Sex differences in mesenteric endothelial function of streptozotocin-induced diabetic rats: a shift in the relative importance of EDRFs

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Zhang R, Thor D, Han X, Anderson L, Rahimian R. Sex differences in mesenteric endothelial function of streptozotocin-induced diabetic rats: a shift in the relative importance of EDRFs. Am J Physiol Heart Circ Physiol 303: H1183–H1198, 2012. First published September 14, 2012; doi:10.1152/ajpheart.00327.2012.—Several studies suggest that diabetes affects male and female vascular beds differently. However, the mechanisms underlying the interaction of sex and diabetes remain to be investigated. This study investigates whether there are 1) sex differences in the development of abnormal vascular responses and 2) changes in the relative contributions of endothelium-derived relaxing factors in modulating vascular reactivity of mesenteric arteries taken from streptozotocin (STZ)-induced diabetic rats at early and intermediate stages of the disease (1 and 8 wk, respectively). We also investigated the mesenteric expression of the mRNAs for endothelial nitric oxide (NO) synthase (eNOS) and NADPH oxidase (Nox) in STZ-induced diabetes in both sexes. Vascular responses to acetylcholine (ACH) in mesenteric arterial rings precontracted with phenylephrine were measured before and after pretreatment with indomethacin (cyclooxygenase inhibitor), Na+/K+-ATPase inhibitor, Nω-nitro-arginine methyl ester (NOS inhibitor), or barium chloride (K+ blocker) plus ouabain (Na+/K+-ATPase inhibitor). We demonstrated that ACh-induced relaxations were significantly impaired in mesenteric arteries from both male and female diabetic rats at 1 and 8 wk. However, at 8 wk the extent of impairment was significantly greater in diabetic females than diabetic males. Our data also showed that in females, the levels of eNOS, Nox2, and Nox4 mRNA expression and the relative importance of NO to the regulation of vascular reactivity were substantially enhanced, whereas the importance of endothelium-derived hyperpolarizing factor (EDHF) was significantly reduced at both 1 and 8 wk after the induction of diabetes. This study reveals the predisposition of female rat mesenteric arteries to vascular injury after the induction of diabetes may be due to a shift away from a putative EDHF, initially the major vasodilatory factor, toward a greater reliance on NO.

Cardiovascular diseases (CVDs) are the major causes of morbidity and mortality in patients with diabetes mellitus. Several reports including our recent studies (30, 31, 36) suggest that hyperglycemia and diabetes affect male and female vascular beds differently. Clinically, premenopausal women have a lower incidence of CVDs compared with age-matched men. However, premenopausal women with diabetes not only lose this sex-based cardiovascular protection but also have a higher risk of CVDs than men (4, 20, 71). Nonetheless, there is insufficient evidence to establish the mechanism(s) underlying the loss of this female-specific cardiovascular protection in premenopausal patients with diabetes.

Endothelial dysfunction is an early sign of diabetic vascular diseases. Endothelial dysfunction can be defined as a reduced endothelium-dependent vasorelaxation (EDV) to vasodilators, such as acetylcholine (ACH) and bradykinin, or flow-mediated vasodilation. Thus EDV is generally used as a reproducible parameter to investigate endothelial function under various pathological conditions. Impaired EDV has been observed in both type 1 and type 2 diabetes (18, 37, 55). However, an impairment of EDV was not observed in the hindlimb of acute streptozotocin (STZ)-induced diabetic rats (9) and in mesenteric vasculature of mice 1 wk after the induction of STZ diabetes (59). Clinical studies also reported an intact EDV in the forearm of type 1 diabetic patients (47) and in renal vasculature from type 2 diabetic patients (19). There are also some reports of enhanced EDV in diabetes (7, 74). Conflicting data were also obtained when responses to vasoconstricting factors were studied (1, 42, 85, 93). The source of these discrepancies is not clear. However, the duration of disease, among other factors, may play an important role in alteration of vascular reactivity to vasodilating or vasoconstricting agents in diabetes (1, 68). Thus the initial aim of our study was to determine whether STZ-induced diabetes impaired the responses to endothelium-dependent and -independent vasodilators as well as vasoconstrictors in mesenteric arteries of rats. Specifically, we studied whether there were sex differences in the development of abnormal vascular responses at two time points in the course of disease (at 1 and 8 wk after induction of diabetes). The 8-wk duration of diabetes in this study was chosen based on reports by others showing that EDV is not impaired until 7 wk after STZ treatment (34, 41, 68). We also included 1-wk duration of diabetes to examine the responses of mesenteric arterial rings at the early stage of the disease. Mesenteric arteries were chosen for analysis because they are important for maintaining vascular tone and contribute to the regulation of blood pressure in the basal state and in response to vasoactive agents.

Impaired EDV can arise from either a decreased synthesis or release of endothelium-derived relaxing factors (EDRFs), including prostacyclin (PGI2), nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF), or an increased release of endothelium-derived contracting factors such as thromboxane A2 and endothelin-1 (ET-1). In addition, inactivation of NO by reactive oxygen species such as superoxide may also be involved. NADPH oxidase (Nox) family is one of the potent cellular sources of superoxide in the cardiovascular system (11, 12, 32, 33). Among the catalytic subunit of NADPH oxidase, high expression of Nox2 and Nox4 appears to...
be a specific characteristic of vascular cells (2, 13, 80), and their upregulation have been implicated in vascular diseases (80, 82).

Although NO has been generally considered as the principal mediator of EDV, it has become clear that EDHF is also an important regulator of vascular tone, especially in small vessels such as mesenteric arteries. The contribution of EDHF to EDV is inversely proportional to the vessel size (87). There are two major categories of EDHF involved in vascular reactivity (25, 45). The classical pathway involves opening of small and intermediate conductance calcium-activated potassium channels on the endothelium, and the subsequent hyperpolarizing of smooth muscle cells via activation of Na\(^+\)-K\(^+\)-ATPase and/or \(K_v\) channels or through myoendothelial gap junctions (24, 66, 73). The second pathway for EDHF activity does not involve endothelial cell hyperpolarization, but is associated with the \(Ca^{2+}\)-dependent release of an eclectic variety of EDHFs including NO, PG12, and epoxideciosatrenio acids followed by the hyperpolarization of smooth muscle cells through the activation of large conductance \(Ca^{2+}\)-activated \(K^+\) or \(K_{ATP}\) channels (67, 84, 91). In rat mesenteric arteries, EDV is mediated by NO, as well as both EDHF pathways (62, 81, 89).

