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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 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 was 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.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: X.H. and R.Z. performed experiments; X.H. analyzed data; X.H. and R.R. interpreted results of experiments; X.H. prepared figures; X.H. and R.R. drafted manuscript; L.A. and R.R. edited and revised manuscript; R.R. conception and design of research; R.R. approved final version of manuscript.

Keywords

Sex difference; diabetes; endothelial dysfunction; nitric oxide; superoxide

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; Goel et al., 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 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 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 (92 ± 2 vs 89 ± 3% 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) using real-time RT-PCR (MyiQ Single-Color Real-Time PCR Detection System, Bio-Rad). Internal variations were normalized to rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin, and expression was analyzed by $2^{-\Delta\Delta Ct}$ 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′ 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 TCA CCCTGA CCT CT-3′ (forward) and 5′-TCC CAG CTC CCA CTA ACA TC-3′ (reverse) for rat Nox2; 5′-CCA GAA TGA GGA TCC CAGAA-3′(forward) and 5′-AGC AGC AGC AGC ATG TAG AA-3′(reverse) for rat Nox4. Specificity was verified by electrophoresis of the PCR products on a 2% agarose gel and staining with ethidium bromide.

2.9 Statistical Analysis

The ACh- and SNP-induced relaxations were expressed as the percentage of relaxation from maximum PE contraction at each concentration. Similarly, the recorded increase in the force of contraction was calculated as the percentage of maximum contraction obtained with PE at the highest dose. EC_{50}, the concentration of the agonist, which produced half of the maximum effect (E_{max}) was calculated by a sigmoidal dose-response model (for variable slope) using GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, CA). The sensitivity of the agonists was expressed as pD_2 values (−log [EC_{50}]), which were normally distributed. The area under the curve (AUC) was determined using GraphPad Prism 5.01 with trapezoidal technique. To compare the effect of pharmacological agents such as L-NAME on the PE response, the PE results were expressed as differences of area under the concentration-response curve (ΔAUC) in control (absence of drug) and experimental (presence of drug) condition. Statistical analysis was measured using SPSS software (SPSS

Inc., Chicago, IL). Data were reported as the mean ± standard error of the mean (S.E.M.). Differences among the four groups were analyzed using three-way analysis of variance (ANOVA), with factors being sex, diabetes and concentration. Comparison of concentration response curves between two groups was done using two-way ANOVA, with one factor being concentration and the others being groups (female vs. male and control vs. diabetic). Comparison of CRCs in a pre/post-test format within a group was done using two-way ANOVA with repeated measures. Student's unpaired t-test was used for comparisons of two group means (e.g., blood glucose level and eNOS mRNA expression). A probability value of less than 5% ($P<0.05$) was considered significant.

3. RESULTS

3.1 Effects of STZ-induced Diabetes on Blood Glucose and Body Weight

Non-fasting blood glucose levels were significantly higher in diabetic rats than those of their respective controls one week after the induction of STZ-diabetes. Body weights of both male and female diabetic rats were significantly lower compared with those of age-matched non-diabetic controls (Table 1).

3.2 Relaxation Responses to ACh

Concentration response curves to ACh were similar in age-matched control male and female groups, but one week after the induction of diabetes, the concentration response curves revealed an attenuated ACh-induced relaxation in both sexes (Figure 1). Specifically, E_{max} to ACh was significantly decreased in diabetic rats compared with controls regardless of sex (Table 1). However, the effect of diabetes by itself in diminishing ACh-mediated aortic vasorelaxation in females was significantly greater than that seen in males ($P<0.05$ vs Δ in diabetic males, Mann-Whitney test). Furthermore, the sensitivity of aortic rings to ACh, as assessed by $-\log EC_{50}$ (pD_2), was significantly decreased only in diabetic females (Table 1).

3.3 Effect of Superoxide Scavenging on ACh-induced Relaxation

Pre-incubation of rat aortic rings for 20 min with 25 μM of MnTMPyP, a SOD mimetic agent which acts as a superoxide scavenger, significantly enhanced the sensitivity to ACh only in the diabetic female group (Figure 2, Table 2). However, prior treatment with MnTMPyP did not change the E_{max} to ACh in this group. Furthermore, pre-incubation of tissues for 20 min with MnTMPyP did not alter the ACh-induced relaxation in male and female controls or in diabetic males, as indicated by no significant differences in E_{max} or pD_2 from the ACh response in the absence of drug.

3.4 Relaxation Responses to BK

BK (10^{-9} to 10^{-4} M), which is a receptor-mediated vasodilator, relaxed aortic rings pre-contracted with a thromboxane A_2 receptor agonist (U46619, 30 nM) in a concentration-dependent manner in both males and females. STZ-induced diabetes significantly impaired the BK-induced relaxation in females, but not in males, as indicated by a decreased E_{max} and pD_2 in aorta of diabetic females compared to those in control females (E_{max}, control female: 100 ± 0.2% vs diabetic female: 81 ± 3%; pD_2, control female: 7.21 ± 0.11 vs diabetic female: 5.67 ± 0.11; $P<0.05$, n=3–4) (Figure not shown).

