Streptozotocin-induced diabetes differentially affects sympathetic innervation and control of plantar metatarsal and mesenteric arteries in the rat

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Abstract

In humans neural control of arterial vessels supplying skin in the extremities is particularly vulnerable to the effects of diabetes. Here the streptozotocin (STZ) rat model of type 1 diabetes was used to compare effects on neurovascular function in plantar metatarsal arteries (PMAs), which supply blood to skin of hind paw digits, with those in mesenteric arteries (MAs). Twelve weeks after STZ (60 mg/kg, i.p.), wire myography was used to assess vascular function. In PMAs, lumen dimensions were unchanged but both nerve-evoked contractions and sensitivity to $\alpha_1$- (phenylephrine, methoxamine) and $\alpha_2$- (clonidine) adrenoceptor agonists were reduced. The density of perivascular nerve fibers was also reduced by ~25%. These changes were not observed in PMAs from STZ-treated rats receiving either a low dose of insulin that did not greatly reduce blood glucose levels or a high dose of insulin that markedly reduced blood glucose levels. In MAs from STZ-treated rats, nerve-evoked increases in force did not differ from control but, because lumen dimensions were ~20% larger, nerve-evoked increases in effective transmural pressure were smaller. Increases in effective transmural pressure produced by phenylephrine or $\alpha,\beta$-methylene ATP in MAs from STZ-treated rats were not smaller than control but the density of perivascular nerve fibers was reduced by ~10%. In MAs, the increase in vascular dimensions is primarily responsible for reducing effectiveness of nerve-evoked constrictions. By contrast, in PMAs decreases in both the density of perivascular nerve fibers and the reactivity of the vascular muscle appear to explain impairment of neurovascular transmission.

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Keywords: Diabetes, Sympathetic innervation, Neurovascular transmission.
Introduction

In humans abnormal control of the vasculature has been implicated in the etiology of many diabetes-related complications such as neuropathy and diabetic foot ulceration (41). These changes involve endothelium-dysfunction (30) as well as deficits in both sympathetic nerve-mediated vasoconstriction (see below) and sensory nerve-mediated vasodilation (1). However, the mechanisms whereby diabetes affects sympathetic and sensory nerve regulation of the vasculature are not well understood, although it is believed that diabetes causes degeneration of these nerve supplies (34).

Many clinical studies have evaluated the effects of diabetes on sympathetic nerve-mediated vasoconstriction of arterial vessels supplying plantar skin of the foot. These studies have demonstrated that both type 1 (insulin dependent) and type 2 (non-insulin dependent) diabetic patients with signs of sensory and/or autonomic neuropathy (e.g. orthostatic hypertension) have increased skin blood flow under basal conditions (2, 33) and attenuated reductions in skin blood flow (i.e. vasoconstriction) in response to sympathetic arousal (e.g. produced by mental arithmetic(2) or postural stimuli (29)). Importantly, deficits in sympathetic vasoconstriction of arterial vessels supplying plantar skin can be demonstrated in both type 1 and type 2 diabetics at an early phase of diabetic neuropathy (31, 35), perhaps suggesting that sympathetic neurons supplying these vessels are particularly vulnerable to the effects of diabetes. Furthermore, it has been suggested that reduced sympathetic nerve-mediated vasoconstriction of these vessels is an early change that contributes to the etiology of microangiopathy in skin of the feet (7). In contrast, while type 1 diabetic patients with orthostatic hypertension had increased basal blood flow in the superior mesenteric artery, posturally-evoked reductions in blood flow in this vessel were not impaired (26), indicating that sympathetic control of arterial vessels in the splanchnic vascular bed was not detectably changed. These findings may indicate that diabetes differentially affects sympathetic neurons supplying arterial vessels in different vascular beds.
There are only a small number of studies that have investigated the effects of diabetes on sympathetic neural control of arterial vessels in animals. Most of these studies have used the streptozotocin (STZ)-treated rat model of type 1 diabetes and have investigated effects in the rat tail artery, a cutaneous thermoregulatory vessel with a function similar to that of digital arteries in man, and mesenteric arteries. In tail artery, Hart et al. (11) reported that eight weeks of diabetes produced a small reduction in sympathetic nerve-mediated vasoconstriction, whereas, Weber and MacLeod (42) and Speirs et al. (32) reported that twelve weeks of diabetes did not reduce sympathetic nerve-mediated vasoconstriction. In the perfused mesenteric arterial bed, diabetes reduced sympathetic nerve-mediated pressor responses after twelve weeks of diabetes (27) but this effect was not present after eight weeks (28). In mesenteric arteries, it is not known if the decrease in the sympathetic nerve-mediated pressor response produced by diabetes is associated with degeneration of the sympathetic nerve supply but in tail artery the perivascular sympathetic innervation is not affected by twelve weeks of diabetes (32).