Diabetes-induced changes to the relative contributions of various EDRFs to EDV in smaller arteries, such as mesenteric arteries, are not fully understood. Kamata et al. (39) demonstrated that EDV to ACh in renal vasculature was regulated by PG12, and an increase of PG12 was a possible compensatory mechanism in STZ-induced diabetic rats. Wigg et al. (95) reported a preserved NO-dependent response, but an impaired EDHF-dependent response in femoral and mesenteric arteries. On the other hand, Shi et al. (75) described a reduced NO-mediated response and an enhanced EDHF-mediated response in mesenteric arteries of diabetic rats. Finally, Leo et al. (45) reported both impaired NO- and EDHF-mediated relaxation in the same arteries.

By comparison there is extensive literature on sex differences in the relative contribution of EDRFs to EDV (61, 86). It is generally agreed that NO release is increased in the aorta and coronary arteries of females compared with those of males (80). In contrast, NO may be of greater importance in tail and mesenteric arteries in males than in females (53, 63). An elevated contribution of EDHF to vascular reactivity in females has also been reported (63, 94). Under NO-deficient conditions flow-mediated responses are reported to be due entirely to EDHF in females, whereas PG12 appears to be the major contributor to EDV in males (96).

Although several studies on the effect of sex on the relative contribution of EDRFs to EDV exist, the influence of both sex and diabetes in modulating the contribution of EDRFs to EDV in small arteries, such as mesenteric arteries, has not been examined. Thus the second aim of this study was to investigate whether sex and STZ-induced diabetes (1 and 8 wk) altered the relative contributions of PG12, NO, and EDHF to mesenteric reactivity in male and female rats. We also investigated the mesenteric mRNA expressions for endothelial NO synthase (eNOS) as well as Nox2- and Nox4-dependent NADPH oxidase in STZ-induced diabetic rat of both sexes.

**MATERIALS AND METHODS**

**Materials**

All chemicals and drugs were purchased from Sigma-Aldrich (St. Louis, MO). All agents were dissolved in distilled water, unless otherwise stated.

**Animals**

Adult male and female Sprague-Dawley rats aged 9—11 wk (Simonsen Laboratories, Gilroy, CA) were divided into four experimental groups: control female, diabetic female, control male, and diabetic male. After an overnight fast, diabetes was induced via the tail vein by single injection of STZ (60 mg/kg). Age-matched control animals were injected with a similar volume of citrate buffer. On the day of euthanization, blood glucose was measured using a standard glucose test meter (OneTouch, LifeScan, CA). Animals were considered diabetic when nonfasting blood glucose levels were higher than 300 mg/dl along with exhibiting polyuria, polydipsia, and polyphagia. One or eight weeks after the injection of STZ, animals were euthanized using a carbon dioxide gas chamber according to the recommendations from the 2007 AVMA Guidelines on Euthanasia and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All animal protocols were approved by the Animal Care Committee of the University of the Pacific and compiled with the Guide for the Care and Use of Laboratory Animals (Eighth Edition, 2011).

**Isometric Force Measurements in Mesenteric Arteries**

The second- or third-order branches of mesenteric arteries were excised from veins and cleared of fatty and adhering tissue and cut into 2-mm rings (internal diameter range, 250—350 \(\mu\)m). Each ring was mounted between two jaws using two tungsten wires (40 \(\mu\)m diameter) in organ baths (model 101M; Danish Myo Technology, Denmark) containing Krebs solution of (in mM) 119 NaCl, 4.7 KCl, 1.6 CaCl\(_2\), 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), 25 NaHCO\(_3\), 0.023 EDTA, and 6 glucose at 37°C, bubbled with 95% O\(_2\)-5% CO\(_2\). The arterial tension was monitored with a computer-based data acquisition system (Chart 5, Powerlab; ADInstruments, Colorado Springs, CO). The rings were normalized to a resting tension of 13.3 kPa and equilibrated for 30 min to obtain a basal tone. Stimulation of rings with 80 mM KCl solution was repeated two times to obtain a stable contractile response. ACh (10 \(\mu\)M)-induced relaxation of the phenylephrine (PE; 2 \(\mu\)M) precontracted vessels was taken as evidence for the preservation of an intact endothelium. For the relaxation studies, the mesenteric arterial rings were precontracted with an equal submaximal concentration of PE (2 \(\mu\)M) since the maximum tension developed in response to PE was similar between the control and diabetic groups as well as the control male and female groups.

**Relaxation Response Curves to ACh**

Mesenteric arterial rings were contracted with PE (2 \(\mu\)M), which produced about 80% of the maximal contraction. The first concentration-response curve (CRC) was obtained by the addition of increasing concentrations of ACh (10\(^{-9}\) to 10\(^{-5}\) M). After incubation with indomethacin (Indo; 10 \(\mu\)M; dissolved in DMSO), a cyclooxygenase (COX) inhibitor for 20 min, rings were again precontracted with PE and the second CRC to ACh was generated. The third CRC to ACh in PE-precontracted rings was obtained after incubation with Indo plus \(N^\text{6}-\text{nitro-L-arginine methyl ester}\) (L-NAME; 200 \(\mu\)M), a nonsselective NO synthase (NOS) inhibitor for 20 min. Finally, the fourth CRC to ACh was obtained after incubation with Indo plus L-NAME plus barium chloride (100 \(\mu\)M), a \(K_{\text{IR}}\) channel blocker, and ouabain (10 \(\mu\)M), a \(Na^+\)-\(K^+\)-ATPase inhibitor for 20 min. After each CRC to ACh in PE-precontracted rings, there was a 5—10-min tissue wash with Krebs solution to allow a return to basal tone before beginning the next CRC. Furthermore, to show that the reactivity to ACh was not significantly altered by the same tissue to four successive experiments being exposed, CRCs to ACh were simultaneously generated in mesenteric arteries from the same animal with no drug (vehicle only) given during incubation. Although a slight shift of second CRC to the right was observed compared with the first CRC over time in the vehicle study, there were no observable...
differences between the second, third, and fourth ACh CRC (data not shown).

**Relaxation Response Curves to Sodium Nitroprusside**

The CRCs to sodium nitroprusside (SNP; 10^{-9} to 10^{-5} M), an endothelium-independent vasodilating agent, were generated in mesenteric arterial rings precontracted with PE (2 μM) from control and age-matched male and female diabetic rats.

**Constrictor Response Curves to PE**

The CRC to PE was obtained by the addition of increasing concentrations of PE (10^{-7} to 3 × 10^{-5} M). The rings were then washed with Krebs solution, and the CRC to PE was repeated before and after incubation of l-NAME (200 μM, 20 min) in the presence of Indomethacin (10 μM). The use of this concentration of l-NAME was based on our previous studies and investigation by others (69, 97). Similarly, the vehicle study was performed simultaneously in mesenteric arteries from the same animal and no drug (vehicle only) was given during incubation. There were no differences between the first and second PE CRC (data not shown).