3.5 Relaxation Responses to SNP

To determine the effects of sex and diabetes on endothelium-independent vasodilatation, responses to the NO donor, SNP (10^{-9} to 10^{-5} M), was measured in pre-contracted aortic rings. Unlike the effects on ACh-induced endothelium-dependent relaxation, there were no

significant statistical differences in SNP-induced relaxations between sexes or diabetic animals and their respective age-matched controls (Figure 3).

3.6 Contractile Responses to PE

To determine whether sex and diabetes affect the contractile responses to α-adrenoceptors, concentration response curves to PE (10^{-8} to 10^{-5} M) were measured. There was a slight, but significant, leftward shift of the PE concentration response curve in aortic rings from control male rats relative to control female rats (Figure 4). STZ- diabetes significantly shifted PE concentration response curves to the left in aortic rings of both sexes. The E_{max} to PE was higher in aortic rings taken from diabetic animals than those from controls regardless of sex (P=0.05). There were no differences in the pD_2 values among the experimental groups (Table 3).

To study the possible role of NO in diabetes-induced vascular dysfunction, NO production was determined indirectly by generating concentration response curves to PE (10^{-8} to 10^{-5} M) in aortic rings before and after pretreatment with L-NAME (200 μM, 20 min) in the presence of indo (10 μM). Significant changes in PE response as a result of L-NAME pretreatment would reveal the extent of NO release from endothelium during smooth muscle contraction to PE (Csanyi et al., 2007; Hayashi et al., 1992).

Incubation of the aortic rings with L-NAME resulted in a significant increase of the contractile responses to PE in all four groups (Figure 5). However, as indicated by ΔAUC (the difference in area under the curve between PE concentration response curve before and after L-NAME) aortae from control females showed a greater potentiation of the PE response after NOS inhibition compared with that observed in other groups. STZ-induced diabetes reduced the ΔAUC in females to the same level as those seen in males (Table 4).

3.7 Analysis of eNOS and Nox mRNA expression

To study a mechanism by which endothelium derived NO production in response to PE might be decreased in diabetic females, the level of eNOS mRNA expression in aortae was determined by real-time PCR. eNOS mRNA expression was significantly higher in aortae from control females than control males (P<0.05, unpaired t-test, n=6 per group) (Figure 6). However, STZ-diabetes reduced the eNOS mRNA expression level in aortae of females to the same levels as seen in male animals. Although eNOS mRNA expression also tended to be lower in diabetic males than in control males, the difference was not statistically significant (Figure 6).

Lastly, to study a possible mechanism for the impairment of the ACh sensitivity in only diabetic female rats, the levels of mRNA expression for Nox subunits (Nox1, 2 and 4, sources of superoxide in the vascular wall) were measured. In males, although the levels of Nox1 and Nox4 mRNA expression tended to be greater in aortae of diabetic animals than in controls, the differences were not found to be statistically significant (Figure 7). However, in females, the levels of mRNA expression for Nox1 and Nox4 were significantly higher in aortae taken from diabetic rats compared to those observed in controls (P<0.05, unpaired t-test, n=3–5 per group). As shown in Figure 7, there were no differences in Nox2 mRNA expression in male and female rat aortae, regardless of health status.

4. DISCUSSION

The main findings of our investigation were that 1) STZ-diabetes caused a greater reduction of E_{max} to ACh, along with a decreased sensitivity to ACh in females and 2) the decrease in sensitivity to ACh in diabetic female aorta was restored by superoxide scavenging. We also observed that the mRNA expression levels of Nox1 & 4, sources of superoxide in vascular

wall, were higher in diabetic female aortae compared to those in other groups. Furthermore, female animals showed higher levels of eNOS expression and basal NO release compared to other groups. However, when females became diabetic, the levels of eNOS and basal NO in aortae were reduced to the same levels as those seen in control and diabetic male animals.

Enhanced endothelium-dependent vasodilation to ACh has been observed in female arteries of rabbits and rats compared to male animals (Aloysius et al., 2012; Gisclard et al., 1988). However, other studies found that estrogenic treatment or sex did not affect receptor-mediated relaxations of arteries (Hayashi et al., 1992; Miller and Vanhoutte, 1991). In the current study, we also did not observe any sex difference in stimulated release of NO by ACh in normoglycemic animals.

It has previously been established that diabetes is associated with endothelial dysfunction (Hink et al., 2001; Vanhoutte et al., 2009), and we observed an impaired endothelium-dependent vasodilation to ACh in rat aortae one week after the induction of diabetes with STZ in both sexes. Although there was no difference in the endothelium-dependent vasodilation between diabetic male and female rat aortae, the effect of diabetes *per se* in diminishing ACh relaxation in females was significantly greater than that observed in males. Furthermore, the sensitivity to ACh decreased in diabetic female rats, but not in diabetic males compared with their respective controls. This is in agreement with data from our previous studies on sex differences in rat or rabbit aorta relaxation after acute exposure to high glucose (Goel et al., 2008; Goel et al., 2007), and on endothelium-dependent vasodilation in rat mesenteric arteries following the induction of diabetes (Zhang et al., 2012).