In the present study we used the STZ-treated rat model of type 1 diabetes to test the hypothesis that sympathetic control of planter metatarsal arteries, which supply blood to skin of the hind paw digits, is particularly vulnerable to the effects of diabetes. In this vessel, we investigated whether diabetes modified sympathetic neurovascular transmission and whether the contributions of the co-transmitters, norepinephrine and ATP, to transmission were changed. We also assessed if any of the effects of diabetes could be attributed to changes in reactivity of plantar metatarsal arteries to \( \alpha \)-adrenoceptor and P2X1-purinoceptor agonists or the effectiveness of the neuronal norepinephrine transporter (NET). In addition, the effects of diabetes on peptidergic sensory nerve-mediated vasodilation and the perivascular innervation of planter metatarsal arteries were determined. In these arteries, the effects of insulin treatment were investigated to confirm that any observed effects were due to diabetes and not to the direct toxic effects of STZ treatment. For comparison, the effects of diabetes on sympathetic and peptidergic sensory nerve-mediated control
and innervation of small mesenteric arteries supplying the ileal segment of the gastrointestinal tract were investigated.

Materials and Methods

All experiments were approved by the Animal Ethics Committees at the University of New South Wales (January 2010 – December 2010) and the University of Melbourne (January 2010 – March 2012) and conformed to the Australian code of practice for the care and use of animals for scientific purposes. Male Wistar rats, 8 - 10 weeks old, were purchased from Animal Resources Center (Canning Vale, WA, Australia). The rats were treated intraperitoneally with 60 mg/kg streptozotocin dissolved in 50 mM citrate buffer (pH 4.5). Age-matched control animals were treated with an equivalent volume of citrate buffer. Blood glucose levels were measured 5 - 7 days post-injection using a glucose meter (Accu-Check Performa, Roche Diagnostics Australia Pty Ltd) to confirm that the rats were diabetic (>15 mmol/L glucose). Blood glucose levels and body weights were monitored weekly thereafter until they were terminated 12 – 13 weeks after the induction of diabetes. Diabetic animals were left untreated or were treated with a low (~1 unit/day) or high dose (~4 units/day) of insulin delivered by sustained-release implants (Linplant, Linshin Canada Inc.) inserted subcutaneously beneath the nuchal skin. At 1 and 7 weeks after the induction of diabetes, the low dose group received half a Linplant pellet and the high dose received 2 Linplant pellets. Animals were maintained on a 12:12 hour light-dark cycle and were provided with food and water ad libitum.

At termination the animals were deeply anaesthetized with isoflurane and exsanguinated by cutting the carotid arteries. To assay long-term glycemic control, approximately 1 ml of blood was collected in EDTA tubes from the STZ-treated rats receiving insulin and their controls to determine the glycated hemoglobin levels by HPLC (CLC330 GHb Analyzer, Primus, Kansas City, MO). The five plantar metatarsal arteries (PMAs) branch from the plantar arch (at the distal end of the median
plantar artery) and the first four PMAs divide in the interdigital spaces to form the plantar digital arteries supplying the adjacent sides of the digits (the 5th PMA supplies the plantar digital artery on the lateral side of the fifth digit). The first and second PMAs or second order mesenteric arteries (MAs) were dissected and placed in physiological saline containing (in mM): NaCl, 133; KCl, 4.7; CaCl₂, 2.0; MgCl₂, 1.2; NaH₂PO₄, 1.3; NaHCO₃, 16.3; glucose 7.8. This solution was bubbled with carbogen (5% CO₂/95% O₂). On each experimental day tissue was collected from both a control and an STZ-treated rat and the experiments described below were performed in parallel.