**Real-Time PCR**

Total RNA was extracted from mesenteric arteries using an RNeasy Mini Kit with on-column DNase treatment (Qiagen, Valencia, CA). The first-strand cDNA was subsequently synthesized by reverse transcription of 2 μg of total RNA using the Omniscript RT Kit (Qiagen), in a total volume of 20 μL according to the manufacturer’s suggestions. The gene fragments were specifically amplified with the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) using real-time RT-PCR (MyiQ Single-Color Real-Time PCR Detection System; Bio-Rad). Internal variations were normalized to rat GAPDH, with the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) using real-time RT-PCR (MyiQ Single-Color Real-Time PCR Detection System; Bio-Rad). Statistical analysis was performed with SPSS software (version 18; SPSS, Chicago, IL). Data were reported as means ± SE. Differences among four groups were analyzed using three-way ANOVA, with factors being sex, diabetes, and concentration. Comparison of CRCs between two groups was done using two-way ANOVA, with one factor being concentration and the other being groups (female vs. male and control vs. diabetic). Comparison of CRCs in a pre/posttest 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 plasma NO level). A probability value of less than 5% (P < 0.05) was considered significant.

**RESULTS**

**Effects of STZ-Induced Diabetes on Body Weight and Blood Glucose Levels**

Final body weights and blood glucose levels are given in Table 1. One or eight weeks after the induction of diabetes, the body weights of both female and male rats were significantly lower than those in age-matched nondiabetic controls (Table 1). In addition, the nonfasting blood glucose levels of diabetic female and male rats were significantly higher than those of their respective nondiabetic controls at both 1 and 8 wk (Table 1). Although there appeared to be a decline in blood glucose level in diabetic males from week 1 to week 8 (509 ± 58 vs. 396 ± 82 mg/dl), the mean difference was not statistically significant. The blood glucose levels in diabetic females were similar at both time periods (549 ± 19 vs. 539 ± 49 mg/dl, 1 and 8 wk, respectively).

**Effects of Sex and Diabetes on ACh-Induced Mesenteric Relaxation**

Concentration-response curves to ACh were similar in age-matched nondiabetic control groups in both sexes, regardless of 1- or 8-wk study (Fig. 1, A and B).

**Table 1. Body weight and blood glucose levels of male and female rats 1 and 8 wk after vehicle or STZ treatment**

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Body Weight, g</th>
<th>Blood Glucose, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control female</td>
<td>12</td>
<td>241 ± 5</td>
<td>156 ± 25</td>
</tr>
<tr>
<td>Diabetic female</td>
<td>11</td>
<td>216 ± 5**</td>
<td>549 ± 19***</td>
</tr>
<tr>
<td>Control male</td>
<td>12</td>
<td>303 ± 12</td>
<td>149 ± 10</td>
</tr>
<tr>
<td>Diabetic male</td>
<td>9</td>
<td>239 ± 8***</td>
<td>509 ± 58**</td>
</tr>
<tr>
<td>8 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control female</td>
<td>9</td>
<td>253 ± 3</td>
<td>161 ± 29</td>
</tr>
<tr>
<td>Diabetic female</td>
<td>7</td>
<td>217 ± 14*</td>
<td>539 ± 49***</td>
</tr>
<tr>
<td>Control male</td>
<td>8</td>
<td>392 ± 9</td>
<td>120 ± 10</td>
</tr>
<tr>
<td>Diabetic male</td>
<td>8</td>
<td>337 ± 18*</td>
<td>396 ± 82**</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 diabetes vs. respective control, analyzed using Student’s unpaired t-test. STZ, streptozotocin.
One week after STZ injection, there was a significant decrease in ACh-induced relaxation in mesenteric arterial rings from diabetic rats compared with those from control rats, regardless of sex (Fig. 1A). In females, the R_{max} to ACh was 99 ± 1% in control and 83 ± 6% in diabetic rats; in males, R_{max} was 99 ± 0% in control and 85 ± 5% in diabetic rats. However, the sensitivity of mesenteric arteries to ACh as assessed by \(-\log[EC_{50}]\) (pD_{2}) was similar in all groups (Table 2, first column).

Eight weeks after the induction of STZ diabetes, the CRC to ACh in mesenteric arterial rings was markedly reduced and shifted to the right in both diabetic males and females (Fig. 1B). Both R_{max} and pD_{2} to ACh were significantly reduced in mesenteric arteries from diabetic rats compared with age-matched control (Table 3, first column). Additionally, when compared with the observations in the 1-wk study, there was a significant difference in mesenteric arterial relaxation between diabetic males and females. The effect of STZ-induced diabetes in blunting ACh-mediated relaxation in females was significantly greater than in males as assessed by the bigger shift of ACh CRC to the right (Fig. 1B). Furthermore, the pD_{2} to ACh was 6.85 ± 0.12 in diabetic females and 7.20 ± 0.09 in diabetic males (Table 3, first column), indicating the lower sensitivity of mesenteric arteries to ACh in diabetic females.

Effects of Sex and Diabetes on the Relative Contributions of PGI_{2}, NO, and EDHF to Mesenteric Relaxation

The relative contributions of PGI_{2}, NO, and EDHF to vasorelaxation induced by ACh were estimated by sequentially inhibiting COX, NOS, and a combination of K_{dep} channel and Na^{+}-K^{+}-ATPase. Specifically, EDV to ACh (10^{-8} to 10^{-5} M) in rat mesenteric arterial rings precontracted with PE (2 μM) was obtained before and after pretreatment with Indo (10 μM), followed by addition of l-NAME (200 μM) and then a combination of barium chloride (100 μM) and ouabain (10 μM).

One-week study. One week after the induction of diabetes, the administration of Indo to block COX activity had no apparent effect on R_{max} to ACh, but it decreased the sensitivity of mesenteric arteries to ACh in diabetic females and control males (Fig. 2, B and C, and Table 2, second column). In females, addition of l-NAME to block NO synthesis reduced Indo-resistant vasorelaxation in both 1-wk control and diabetic animals (Fig. 2, A and B). However, when compared with that of 1-wk control females, the effect of l-NAME was much greater in 1-wk diabetic females as measured by reduction of ACh R_{max} (81 ± 6% and 38 ± 11%, respectively; Fig. 2, A vs. B, and Table 2, third column). In males, l-NAME substantially