These data stand in contrast to the findings of Aloysius et al. (Aloysius et al., 2012) who observed that ACh relaxation was impaired in aortae from STZ-male rats, while it was enhanced in the STZ-females. The reason for this discrepancy is not clear, but it may be explained in part by experimental conditions, such as duration of the diabetic state.

In general, our findings are consistent with those who showed impairment in endothelium-dependent vasodilation in acute STZ-diabetes (Csanyi et al., 2007; Hink et al., 2001; Otter and Chess-Williams, 1994; Utkan et al., 1998). However, they are contrast with Pieper's results (Pieper, 1999). The basis for this difference is unclear, but to verify our ACh results, we also used BK, another receptor-mediated endothelium-dependent vasodilation agent. Similar to the ACh findings, aortic relaxation to BK was impaired to a much greater extent in females than males, suggesting that impairment of ACh-induced relaxation by STZ-diabetes is a general phenomenon of endothelial dysfunction.

In conduit arteries, NO is the major EDRF (Gao et al., 2011; Shimokawa et al., 1996). The modulation of endothelium-dependent vasodilation to ACh may result from an alteration of NO degradation, NO production and/or NO interaction with smooth muscle. It was observed, however, that aortic relaxation to SNP, a NO donor, was not affected by STZ-diabetes. This suggests that smooth muscle responsiveness to NO in female rat aorta was not affected by STZ-diabetes, and leaves NO degradation and production as likely candidates. It is also possible that the contractile prostanoids are elevated in diabetic female rat aorta as reported by Aloysius et al (Aloysius et al., 2012).

It has been noted that ROS may play an important role in reducing endothelium-dependent vasodilation responses in diabetes (Kalinowski and Malinski, 2004). Therefore, we examined the role of superoxide in mediating the greater impairment of endothelium-dependent vasodilation in diabetic female rat aortae. Prior incubation of aortic rings with MnTMPyP, a superoxide scavenger, significantly increased the sensitivity to ACh-induced

relaxation, but only in diabetic females, suggesting the enhancement of superoxide in this group. Along similar lines, prior treatment of female rat aorta with an SOD mimetic agent significantly improved ACh responses following acute exposure to high glucose (Goel et al., 2007).

We hypothesized that enhanced superoxide production resulted from an increased expression of Nox, which is an important source of superoxide in the vascular system (Cai and Harrison, 2000; Griendling et al., 2000). Vascular walls express high levels of Nox1, Nox2 and Nox4 (Griendling, 2004). Nox1 is mainly expressed in large conduit vessels (Lassegue et al., 2001), whereas Nox2 is more strongly expressed in resistance vessels (Touyz et al., 2002). We did not directly measure superoxide production, but our results demonstrated a significant elevation of Nox1 and Nox4 expression only in diabetic female rat aorta. These data are in accordance with our recent report (Zhang et al., 2012) demonstrating that the Nox2 mRNA expression was significantly enhanced in 1-week diabetic female mesenteric arteries.

The effects of sex on vascular smooth muscle reactivity are less clear. In accordance with Robert et al's report (Robert et al., 2005), in the current study, the maximal PE tension in the aortae from control rats tended to be greater in males than in females.

Similarly, the effects of diabetes on vascular smooth muscle are also ambiguous. Diabetes has been reported to result in decreased (Myers and Messina, 1996; Ramanadham et al., 1984), increased (Abebe et al., 1990; White and Carrier, 1988) or no change (Chang and Stevens, 1992; Mulhern and Docherty, 1989) in the vascular contraction. In the present study, STZ-diabetes significantly shifted PE responses to the left in aortic rings of both sexes. Our observations are in line with previous findings that reactivity to vasoconstrictors was enhanced early in the disease (Chang et al., 1993; Davel et al., 2000).

The enhanced responsiveness of male or diabetic aortae to PE may have been the result of a decreased release of NO and/or an enhanced release of contractile factors. The differences in basal NO could be monitored by observing differences in the degree of PE-induced contraction in the absence and presence of L-NAME (Hayashi et al., 1992). Aortic rings from female rats showed a significantly greater potentiation of PE contraction after L-NAME compared with those in male rats. These data are consistent with previously published reports (Aloysius et al., 2012; Hayashi et al., 1992; Rahimian et al., 1997), which showed greater basal NO release from aortic rings in female than in male animals. In this context, it might seem peculiar that ACh-induced relaxation was not affected by sex in normoglycemic animals. However, these results are consistent with our previous report demonstrating that, while both basal NO and eNOS expression were enhanced in aortae of female rats compared to males, ACh-stimulated NO was not affected in females (Rahimian et al., 2002).

Interestingly, STZ-diabetes reduced the level of NO in aortae of females to the same levels as those seen in control or diabetic males. This conclusion is supported by our observation that the potentiation of PE responses by L-NAME is significantly reduced in aortae from diabetic female rats to that seen in control or diabetic males.

