., 2009); and a SHAM group.

The estrous cycle in the SHAM-operated females was monitored by microscopic investigation of vaginal smears (Kauser et al., 1997), and only females in the estrus phase were used in the experiments.

Eight weeks after the surgery and 24 h before the experiments, under ketamine–xylazine anesthesia, a polyethylene catheter was inserted into the femoral artery and vein and externalized dorsally to record MAP and heart rate (HR) during a conscious state, according to previous studies (Ariza et al., 2015; da Cunha et al., 2014).

2.3. Measurement of cardiovascular parameters

Twenty-four hours after catheterization, the animals were kept in their cages, and basal recordings were obtained for at least 20 min before starting the protocol. MAP and HR were recorded by an MLT0380 blood pressure transducer connected to a Powerlab system 4/20 T (ADInstruments®) while the animals were awake and freely moving (da Cunha et al., 2014).

After the basal recording, NOS isoforms were inhibited by a *bolus* injection (i.v.) of either L-NAME (10 mg/kg), a selective inhibitor of calcium-dependent isoforms (i.e., cNOS) (Vitecek et al., 2012), or of SMT (3 mg/kg), a potent inhibitor of iNOS (Su et al., 2007; Szabo et al., 1994), both from Santa Cruz Biotechnology®, Texas, USA. Control groups received physiological saline 0.9% (1 mL/kg).

Cardiovascular parameters were recorded for 2 h (Mehanna et al., 2007). At the end of recording, plasma samples were collected for measurement of the nitrite and lipoperoxidation levels, the plasma activity of paraoxonase 1 (PON1) and the total antioxidant capacity of plasma. A standard laboratory scale was used to measure body weight, tibia length and uterus weights (Gore et al., 2002; Voltera et al., 2008).

2.4. Heart rate and systolic arterial pressure variability

The recordings of arterial pressure, 10 min from basal and the last 3 min of the 2 h post-treatment, were processed using a specific computer program (LabChart 7 Pro®, ADInstruments, Bella Vista, Australia) that was able to detect inflection points in pressure pulses to generate a beat-by-beat time series of pulse interval (PI) and systolic pressure (SAP). The frequency domain (PI and SAP variability) spectral analysis was performed using custom software (CardioSeries® v2.4). For power spectral analysis of the PI and SAP variability, the beat-by-beat series of these parameters were resampled at data points every 100 ms by cubic spline interpolation (10 Hz). Next, the interpolated series was divided into half-overlapping segments. All segments were visually inspected, and those with artifacts or nonstationary data were excluded from analysis. Then, a Hanning window was used to attenuate the side effects, and spectra were calculated for all segments using a fast Fourier transform (FFT) algorithm for a discrete time series. Finally, the spectra were integrated in low (LF: 0.2–0.75 Hz) and high frequency (HF: 0.75–3.0 Hz) bands. The relative power of the LF and HF bands of the PI spectra were calculated taking into account the total power of the spectra minus the power of the very low oscillations (< 0.2 Hz). To assess cardiac sympathovagal balance, we also calculated the ratio between the power of the LF and the HF bands (LF/HF ratio) of the PI spectrum (Dutra et al., 2013).

2.5. Biochemical analysis

Sample nitrite was measured as an estimate of NO levels and