Table 2. pD_{2} and R_{max} to ACh at 1 wk

<table>
<thead>
<tr>
<th>Groups</th>
<th>No Drug</th>
<th>Indo</th>
<th>Indo + l-NAME</th>
<th>Indo + l-NAME + Barium + Ouabain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control female</td>
<td>7.33 ± 0.10</td>
<td>99 ± 1</td>
<td>7.10 ± 0.10</td>
<td>98 ± 1</td>
</tr>
<tr>
<td>Diabetic female</td>
<td>7.18 ± 0.09</td>
<td>83 ± 6</td>
<td>6.68 ± 0.07</td>
<td>91 ± 4</td>
</tr>
<tr>
<td>Control male</td>
<td>7.38 ± 0.12</td>
<td>99 ± 0</td>
<td>7.03 ± 0.12</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>Diabetic male</td>
<td>6.97 ± 0.24</td>
<td>85 ± 5</td>
<td>6.80 ± 0.15</td>
<td>77 ± 11</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. A comparison of the sensitivity (pD_{2}) and maximum response (R_{max}) to ACh in the absence (no drug) or in the presence of indomethacin (Indo), Indo + N^{6}-nitro-l-arginine methyl ester (l-NAME), and Indo + l-NAME + barium + ouabain in mesenteric arteries from male and female rats 1 wk after vehicle or STZ treatment is shown. *P < 0.05 vs. control female within the respective treatment; †P < 0.05 vs. control male within the respective treatment; ‡P < 0.05 vs. diabetic female within the respective treatment (Student’s unpaired t-test); §P < 0.05 vs. no drug control within each group; ¶P < 0.05 vs. Indo within each group; ††P < 0.05 vs. Indo + l-NAME within each group (Student’s paired t-test). ND, not determined.
blocked the remaining relaxation in both 1-wk control and diabetic animals (Fig. 2, C and D). However, its effect was greater in 1-wk control males than that observed in 1-wk diabetic male rats. Finally, the remaining Indo- and L-NAME-resistant vasorelaxation was almost completely abolished in all groups by simultaneously blocking Kir channels and Na+/K+-ATPase with barium (100 μM) and ouabain (10 μM), respectively (Fig. 2). There were no significant statistical differences in the slight remaining resistant vasorelaxation between sexes or diabetic animals and their respective age-matched controls (Table 2, last column).

**Eight-week study.** Eight weeks after the induction of diabetes, inhibition of COX had no apparent effect on ACh R_{\text{max}} but reduced sensitivity to ACh in all four experimental groups (Table 3).

### Table 3. pD\textsubscript{2} and R\textsubscript{max} to ACh at 8 wk

<table>
<thead>
<tr>
<th>Groups</th>
<th>No Drug</th>
<th>Indo</th>
<th>Indo + L-NAME</th>
<th>Indo + L-NAME + Barium + Ouabain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control female</td>
<td>7.69 ± 0.10</td>
<td>7.32 ± 0.11\textsuperscript{d}</td>
<td>ND 66 ± 13\textsuperscript{d,e}</td>
<td>ND 9 ± 2\textsuperscript{d,e}</td>
</tr>
<tr>
<td>Diabetic female</td>
<td>6.85 ± 0.12\textsuperscript{a}</td>
<td>6.31 ± 0.12\textsuperscript{d}</td>
<td>ND 15 ± 7\textsuperscript{d,e}</td>
<td>ND 8 ± 2\textsuperscript{d,e}</td>
</tr>
<tr>
<td>Control male</td>
<td>7.73 ± 0.03</td>
<td>7.25 ± 0.19\textsuperscript{d}</td>
<td>ND 81 ± 8</td>
<td>ND 14 ± 2\textsuperscript{d,e}</td>
</tr>
<tr>
<td>Diabetic male</td>
<td>7.20 ± 0.09\textsuperscript{b,c}</td>
<td>6.25 ± 0.23\textsuperscript{b,d}</td>
<td>ND 34 ± 13\textsuperscript{b,d,e}</td>
<td>ND 11 ± 2\textsuperscript{d,e}</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. A comparison of pD\textsubscript{2} and R\textsubscript{max} to ACh in the absence (no drug) or in the presence of Indo, Indo + L-NAME, and Indo + L-NAME + barium + ouabain in mesenteric arteries from male and female rats 8 wk after vehicle or STZ treatment is shown. \textsuperscript{a}P < 0.05 vs. control female within the respective treatment; \textsuperscript{b}P < 0.05 vs. control male within the respective treatment; \textsuperscript{c}P < 0.05 vs. diabetic female within the respective treatment (Student’s unpaired t-test); \textsuperscript{d}P < 0.05 vs. no drug control within each group; \textsuperscript{e}P < 0.05 vs. Indo within each group; \textsuperscript{f}P < 0.05 vs. Indo + L-NAME within each group (Student’s paired t-test).

Fig. 2. Effects of inhibiting cyclooxygenase, nitric oxide (NO) synthase, K\textsubscript{ir} channel, and Na\textsuperscript{+}/K\textsuperscript{+}-ATPase on ACh-induced vasorelaxation in mesenteric arteries taken from control female (A), diabetic female (B), control male (C), and diabetic male (D) rats at 1 wk. ACh relaxation was measured in the presence of indomethacin (Indo; 10 μM), followed by addition of N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME; 200 μM), and then with a combination of barium chloride (100 μM) and ouabain (10 μM). Data are expressed as means ± SE. \*P < 0.05 vs. no drug; \#P < 0.05 vs. Indo; \^P < 0.05 vs. Indo + L-NAME, analyzed using 2-way ANOVA with repeated measures followed by Bonferroni post hoc test. EDHF, endothelium-derived hyperpolarizing factor.
In females, addition of L-NAME resulted in a further reduction in ACh-induced relaxation of mesenteric arteries from both 8-wk control and diabetic rats (Fig. 3, A and B). Similar to the results of the 1-wk study, the effect of L-NAME on vasorelaxation was more prominent in diabetic females compared with age-matched control rats (Fig. 3, B vs. A). The R_max to ACh in control females and diabetic females was 66 ± 13% and 15 ± 7%, respectively (Table 3, third column). In males, adding L-NAME significantly reduced the Indo-resistant relaxation in the mesenteric arteries from both 8-wk control and diabetic rats (Fig. 3, C and D). Similar to 8-wk females, the effect of L-NAME was greater in 8-wk diabetic males than that observed in 8-wk control males (Fig. 3, D vs. C). The R_max to ACh in control males and diabetic males was 81 ± 8% and 34 ± 13%, respectively (Table 3, third column). Thus the addition of L-NAME had a more significant effect in diabetic animals compared with their respective controls in both sexes in the 8-wk study. Finally, pretreatment of mesenteric arteries with barium and ouabain in the presence of Indo and L-NAME almost completely abolished the remaining ACh-induced vasorelaxation in both sexes (Fig. 3 and Table 3, last column).