Decreased NO in diabetic female aorta may result, in part, from a lower expression of eNOS mRNA and/or activity of eNOS. Although we did not directly measure eNOS activity, we observed that the level of eNOS mRNA expression was decreased in diabetic female aorta to the same level as seen in males. Previously published data demonstrated both a decrease (Fu et al., 2007) and an increase (Hink et al., 2001) in eNOS expression in diabetic rat aortae. Our recent findings demonstrating elevated NO-dependent responses and eNOS expression in female diabetic rat mesenteric arteries (Zhang et al., 2012) would appear to be

contradictory, but clearly, this could be related to differences in the vascular beds being studied. It is known that endothelium from different vascular beds respond differently to the pathological insult (Rosenberg and Aird, 1999), and it is highly likely that in diabetes NO regulation could vary from one vascular bed to another. Furthermore, it should be noted that in small resistance arteries EDHF also contributes to relaxation. Interestingly, we reported that the role of EDHF was reduced in female mesenteric arteries, one week after induction of diabetes (Zhang et al., 2012).

Finally, although we used a non-selective NOS inhibitor and we did not evaluate the role of other NOS isoforms, the endothelial dysfunction in diabetic animals regardless of sex may result in part from an enhanced of inducible NOS (iNOS) or uncoupled eNOS. Activation of iNOS (Maggi et al., 2003) or uncoupled eNOS (Leo et al., 2010) have been described in diabetes. Accordingly, in the current study, one week STZ-diabetes increased iNOS expression in rat aortae from both sexes (data not shown). However, the sustained accumulation of NO levels generated by iNOS does not compensate for the decreased NO bioavailability (Beckman and Koppenol, 1996). In fact, it can be toxic through reaction with superoxide to form peroxynitrite (Beckman and Koppenol, 1996).

5. CONCLUSION

In conclusion, we have shown that STZ-diabetes at a very early stage (one week) causes a more pronounced impairment of endothelial function in aortae from female compared to male rats. A role for superoxide was suggested by the fact that a superoxide scavenger could significantly enhance the sensitivity to the endothelium-dependent vasodilation, and the mRNA expression levels of Nox subunits were elevated in aortae of diabetic females. Furthermore, our data also suggests that a decrease in NO levels resulting from either decreased eNOS expression or elevated superoxide due to the higher Nox subunits may partially contribute to the predisposition of the female aorta to injury early in diabetes.

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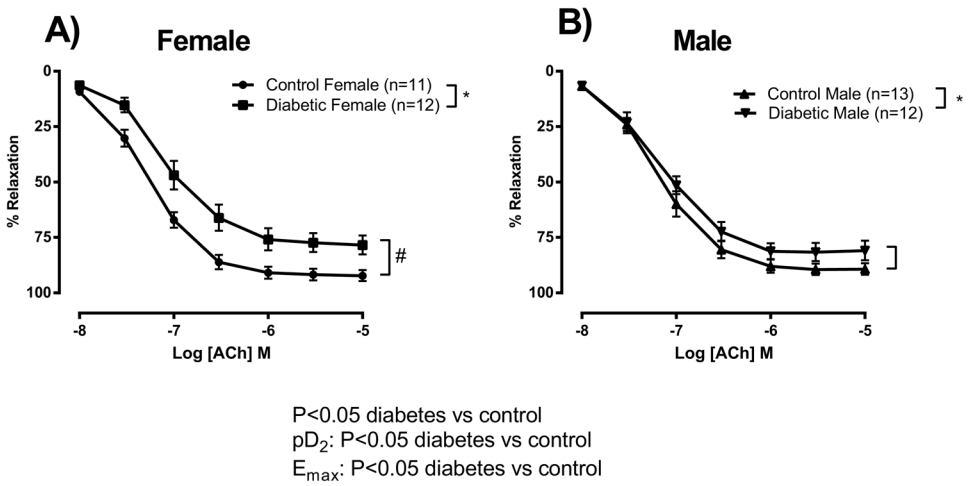

Figure 1.

Relaxation response to cumulative concentrations of acetylcholine (ACh) in intact aortic rings pre-contracted with phenylephrine (PE, 2 μM) from male and female rats analyzed at 1 week after vehicle or STZ treatment. Relaxation to ACh is expressed as a percentage of PE (2 μM) maximum contraction. Data are expressed as mean ± S.E.M., analyzed using three-way ANOVA. * P<0.05 between two groups using two-way ANOVA. # P<0.05 between male and female rats using Mann-Whitney nonparametric test. pD_2, sensitivity; E_{max}, maximum response.

