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Sexual dimorphism in rat aortic endothelial function of streptozotocin-induced diabetes: Possible involvement of superoxide and nitric oxide production

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ABSTRACT

Little is known of the interactions between diabetes and sex hormones on vascular function. The objectives of this study were to investigate whether there were sex differences in rat aortic endothelial function one week after the induction of streptozotocin (STZ)-diabetes, and to examine the potential roles of superoxide and nitric oxide (NO) in this sex-specific effect. Endothelium-dependent vasodilatation to acetylcholine (ACh) was measured in rat aortic rings before and after treatment with MnTMPyP (25 μM), a superoxide dismutase. Contractile responses to phenylephrine (PE) were generated before and after treatment with L-NAME (200 μM), a nitric oxide synthase (NOS) inhibitor. The mRNA expression of NADPH oxidase (Nox) and endothelial nitric oxide synthase (eNOS) were also determined. We demonstrated that (1) STZ-diabetes impaired endothelium-dependent vasodilatation to ACh to a greater extent in female than male aortae, (2) inhibition of superoxide enhanced sensitivity to ACh only in diabetic females, and (3) Nox1 and Nox4 mRNA expression were significantly elevated only in aortic tissue of diabetic females. Furthermore, incubation of aortic rings with L-NAME potentiated PE responses in all groups, but aortae from control females showed a greater potentiation of the PE response after NOS inhibition compared with others. STZ-diabetes reduced the extent of PE potentiation after L-NAME and the aortic eNOS mRNA expression in females to the same levels as seen in males. These data suggest that a decrease in NO, resulting from either decreased eNOS or elevated superoxide, may partially contribute to the predisposition of the female aorta to injury early in diabetes.

1. Introduction

Premenopausal women have a lower incidence of cardiovascular diseases compared to age-matched men (Kannel and Belanger, 1991; Kannel et al., 1998; Lerner and Kannel, 1986). Premenopausal women with diabetes not only lose this sex-based cardiovascular protection, they also experience a higher risk of cardiovascular diseases compared to diabetic men (Huxley et al., 2006; Pilote et al., 2007; Zuanetti et al., 1993). However, there is insufficient evidence to establish the mechanism(s) underlying the loss of this female-specific cardiovascular protection in diabetes.

Acute hyperglycemia may affect male and female vascular beds differently (Goel et al., 2008, 2007). Previously, we observed a sex difference in the development of impaired endothelium-dependent vasodilation in mesenteric arteries from streptozotocin (STZ)-treated rats (Zhang et al., 2012). Nevertheless, it remains to be established whether the above-mentioned sexual dimorphism is specific to the mesenteric vascular bed or whether it is a generalizable effect extending to larger conduit arteries. Thus, our first objective was to investigate whether there were sex differences in the development of abnormal vascular responses following the induction of STZ-diabetes in rat aortae. Because published data on short term (1–2 weeks) diabetes is inconsistent (Hink et al., 2001; Pieper, 1999; Rodriguez-Manas et al., 2003), one week was chosen to examine whether the responses of aortic rings are impaired at very early stage of the diabetes. Endothelium-dependent vasodilation is a reproducible parameter used to measure endothelial function and is dependent on a variety endothelium-derived relaxing factors (EDRF), such as nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor (EDHF). In rat mesenteric arteries, we reported that the predisposition of female to vascular injury after the induction of diabetes may be due to a shift away from a putative EDHF toward a greater reliance on NO (Zhang et al., 2012). On the other hand, in conduit arteries NO is critical to the regulation of vascular responses under physiological conditions (Félétou, 2011).

It is widely accepted that NO level is reduced in diabetes (Endemann and Schiffrin, 2004; Hink et al., 2001) and that changes in the level of endothelial NO synthase (eNOS) may contribute to

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the reduction of NO production. However, previous studies demonstrated both an increase (Ikubo et al., 2011; Kazuyama et al., 2009) and a decrease (Fu et al., 2007; Olukman et al., 2010) in eNOS expression in diabetic rat aortae. Therefore, the second aim of this study was to investigate whether sex and STZ-diabetes altered NO and eNOS expression in rat aorta. Impaired endothelium-dependent vasodilation may result from either a decreased NO release or an increased inactivation of NO by reactive oxygen species (ROS). Thus, experiments were carried out to examine the role of superoxide in the abnormal aortic responses to STZ-diabetes in rats. Specifically, we determined whether scavenging superoxide would fully or partially reverse the impairment of endothelium-dependent vasodilation. Because the NADPH oxidase (Nox) family is one of the potent cellular sources of superoxide in the vascular system (Griendling et al., 2000), we sought to determine whether sex and STZ-diabetes altered the aortic mRNA expressions of Nox subunits.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), and dissolved in water, unless otherwise stated.