Fig. 3. Effects of inhibiting cyclooxygenase, NO synthase, Kᵢᵣ channel, and Na⁺-K⁺-ATPase on ACh-induced vasorelaxation in mesenteric arteries taken from control female (A), diabetic female (B), control male (C), and diabetic male (D) rats at 8 wk. ACh relaxation was measured in the presence of Indo (10 μM), followed by addition of L-NAME (200 μM), and then with a combination of barium chloride (100 μM) and ouabain (10 μM). Data are expressed as means ± SE. *P < 0.05 vs. no drug; #P < 0.05 vs. Indo; ^P < 0.05 vs. Indo + l-NAME, analyzed using 2-way ANOVA repeated measures followed by Bonferroni post hoc test.

Effects of Sex and Diabetes on SNP-induced Mesenteric Relaxations

Endothelium-independent relaxation was investigated by performing CRCs to SNP (10⁻⁹ to 10⁻⁵ M). CRCs to SNP were similar in age-matched control groups in both sexes, regardless of 1- or 8-wk study (Fig. 4, A and B). At 1 wk, STZ-induced diabetes had no effect on SNP-induced relaxation of PE precontracted rings in males (Fig. 4A). However, there was a slight, but significant, rightward shift of SNP CRC in mesenteric arteries from 1-wk diabetic female rats relative to other groups (Fig. 4A). At 8 wk (Fig. 4B), diabetes significantly reduced both sensitivity, as assessed by pD2 values, and R_max to SNP in mesenteric arteries of both sexes (Table 4, 8 wk).

Effects of Sex and Diabetes on the Vascular Responses to PE

To examine whether sex and diabetes affect the sensitivity to α-adrenoceptors, CRCs to PE (10⁻⁷ to 3 × 10⁻⁵ M) were generated in rat mesenteric arterial rings from male and female STZ-induced diabetic rats and their age-matched controls in the 1- and 8-wk studies. The sensitivity of α-adrenoceptors as well as maximal response was not significantly affected by either
sex or diabetes of 1 wk duration (Fig. 5A and Table 5, 1 wk). At 8 wk, there was a slight, but significant, leftward shift of PE CRC in mesenteric arteries from STZ-induced diabetic rats relative to age-matched control females. The tension developed in response to PE in the arteries from 8-wk control rats tended to be slightly higher in males than in females (22.18 ± 1.60 vs. 18.57 ± 1.33 mN, in males and females, respectively; Table 5, 8 wk). However, no significant differences were observed in either sensitivity or maximal response to PE between sexes. In males, STZ-induced diabetes did not affect vasoconstrictor responses to PE irrespective of the time of study. However, at 8 wk, there was a significant increase in the sensitivity to PE in mesenteric arteries of diabetic females compared with age-matched control females and diabetic males (Fig. 5B). The pD2 to PE was 6.28 ± 0.12 in diabetic females, 5.68 ± 0.06 in control females, 5.75 ± 0.10 in diabetic males, and 5.84 ± 0.14 in control males (Table 5, 8 wk).

Significant changes as a result of t-NAME pretreatment would reveal the extent of NO release from endothelium during smooth muscle contraction to PE (21, 22). To investigate this, CRCs to PE (10⁻⁷ to 3 × 10⁻⁵ M) were obtained in mesenteric arterial rings before and after pretreatment with L-NAME (200 μM, 20 min) in the presence of Indo (10 μM).

In the 1-wk study, t-NAME significantly potentiated the contractile responses (mainly at lower concentrations of PE) and shifted the CRC to PE to the left in the mesenteric arteries of all four groups (Fig. 6). Although the mesenteric arteries from 1-wk diabetic rats showed a slightly greater potentiation of the PE responses after inhibition of NO than those from 1-wk control animals, the extent of their potentiation were not significantly different from 1-wk control groups in either sex (Table 6, ΔAUC, 1 wk).

Similarly, t-NAME significantly potentiated contraction to PE (mainly at lower concentrations) in mesenteric arteries of all four groups in the 8-wk study (Fig. 7). However, when compared with 8-wk control females, 8-wk diabetic females showed a significantly greater potentiation, as assessed by the larger shift of PE CRC to the left and ΔAUC, of the PE responses after inhibition of NO (Fig. 7, A vs. B, and Table 6, ΔAUC, 8 wk). In females, the ΔAUC (in mN) was 9.03 ± 1.45 in 8-wk control and 16.80 ± 1.69 in 8-wk diabetic rats. On the other hand, the extent of PE potentiation was similar in 8-wk diabetic and control male groups (Fig. 7, C vs. D, and Table 6, ΔAUC, 8 wk). In males, the ΔAUC (in mN) was 11.32 ± 2.92 in 8-wk control and 10.53 ± 2.15 in 8-wk diabetic rats.

**Effects of Sex and Diabetes on mRNA Expression of eNOS and Nox**

To investigate a mechanism by which NO release in response to PE might have been increased in 8-wk diabetic female rats, eNOS mRNA expression in mesenteric arteries was measured using real-time RT-PCR. As shown in Fig. 8, A and B, eNOS mRNA level was significantly elevated in mesenteric arteries taken from female rats both at 1 and 8 wk after STZ treatment (by 83% and 124%, respectively) compared with those seen in the mesenteric arteries of age-matched female control rats. Although eNOS mRNA expression tended to be greater in 8-wk diabetic males than in age-matched nondiabetic control males, the difference was not statistically significant.

To further investigate the possible mechanisms underlying the greater impairment of the ACh responses in 8-wk diabetic female rats (Fig. 1B), the mRNA expression of NADPH oxidase (Nox) subunits, Nox2 and Nox4, major sources of superoxide in the vessel wall, were also measured. Real-time

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**Table 4. pD2 and Rmax to sodium nitroprusside at 1 and 8 wk**

<table>
<thead>
<tr>
<th>Groups</th>
<th>1 Wk</th>
<th>8 Wk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pD2</td>
<td>Rmax, %</td>
</tr>
<tr>
<td>Control female</td>
<td>7.27 ± 0.16</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>Diabetic female</td>
<td>6.88 ± 0.19</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Control male</td>
<td>7.31 ± 0.12</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>Diabetic male</td>
<td>7.24 ± 0.14</td>
<td>6 ± 2</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. A comparison of pD2 and Rmax to sodium nitroprusside in mesenteric arteries from male and female rats 1 and 8 wk after vehicle or STZ treatment is shown. *P < 0.05 vs. respective control, analyzed using Student’s unpaired t-test.
PCR analysis revealed that the mRNA expression of Nox2 and Nox4 showed no significant difference between the diabetic male group and the age-matched control group in either the 1- or 8-wk study (Fig. 9). However, the levels of Nox2 and Nox4 mRNA expression were significantly elevated in mesenteric arteries taken from female rats at both 1 wk (for Nox2, approximately by 3-fold; Fig. 9A) and 8 wk (for Nox2, approximately by 3-fold and Nox4 by 2.3-fold; Fig. 9, C and D) after STZ treatment. This is in comparison with age-matched non-diabetic control females.