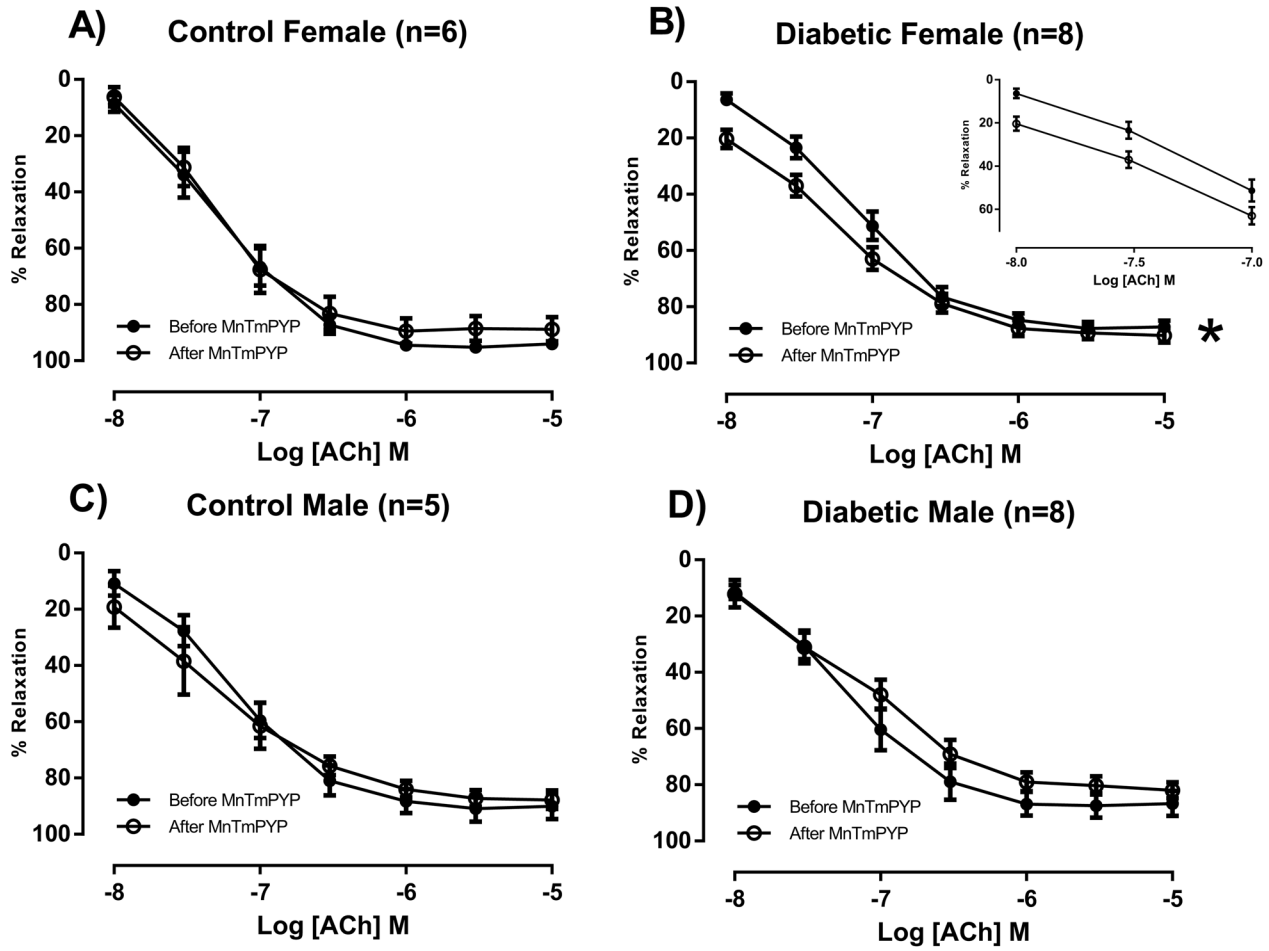

Figure 2.

Relaxation response to cumulative concentrations of acetylcholine (ACh) in intact aortic rings pre-contracted with phenylephrine (PE, 2 μM) from control female (A), diabetic female (B), control male (C) and diabetic male (D) rats at 1 week after vehicle or STZ treatment. Relaxation to ACh was measured before and after incubation with MnTmPYP (25 μM). Data are expressed as mean ± S.E.M., with * P<0.05 vs before MnTmPYP, as analyzed using two-way ANOVA with repeated measures.

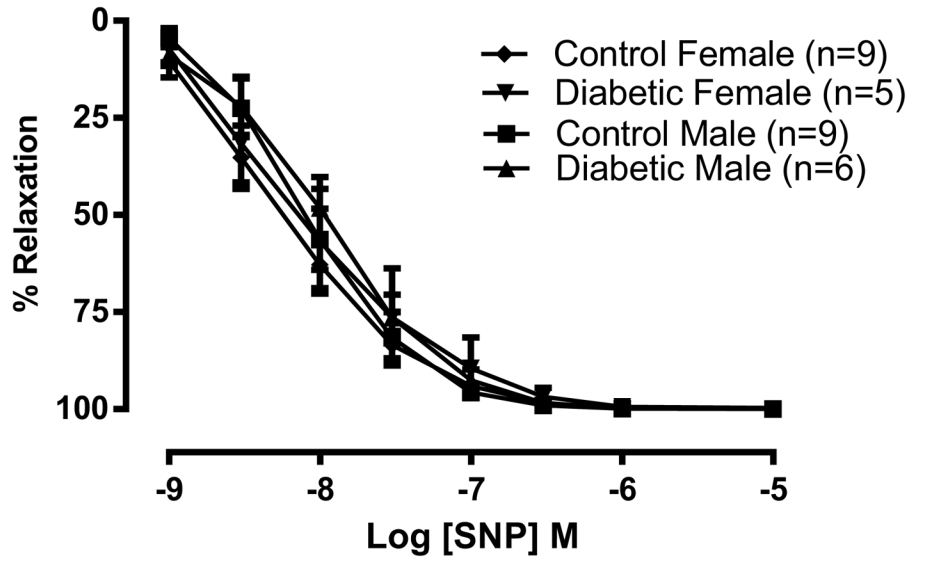

Figure 3.