**Wire Myography**

Two four-channel myographs (Multi Myograph model 610M, Danish Myo Technology, Aarhus, Denmark) were used to record mechanical responses of the arteries. In each device, 2 artery segments (~1.5 mm in length) from a control and an STZ-treated rat were mounted isometrically between two stainless steel wires (40 µm diameter). Arterial segments were bathed in 6 ml of physiological saline that was continuously bubbled with carbogen and maintained at 36 - 37 °C. The basal conditions were normalized by gradually stretching the vessel in small steps until the effective transmural pressure (ETP) calculated using Laplace’s equation (transmural pressure = wall tension/(internal circumference/2π), where wall tension = force/2 x vessel segment length) was 13.3 mN/mm² (100 mmHg)(see 23). For MAs, the circumference was then adjusted to 90% of that determined at 13.3 mN/mm² (see (23). For PMAs, the ETP was set initially at 13.3 mN/mm². The vessels were then left to equilibrate for at least 30 minutes. After this period, the basal ETP in MAs stabilized at ~6.5 mN/mm² (~50 mmHg) and in PMAs at ~10.0 mN/mm² (~75 mmHg). Under these conditions, MAs (see (23) and PMAs (Figure 1) are at the peak of their length-tension relationship. Because diabetes has been reported to change the diameter of MAs (43), the responses to nerve stimulation and to agonists were also converted to ETP to account for any changes in vascular dimensions.
After equilibration, all tissues were exposed to three (PMAs) or four (MAs) applications of phenylephrine (PE; 3 μM, Sigma-Aldrich, Castle Hill, Australia). The first 2 applications of PE were for ~4 minutes and confirmed the viability of the vessels. Following the third addition of PE, when the contraction had plateaued, relaxation to carbachol (0.1 and 1 μM added cumulatively; Sigma-Aldrich) was determined to assess the function of the endothelium. In MAs, when the contraction to the fourth application of PE had plateaued, the relaxation to capsaicin (0.01 and 0.1 μM added cumulatively; Sigma-Aldrich) was determined to assess the vasodilator effect of activating the perivascular peptidergic primary afferent axons. In MAs, after washout of the forth application of PE, capsaicin (1 μM) was applied for 10 minutes to prevent the inhibitory effects of activating the peptidergic primary afferent axons (14). After wash out of capsaicin, the MAs were left for a further 30 minutes before starting the experiments described below.

Contractions to neural stimuli

Tissues mounted in one four-channel myograph were used to assess contractions to electrical stimulation of the perivascular axons. Electrical stimuli (15 V, 0.2 ms pulse width) were generated by a 4-channel stimulator (EXP-ST-CH4; Experimetria Ltd, Balatonfüred, Hungary) and delivered via platinum plate electrodes mounted either side of the tissue. In preliminary experiments, it was established that contractions to these stimuli were blocked by tetrodotoxin (0.5 μM), confirming that they are due to action potential-evoked release of neurotransmitter from the perivascular axons.

To construct frequency response curves, the PMAs were stimulated with 25 pulses at 0.1, 0.3, 0.5 and 1.0 Hz whereas the MAs were stimulated with 100 pulses at 1, 2, 3, 5 and 10 Hz. These different stimulus parameters have been selected because PMAs are more responsive to electrical stimulation of their perivascular nerves than MAs, which are only weakly activated by trains of stimuli at 1 Hz (cf. Figures 3A and 8A). In PMAs from the STZ rats that received no insulin support and their controls, the effects of a 10 minute application of capsaicin (1 μM) on
contractions to 10 pulses at 1 Hz were then assessed. Following washout of capsaicin tissues were left for 30 minutes before any further assessments.