Furthermore, there was a sex difference in the Nox4 mRNA expression in the 8-wk study. Level of mRNA expression for Nox4 was significantly lower in mesenteric arteries of 8-wk control females than in the 8-wk control males (Fig. 9B). By contrast, Nox2 expression did not differ between sexes in nondiabetic control rats.

Comparison of NOx Levels in Plasma

To examine whether sex and diabetes affect the basal level of NO in the plasma, the NOx (as a marker of basal NO levels) was measured. Analysis of the plasma NOx levels showed a sex difference in the 8-wk study. The NOx level in control females was significantly higher than that of control males (approximately by 3-fold; Fig. 10, A and B). In the 8-wk study, diabetic males also reached that of control males by 3.3-fold (Fig. 10B).

DISCUSSION

This study reveals a sex difference in the endothelial function in the mesenteric arteries of STZ-induced diabetic rats and suggests some potential underlying mechanisms.

Several reports suggest that endothelial dysfunction represents an early step in the development of vascular complications in diabetes. In agreement with previous reports demonstrating compromised EDV in different vascular beds in STZ-induced diabetes (18, 34, 41), we observed impairment of EDV in rat mesenteric arteries after the induction of diabetes. Specifically, we demonstrated a significant impairment in the responses to ACh at 1 wk, no changes were detected in sensitivity to ACh in both sexes 1 and 8 wk after the induction of diabetes. However, despite a decrease in maximum relaxation to ACh at 1 wk, no changes were detected in sensitivity to ACh in either sex. Our findings are in agreement with those of Davel et al. (16) who showed an impairment of the EDV in the tail vascular bed from 1-wk STZ-induced diabetic rats. On the other hand, our observations contrast with the findings of Pieper (68) and others (34, 41) who showed that EDV is not impaired until 7 wk after STZ treatment.

Alteration of EDV in diabetes might be dependent on the duration of the diabetic state and/or vascular bed. It has been shown that some vascular beds, such as basilar, tail, or mes-

Table 5. pD2 and Rmax to PE at 1 and 8 wk

<table>
<thead>
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<th>Groups</th>
<th>1 Wk</th>
<th>8 Wk</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>pD2</td>
<td>Rmax, mN</td>
</tr>
<tr>
<td>Control female</td>
<td>5.77 ± 0.11</td>
<td>17.22 ± 1.34</td>
</tr>
<tr>
<td>Diabetic female</td>
<td>5.99 ± 0.14</td>
<td>15.18 ± 1.01</td>
</tr>
<tr>
<td>Control male</td>
<td>5.63 ± 0.10</td>
<td>16.44 ± 1.25</td>
</tr>
<tr>
<td>Diabetic male</td>
<td>5.70 ± 0.14</td>
<td>18.86 ± 1.46</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. A comparison of pD2 and Rmax to phenylephrine in mesenteric arteries from male and female rats 1 and 8 wk after vehicle or STZ treatment is shown. **P < 0.01 vs. control female, analyzed using Student’s unpaired t-test.
enteric arteries, may present an endothelial dysfunction before other vessels such as the aorta (18). The onset of endothelial dysfunction may also vary widely due to differences in the type of vasoconstrictor or vasodilator used. In the current study, the relaxation of mesenteric arterial rings to bradykinin, another type of receptor-mediated endothelium-dependent vasodilator, was also affected by diabetes in females at 8 wk of duration (data not shown). These data suggest that the impaired response to ACh by diabetes may be representative of a general phenomenon of endothelial dysfunction.

In accordance with our recent reports on the sex difference in rat or rabbit aorta vasodilation after acute exposure to high glucose (30, 31), we showed a sex-based difference in the mesenteric arterial responses to ACh in diabetic rats. Eight weeks after the induction of STZ-diabetes, mesenteric arteries from female rats demonstrated a suppression of EDV that was greater than that observed in arteries from male rats. Specifically, mesenteric arteries from diabetic females showed a larger shift of ACh CRC to the right (Fig. 1B), along with a greater reduction of sensitivity to ACh. This reinforces our hypothesis that sex influences the effect of diabetes on endothelial function. Our data contrast with the findings of Tak-enouchi et al. (83) who reported that STZ-induced diabetes impaired ACh-induced relaxation in the male but not female rats.

### Table 6. pD2 and ΔAUC to phenylephrine before and after L-NAME at 1 and 8 wk

<table>
<thead>
<tr>
<th>Groups</th>
<th>1 Wk</th>
<th>8 Wk</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td></td>
<td>ΔAUC, mN</td>
<td></td>
</tr>
<tr>
<td>Control female</td>
<td>5.84 ± 0.11</td>
<td>6.20 ± 0.19**</td>
</tr>
<tr>
<td>Diabetic female</td>
<td>6.04 ± 0.17</td>
<td>6.76 ± 0.28**</td>
</tr>
<tr>
<td>Control male</td>
<td>5.72 ± 0.11</td>
<td>6.19 ± 0.21*</td>
</tr>
<tr>
<td>Diabetic male</td>
<td>5.58 ± 0.12</td>
<td>6.02 ± 0.15*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. A comparison of pD2 and differences of area under the concentration-response curve (ΔAUC) to phenylephrine before and after incubation of L-NAME (200 μM) in the presence of Indo (10 μM) in mesenteric arteries from male and female rats 1 and 8 wk after vehicle or STZ treatment is shown. *P < 0.05, **P < 0.01 vs. respective control, analyzed using Student’s paired t-test; #P < 0.05 vs. control female, analyzed using Student’s unpaired t-test.
mouse aorta. The reason for the discrepancy between the work of Takenouchi et al. and our results may be attributed to the fact that their observations were made using a different vascular bed. Endothelial function is regulated differently in large compared with small vessels.

It has previously been shown that both NO-dependent and NO-independent mechanisms are involved in vasorelaxation in rat mesenteric arteries (98). However, the specific roles of NO- and NO-independent pathways (COX and EDHF) in physiological and pathophysiological conditions such as diabetes in relation to sex are not clear.

In the present study, we showed that the effects of Indo and L-NAME on mesenteric artery vasorelaxation were dependent on the duration of diabetes and sex. The inhibition of COX metabolites by Indo reduced sensitivity to ACh-mediated mesenteric relaxation in control males and diabetic females at 1 wk. At 8 wk, preincubation with Indo slightly but significantly reduced the sensitivity to ACh in mesenteric arteries of all four experimental groups. However, because the slight rightward shift of second ACh CRC in the presence of Indo was comparable with the vehicle study (except for the 8-wk diabetic females), this suggests that metabolites in the COX pathway play only a minor role in EDV of mesenteric arteries. Consistent with this interpretation are data demonstrating that COX metabolites have a less important role in the relaxation of smaller arteries, such as mesenteric arteries (78).