Relaxation response to cumulative concentrations of sodium nitroprusside (SNP) in intact aortic rings pre-contracted with phenylephrine (PE, 2 μM) from male and female rats at 1 week after vehicle or STZ treatment. Relaxation to SNP is expressed as a percentage of PE (2 μM) maximum contraction. Data are expressed as mean ± S.E.M..

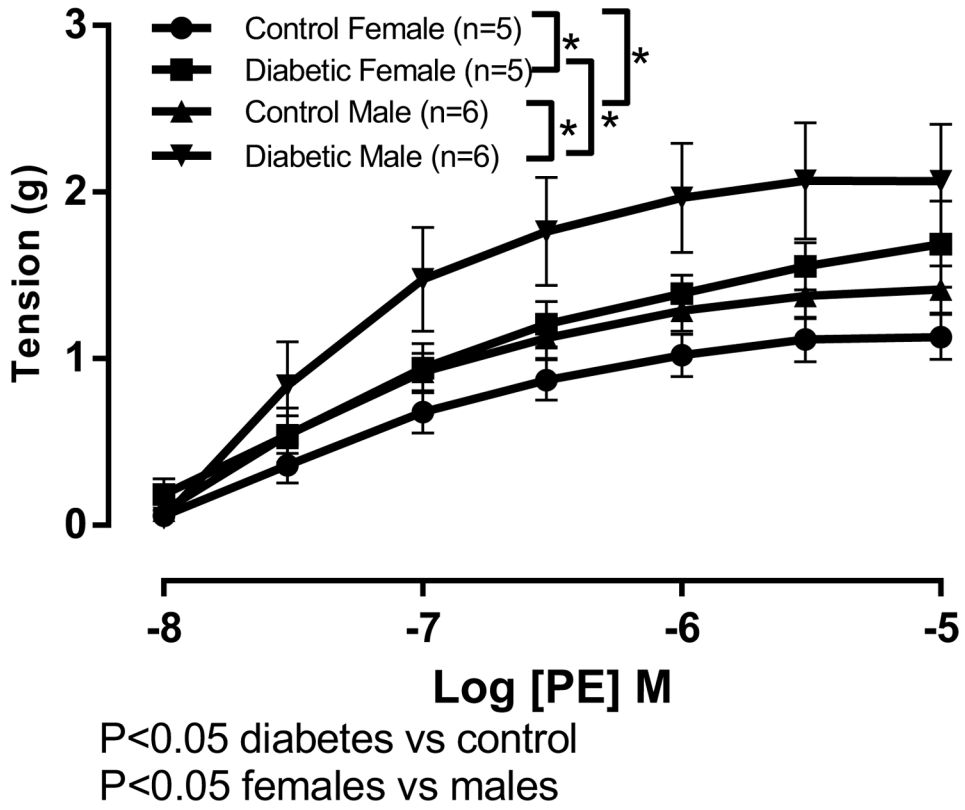

Figure 4.

Contractile response to cumulative concentrations of phenylephrine (PE) in intact aortic rings from male and female rats at 1 week after vehicle or STZ treatment. Data are expressed as mean ± S.E.M., analyzed using three-way ANOVA. * P<0.05 between two groups using two-way ANOVA.