2.2. Experimental animals

Adult male and female Sprague-Dawley rats, 9–11 weeks of age (Simonsen Laboratories, CA) were divided into four groups: control female, diabetic female, control male and diabetic male. Diabetic groups received a single i.v. injection of streptozotocin (STZ, 60 mg/kg). Age-matched control animals were injected with a similar volume of citrate buffer. Only animals demonstrating non-fasting glucose levels higher than 300 mg/dl (about 72 h after STZ treatment) were considered diabetic. Rats were euthanized using CO_2 one week after STZ treatment according to the recommendations from the 2013 AVMA Guidelines on Euthanasia and the NIH Guidelines for the Care and Use of Laboratory Animals. On the day they were euthanized, blood glucose and body weight were measured. All animal protocols were approved by the Animal Care Committee of the University of the Pacific and complied with the Guide for the Care and Use of Laboratory Animals: Eighth Edition (2011).

2.3. Measurement of arterial tension

The thoracic aorta was excised and cleaned of fatty and adhering connective tissues and then cut into 2 mm rings. To measure isometric tension, the rings were suspended horizontally between two stainless steel hooks in individual organ baths containing 20 ml of Krebs buffer (in mM: 119 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 24.9 $NaHCO_3$, 0.023 EDTA, 1.6 $CaCl_2$, and 6.0 glucose) at 37 °C bubbled with 95% O_2–5% CO_2. Isometric tension was continuously monitored with a computer based data acquisition system (PowerLab, ADInstruments). The rings were equilibrated for 40 min under a resting tension of 1 g to allow development of a stable basal tone. Stimulation of rings with 80 mM KCl was repeated two times every 20 min until contractile responses were stable and uniform. The ability of acetylcholine (ACh, 10 μM) to induce relaxation of phenylephrine (PE, 2 μM) pre-contracted vessels was taken as evidence for the preservation of an intact endothelium. For the relaxation studies, we used an equal submaximal concentration of PE (2 μM) in both males and females, although the absolute maximum tension was higher in males than females ($P=0.05$). This decision was based on the fact that aortic rings taken from control males showed the same level of relaxation to ACh compared with control female rings (89 ± 3% vs. 92 ± 2% of maximal relaxation, respectively), despite the higher level of tension in control males.

2.4. Relaxation responses to ACh

Aortic rings were contracted with PE (2 μM), which represented a concentration that produced 80% of the maximal effect (EC_{80}). The vasodilator concentration response curves were obtained by the addition of increasing concentrations of ACh (10^{-8} to 10^{-5} M) before and after incubation with 25 μM of MnTMPyP, a membrane permeate mimetic of superoxide dismutase (SOD) for 20 min. Between each concentration response curve run, tissues were washed with Krebs buffer to allow the rings to return to the basal tone.

2.5. Relaxation responses to sodium nitroprusside (SNP)

Responses to SNP (10^{-9} to 10^{-5} M), a NO-donor, were generated in the aortic rings pre-contracted with PE (2 μM) from all groups.

2.6. Relaxation responses to bradykinin (BK)

The concentration response curves to BK, a receptor-mediated vasodilator, were measured following the addition of increasing concentrations of BK (10^{-9} to 10^{-4} M) in U46619 (30 nM) pre-contracted aortic rings taken from all groups.

2.7. Contractile responses to PE

The constrictor concentration response curves to PE (10^{-8} to 10^{-5} M) were generated before and after incubation with N^{ω}-Nitro-L-arginine methyl ester (L-NAME, 200 μM), a NOS inhibitor in the presence of indomethacin (indo, 10 μM, dissolved in DMSO), a cyclooxygenase (COX) inhibitor. Between each concentration response curve, tissues were washed with Krebs buffer to allow the rings to return to the basal tone. In a second set of experiments, we determined the role of vehicle on PE induced contraction. The vehicle study was performed simultaneously in aortic rings from the same animal and no drug was given during incubation. There was no difference between the first and second concentration response curve to PE in vehicle study (data not shown).

2.8. Real-time PCR

The thoracic aorta was isolated as described above and cut into 12 mm segments. RNA was extracted from male and female rat aortic segments using RNeasy mini kit (QIAGEN, Valencia, CA). First-strand cDNA was synthesized by reverse transcription of 2 μg of total RNA using the Omniscript RT kit (QIAGEN, Valencia, CA), in a total volume of 20 μl, according to the manufacturer's instructions. The gene fragments were then specifically amplified with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) using real-time RT-PCR (MyiQ Single-Color Real-Time PCR Detection System, Bio-Rad, Hercules, CA). Internal variations were normalized to rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin, and expression was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak, 2001). The following primers were used for detection of gene expression: 5′-TGG GTG TGA ACC ACA AGA AA-3′ (forward) and 5′-GTG GCA GTG ATG ACA TGG AC-3′ (reverse) for rat GAPDH; 5′-CTG GGT ATG GAA TCC TGT GG-3′ (forward) and 5′-TCA TCG TAC TCC TGC TTG CTG-3′ (reverse) for rat β-actin; 5′-ACT GCG TCG CTT CAT TAG GT-3′ (forward) and 5′-TAG GCA AGC GCT TTA CCA CT-3′ (reverse) for rat eNOS; 5′-GGC AAC ATG AGA GCT GCA TA-3′ (forward) and 5′-GCA AGT GTC AAC CAG CAA GA-3′(reverse) for rat Nox1; 5′-ACC CTT