When Indo is administrated and then followed by L-NAME, the reduction in EDV is generally considered to represent the role of NO, and the remaining EDV to ACh-mediated vasodilation was much more prominent in diabetic female arteries when compared with age-matched control females at both 1 (Fig. 2, B vs. A) and 8 (Fig. 3, B vs. A) wk, indicating a reduced contribution of EDHF-type relaxation or an enhanced role of NO.

In males, addition of L-NAME caused a greater reduction of EDV in control than in diabetic male arteries at 1 wk (Fig. 2, C vs. D), suggesting that under physiological conditions, NO is the predominant mediator of mesenteric vasorelaxation. Both NO and EDHF remained important for EDV in control males.

Fig. 7. Concentration-response curves for PE in mesenteric arteries taken from control female (A), diabetic female (B), control male (C), and diabetic male (D) rats before and after pretreatment with L-NAME (200 μM) at 8 wk. Responses were performed in the presence of Indo (10 μM). Data are expressed as means ± SE, with *P < 0.05 vs. before L-NAME in all groups analyzed using 2-way ANOVA with repeated measures.
at 8 wk (Fig. 3C). Furthermore, our data indicated that the role of NO is substantially enhanced in diabetic male arteries compared with age-matched control males at 8 wk (Fig. 3, D vs. C).

Our data show that EDHF also serves as the major backup vasodilator in females under physiological conditions and is consistent with the increased contribution of EDHF to vascular reactivity that has been reported in female arteries (63, 94). Along similar lines, we previously reported a greater contribution of EDHF to parasympathetic vasodilatation in females than in males (3). However, it should be noted that NO may be of greater importance in tail and mesenteric arteries in males than females (53, 63). Nawate et al. (60) has reported that estrogen deficiency can lead to a reduced EDHF-type relaxation while increasing NO release. Our observations demonstrated that NO is the main contributor to mesenteric relaxation in healthy males at 1 wk, with both NO and EDHF playing prominent roles 8 wk later.

Alterations in EDHF-mediated responses have been reported in aging and under pathological conditions such as hypertension, atherosclerosis, and diabetes (27). Specifically, in diabetic animals evidence suggests both increased and decreased EDHF-mediated response (29). There are reports of increased EDHF activity in the mesenteric arteries of diabetic animals (64, 75), and it has been proposed that EDHF may serve as a backup vasodilator in situations associated with an altered bioavailability of NO (8, 52). Nevertheless, a decrease in EDHF-mediated response has also been reported in the mesenteric and renal arteries (17, 28, 49, 95). In agreement with the studies demonstrating impaired EDHF vasodilation in resistance vessels of diabetic subjects, our data from female rats show that after the induction of STZ diabetes the role of EDHF was reduced at both 1 and 8 wk, shifting to a greater dependency on NO for vasorelaxation.

EDHF has been reported to have vasoprotective effects, especially in females. Its involvement in cardiovascular inflammation, platelet function, and vascular repair may represent an endogenous protective mechanism against atherosclerosis that is more active in females than males (90). In the current study, the EDHF-type relaxation was completely lost only in the arteries of diabetic females at 8 wk. The reasons for the shift in the role of EDHF in vasorelaxation of mesenteric arteries in the current study are not clear. However, because the role of EDHF is not limited only to the regulation of vasomotor tone but also has an anti-inflammatory function, one might speculate that the loss of sex-based protection for females when they develop diabetes may be partially attributable to a reduced response to EDHF. Also consistent with our working hypothesis are the data demonstrating that the reduced contribution of EDHF (or the elevated contribution of NO) to EDV in 8-wk diabetic males and females (Fig. 3, B and D) was associated with increased impairment of EDV (Fig. 1B). It is important to note that the suppression of EDV in arteries of 8-wk diabetic females was greater than in 8-wk diabetic males (Fig. 1B), possibly due to complete loss of EDHF-type relaxation.

Sex differences in vascular function are well documented (57, 86), and there are also reports on the effects of sex together with diabetes on the mesenteric and aortic endothelial function in mice and rats (50, 51, 65, 83). Nevertheless, to our knowledge we are the first to report on the relative importance of NO and EDHF in regulating mesenteric vascular tone under diabetic conditions with respect to sex.

In addition to a possible role for altered relative contributions of endothelial NO and EDHF, other mechanisms that could explain vascular dysfunction of diabetes may include factors such as a decreased sensitivity of smooth muscle to NO or an enhanced vasoconstrictor response to agents such as PE and ET-1. A majority of previous studies have shown an impairment of EDV in the presence of preserved endothelium-independent relaxation in diabetes (44, 83). However, we observed that endothelium-independent relaxation to SNP, an indicator of smooth muscle sensitivity to NO, was also clearly impaired 8 wk after the STZ injection in both sexes. This finding is in agreement with that of other investigators who also reported an impaired SNP-induced relaxation in diabetes (10, 15). These data suggest that the responsiveness of vascular smooth muscle to NO is affected by STZ injection after 8 wk.
in both sexes. Therefore, the impaired responses to ACh at 8 wk in both sexes may in part occur at NO interaction with smooth muscle. We also examined the vasoconstrictor response to PE, an α-adrenoceptor agonist, in greater detail. We showed no changes in the sensitivity or maximum contractile responses to PE in control male and female rat mesenteric arteries at either 1 wk or 8 wk. Along similar lines, McKee et al. (54) reported no sex differences in sensitivity to PE, 5-HT, or KCl depolarization in intact mesenteric arteries from rats. Maximal contractile responses were also not significantly different between stimuli and sexes. Other investigators reported an increase, decrease, and no change in vasoconstriction of arteries of male rats compared with females (40, 46, 51).

There is also conflicting data regarding diabetes and its action on the contractile responses in vasculature (51, 65, 92). The effects of diabetes on vascular smooth muscle cell function were not the major focus of our current study. Nevertheless, we showed that in males, STZ-induced diabetes did not affect vasoconstrictor responses to PE irrespective of the time of study. Interestingly, an enhanced sensitivity to PE was observed in mesenteric arteries taken from females 8 wk after STZ treatment compared with age-matched control females and diabetic males, further suggesting a sex disparity in the development of diabetic vascular disease. Matsumoto et al. (51) provided the first evidence for a sex-specific augmentation of ET-1-mediated contraction in a diabetic condition (in the mesenteric arteries of female mice). In the current study, we also found that diabetes enhanced the vasoconstrictor reactivity in response to ET-1 in the mesenteric arteries of female rats, but not in males, 8 wk after STZ treatment (data not shown). This provides strong evidence that the observed sex differences in diabetic mesenteric reactivity in the current study may not be limited to responses to PE.