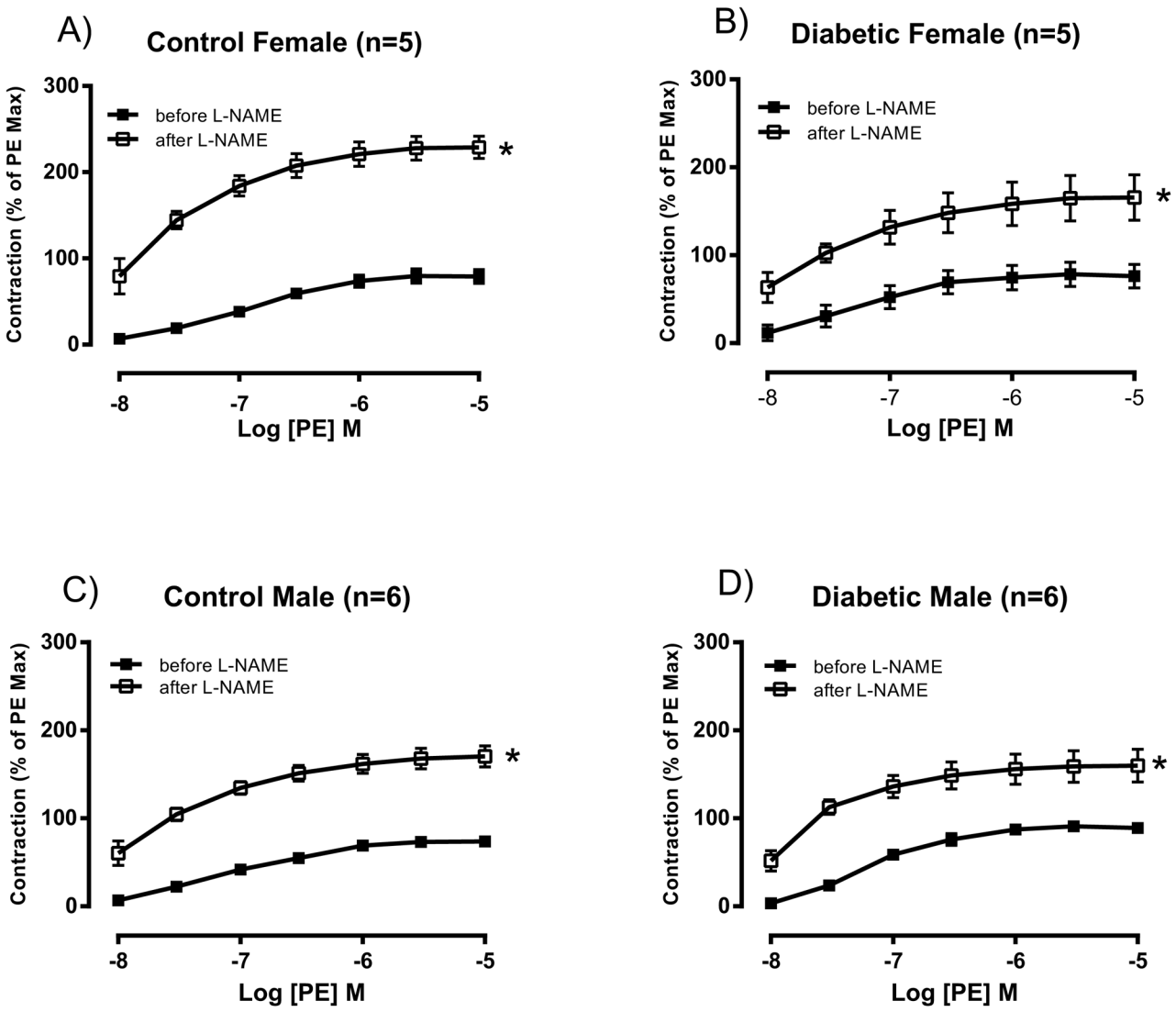

Figure 5.

Contractile response to cumulative concentrations of phenylephrine (PE) in intact aortic rings from control female (A), diabetic female (B), control male (C) and diabetic male (D) rats at 1 week after vehicle or STZ treatment. Contraction to PE was measured before and after incubation with N^{ω}-Nitro-L-arginine methyl ester (L-NAME, 200 μM). Responses were performed in the presence of indomethacin (10 μM). Data are expressed as mean ± S.E.M., with * $P<0.05$ vs before L-NAME in all groups as analyzed using two-way ANOVA with repeated measures.

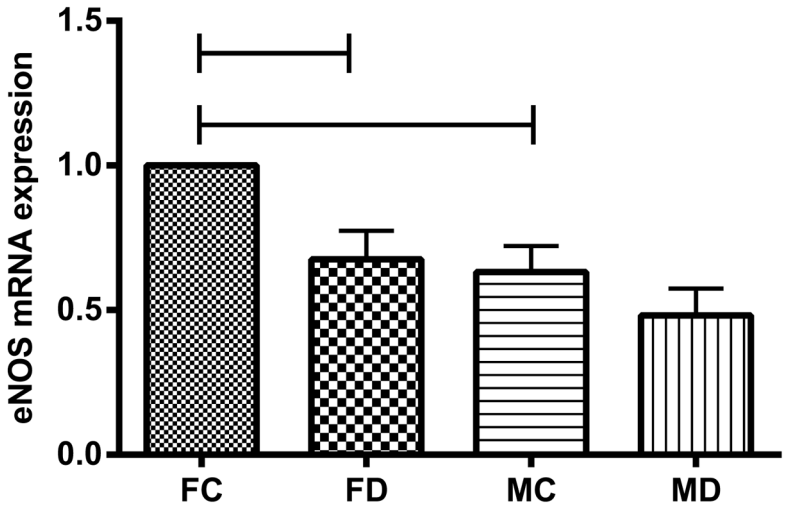

Figure 6.

Real-time PCR analysis of eNOS mRNA expression in rat thoracic aortae of male and female rats at 1 week after vehicle or STZ treatment. Data are expressed as mean ± S.E.M.. The eNOS mRNA expression levels of control female rats were normalized as one. Capped lines indicate statistical differences ($P<0.05$) between two groups, as analyzed by Student's unpaired t-test. (FC: female control; FD: female diabetic; MC: male control; MD: male diabetic).