Theoretically, the enhanced sensitivity to PE observed in 8-wk diabetic females (Fig. 5B) may partially result from a decreased release of relaxing factors (NO or EDHF), a decreased sensitivity of smooth muscle to NO, or an enhanced release of contracting factors.

In the current study, we measured endothelium-derived NO in response to PE by monitoring the effects of L-NAME on the concentration-response curve to PE (69, 70). Pretreatment with L-NAME caused a significantly greater potentiation of the PE
response in mesenteric arteries from 8-wk diabetic females compared with that observed in the other experimental groups (Fig. 7B). This excludes diminished NO as the cause of the increased PE contractile responsiveness.

Along similar lines, Bardell and MacLeod (6) observed an elevated contractile response to norepinephrine in mesenteric arteries of male STZ-diabetic rats (females were not examined) along with a greater potentiation of the norepinephrine response (by nonselective NO inhibition) compared with control arteries. In our study, we observed no significant changes in the PE-induced contraction in mesenteric arteries from diabetic males compared with age-matched controls. Nevertheless, both studies disclose an elevation in α-adrenoceptor agonist contraction in diabetic vasculature along with an increase in the response after pretreatment with l-NAME.

Bardell and MacLeod (6) reported that the augmented NO responses in diabetic arteries may have involved inducible NOS in vascular smooth muscle. Any increase in NO production has the potential for free radical-mediated damage, particularly under conditions of oxidative stress where peroxynitrite is formed more easily (79). Peroxynitrite is a powerful oxidant, which in turn leads to eNOS uncoupling and contributes to vascular disease.

It is important to note that l-NAME, which was used for blocking NOS in our study, is a nonselective inhibitor of NOS and also blocks the uncoupled action of NOS leading to the production of superoxide. Thus, the augmented l-NAME responses in our 8-wk diabetic females (Fig. 7B) may have also involved uncoupled eNOS, a major source of vascular superoxide in diabetes (11).

In diabetes, superoxide production may play an important role in activating endothelium-derived contracting factors-mediated responses (76, 77, 88). Clearly, the scavenging action of superoxide on NO, thus decreasing its bioavailability (72), will also favor endothelium-dependent contractions.

Nevertheless, the fact that the SNP-induced relaxation was affected by 8-wk STZ-induced diabetes suggests that the decreased sensitivity of smooth muscle to NO may also contribute to the increased PE contractile responsiveness (Fig. 5B). Furthermore, EDHF-type relaxation was completely lost in mesenteric arteries of 8-wk diabetic female rats (Fig. 3B), suggesting that the diminished EDHF may be involved as well.

Taken together, our data suggest that elevated sensitivity to adrenergic vasoconstriction observed in the mesenteric vasculature of 8-wk diabetic females is likely due to the loss of EDHF, decreased sensitivity of smooth muscle to NO, and/or decreased NO bioavailability rather than reduced basal NO production.

On the other hand, the elevated NO-dependent responses to PE (Fig. 7B) in mesenteric arteries from 8-wk diabetic females would appear to contradict the significant suppression of ACh-induced EDV in this group (Fig. 1B). However, further examination of ACh responses revealed that NO-type relaxation was not reduced. In fact, it was elevated in the 8-wk diabetic female rats (Fig. 3B). It is also important to note that an impaired EDV to ACh may not always be mediated by a reduction in NO, since the ACh relaxation in the smaller resistance arteries is dependent on both EDHF and NO (53).

Although the functional consequence of elevated NO-dependent responses in mesenteric arteries of diabetic female rats is unclear, these data are consistent with studies by Wigg et al. (95) and Makino et al. (49) showing that NO-dependent responses were preserved in mesenteric and femoral arteries in diabetic rats. Several studies have also shown that diabetes is associated with an increase, rather than a decrease, in eNOS expression, as well as increased NO (14, 35). In agreement with these previous findings, we demonstrated higher eNOS mRNA expression in female mesenteric arteries both at 1 and 8 wk after STZ treatment compared with their age-matched controls. Finally, increased basal levels of plasma NOx concentrations have been reported (48), and we also observed that diabetic female rats had higher levels of plasma NOx concentrations than controls at both 1 and 8 wk.
In our study, the relative increase in NO-mediated responses in mesenteric arteries of diabetic female rats may actually exert a deleterious effect, possibly through an interaction with enhanced superoxide associated with diabetes (38). Consistent with this hypothesis are data demonstrating that mRNA levels of Nox2 and Nox4 catalytic subunits of NADPH oxidase were significantly elevated in mesenteric arteries taken from female rats both at 1 (for Nox2) and 8 (for Nox2 and Nox4) wk after STZ treatment.

We did not directly measure superoxide production or measure uncoupled eNOS. However, our results on elevated NO-mediated responses and the overexpression of Nox2 and Nox4 mRNA in 8-wk diabetic female rats suggest that the enhanced vascular reactivity observed in this group may be due, in part, to the interaction of oxidative stress with elevated NO in their mesenteric arteries. Additional studies will be needed to document the direction and magnitude of these interactions along with the relative importance of NO to sex-specific endothelial health and function in diabetes.

Finally, we report for the first time that nondiabetic control female rats had a lower mesenteric Nox4 mRNA expression than nondiabetic males in the 8-wk study. In contrast, mRNA expression of the Nox2 catalytic subunit was similar between sexes. These results are in accordance with observations of Miller et al. (56) who reported that sex influenced the expression and activity of Nox4 in cerebral arteries. Similarly, they found that Nox4, but not Nox2, protein was expressed at lower levels in the basilar artery of female rats. It is important to note that despite the high level of Nox4 mRNA expression (Fig. 9D) and a lower level of plasma NO (Fig. 10B) in 8-wk control males (compared with 8-wk control females), ACh induced full relaxation (Fig. 1B). This could possibly be due to the elevated contribution of NO-independent (or EDHF)-relaxation type in healthy males at 8 wk (Fig. 3C).

In summary, we have shown that mesenteric endothelial function in rats was impaired both at 1 and 8 wk after the induction of STZ-diabetes. However, the impact of STZ-induced diabetes on the vascular responses of mesenteric arteries from male and female rats varied with duration of the disease. Furthermore, the extent of impairment was significantly greater in diabetic females than diabetic males at 8 wk. The basis for the sex differences in the development of diabetic vascular diseases may be partly attributed to changes in the relative importance of NO and EDHF to the regulation of vascular reactivity. This study suggests that the predisposition of female rat mesenteric arteries to vascular injury after the induction of diabetes may be due to a shift away from a putative EDHF, initially the major vasodilatory factor, toward a greater reliance on NO.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

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

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