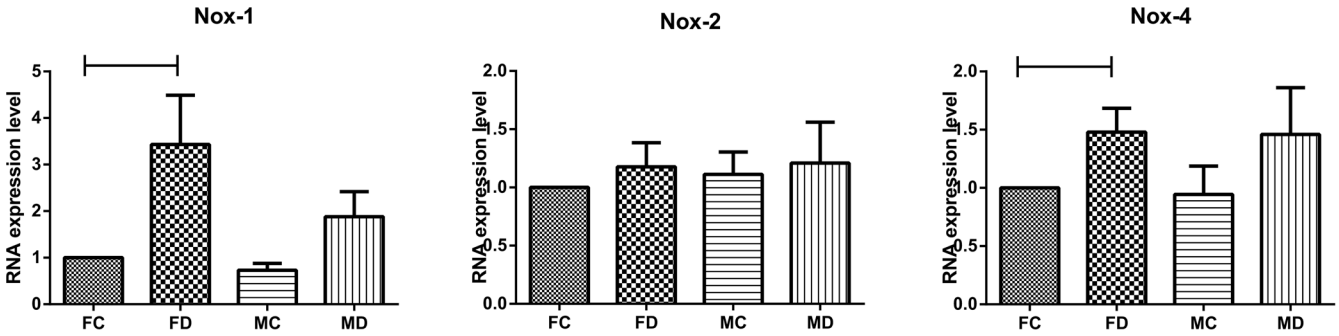

Figure 7.

Real-time PCR analysis of NADPH oxidase (Nox) subunits Nox1, Nox2, and Nox4 mRNA expression in rat thoracic aortae of male and female rats at 1 week after vehicle or STZ treatment. Data are expressed as mean ± S.E.M.. The Nox mRNA expression levels of control female rats were normalized as one. Capped lines indicate statistical differences ($P<0.05$) between two groups, as analyzed by Student's unpaired t-test. (FC: female control; FD: female diabetic; MC: male control; MD: male diabetic)

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Table 1

Body weight, blood glucose levels, E_{max} and pD_2 to acetylcholine (ACh) of male and female rats 1 week after vehicle or STZ treatment.

Group	n	Weight (g)	Blood Glucose (mg/dl)	E_{max} (%)	pD_2
Control Female	11	233.4±3.8	168±20	92±2	7.19±0.06
Diabetic Female	12	201.3±4.5[b]	555±18[b]	78±4[b]	6.78±0.18[b]
Control Male	13	304.5±14.9[a]	147±14	89±3	7.04±0.09
Diabetic Male	12	263.8±11.7[b]	492±40[b]	81±4[b]	6.96±0.10

Data are expressed as mean ± S.E.M..

[a]Statistic significance: P<0.05 (vs control female in the respective study), analyzed using Student's unpaired t-test;

[b]Statistic significance: P<0.05 (vs non diabetic, same gender in the respective study), analyzed using Student's unpaired t-test.

STZ, streptozotocin

Table 2

pD_2 to acetylcholine (ACh) before and after MnTmPyP in male and female rat aortae at 1 week after vehicle or STZ treatment.

Group	n	pD_2	
		before MnTmPYP	after MnTmPYP
Control Female	6	7.25±0.11	7.15±0.16
Diabetic Female	8	6.97±0.10	7.23±0.09[a]
Control Male	5	7.10±0.10	7.24±0.22
Diabetic Male	9	7.06±0.18	6.88±0.15

Data are expressed as mean ± S.E.M..

[a]Statistic significance: P<0.05 (vs before), as analyzed using Student's paired t-test.

STZ, streptozotocin

Table 3

Tension$_{max}$ and pD$_2$ to phenylephrine (PE) in male and female rat aortae at 1 week after vehicle or STZ treatment. (FC: female control; MC: male control)

Group	n	Tension$_{max}$ (g)	pD$_2$
Control Female	5	1.13±0.14	7.13±0.09
Diabetic female	5	1.69±0.26 (P=0.05 vs FC)	7.01±0.34
Control Male	6	1.42±0.14 (P=0.05 vs FC)	7.23±0.09
Diabetic Male	6	2.07±0.34 (P=0.06 vs MC)	7.27±0.08

Data are expressed as mean ± S.E.M..

STZ, streptozotocin

Table 4

E_{max}, $Tension_{max}$, pD_2 and ΔAUC to phenylephring (PE) in male and female rat aortae at 1 week after vehicle or STZ treatment.

	n	E_{max} (%)	$Tension_{max}$ (g)	pD_2	ΔAUC
Control Female	5				
Before L-NAME		78.93±8.03	0.87±0.10	7.03±0.11	-
After L-NAME		228.85±12.98[b]	2.35±0.24[b]	7.82±0.11[b]	414.16±41.24
Diabetic female	5				
Before L-NAME		76.30±13.30	1.22±0.23	7.28±0.20	-
After L-NAME		165.73±25.80[b]	2.63±0.28[b]	8.02±0.25[b]	235.93±63.41[a]
Control Male	6				
Before L-NAME		73.69±4.76	1.05±0.13	7.05±0.09	-
After L-NAME		170.44±29.23[b]	2.39±0.28[b]	7.67±0.11[b]	267.52±36.78
Diabetic Male	6				
Before L-NAME		89.06±4.75	1.88±0.34[a]	7.16±0.08	-
After L-NAME		159.95±18.62[b]	3.02±0.20[b]	7.89±0.11[b]	217.87±51.31

Data are expressed as mean ± S.E.M..

[a]Statistic significance: P<0.05 (vs non diabetic, same gender), as analyzed using Student's unpaired t-test;

[b]Statistic significance: P<0.05 (vs before L-NAME), as analyzed using Student's paired t-test.

STZ, streptozotocin