To assess the effects of neurotransmitter antagonists, the responses of PMAs to 100 stimuli at 1 Hz, and of MAs to 20 stimuli at 10 Hz, were compared before and during the application of the antagonists; the antagonists were in contact with the tissue for at least 20 minutes before changes to the contractions were determined. For PMAs, the effects of the $\alpha_1$-adrenoceptor antagonist prazosin (10 nM; Sigma-Aldrich) were assessed on one vessel segment and the effects of the $\alpha_2$-adrenoceptor antagonist idazoxan (0.1 $\mu$M; Sigma-Aldrich) were assessed on the other. The effects of these antagonists were tested because in other arteries supplying blood to skin both $\alpha_1$- and $\alpha_2$-adrenoceptors contribute to neurovascular transmission (19) and the concentrations used are 10-50 times higher than those corresponding to the pA$_2$ values for prazosin at $\alpha_1$-adrenoceptors (see 18) and idazoxan at $\alpha_2$-adrenoceptors (see 6). Subsequently, both $\alpha$-adrenoceptor antagonists were combined to assess the effects of blocking of all $\alpha$-adrenoceptors. In addition, in a separate group of PMAs from the STZ rats that received no insulin support, the effects of the P2X1-purinoceptor antagonist NF449 (10 $\mu$M, Tocris Bioscience, Bristol, UK) were assessed. For MAs, where neurotransmission is mediated via $\alpha_1$-adrenoceptors and P2X-purinoceptors (17), the effects of prazosin (10 nM) were assessed in one vessel segment and those of the P2-purinoceptor antagonist suramin (0.1 mM; Sigma-Aldrich) were assessed in the other. Subsequently, both antagonists were combined to assess the effects of blocking of both $\alpha_1$-adrenoceptors and P2-purinoceptors.

Contraction to chemical stimuli

Tissues mounted in the other four-channel myograph were used to assess reactivity to exogenously applied agents. For both PMAs and MAs, cumulative concentration-response curves were constructed for the $\alpha_1$-adrenoceptor agonist, PE (0.01 - 100 $\mu$M). To determine the effects of
blocking NETs, concentration-response curves for PE were obtained in the absence and in the presence of desmethylimipramine (DMI, 30 nM; Sigma-Aldrich). In PMAs, cumulative concentration-response curves were also acquired for the \( \alpha_1 \)-adrenoceptor agonist methoxamine (0.01 – 100 \( \mu \)M; Sigma-Aldrich), which is not a substrate for NET (38), and the relatively selective \( \alpha_2 \)-adrenoceptor agonist, clonidine (0.001 - 3.0 \( \mu \)M; Sigma-Aldrich). In PMAs from the STZ rats that received no insulin support and MAs, reactivity to \( \alpha,\beta \)-methylene ATP (0.5-1 \( \mu \)M; Sigma-Aldrich) was assessed. At the end of these experiments, contractions to depolarization of the smooth muscle with physiological saline containing 60 mM K\(^+\) (equimolar substitution of KCl for NaCl) were assessed. Prior to raising the K\(^+\) concentration, prazosin (10 nM) and idaxozan (0.1 \( \mu \)M) were applied to the tissues to prevent the contractile effects of norepinephrine released from the nerve terminals by K\(^+\)-induced depolarization.

**Immunohistochemistry**

PMAs and MAs were fixed at their *in vivo* length in Zamboni’s Fixative overnight at 4°C. The next day tissues were washed in dimethyl sulfoxide (3 x 10 minutes) to allow for better antibody penetration and then in phosphate buffered saline (PBS, pH 7.1-7.2; 3 x 10 minutes). Tissues were stored in PBS containing 0.1% (w/v) sodium azide at 4°C. Arteries were blocked for 1 hour at room temperature in 10% normal horse serum in PBS containing 1% (v/v) Triton™ X-100 (Sigma-Aldrich), and then incubated overnight at 4°C in antibody diluent containing mouse anti-tyrosine hydroxylase (TH) antibody (1:1000, Cat. No. 22941, ImmunoStar Inc, Hudson, WI, USA) and goat anti-calcitonin gene related peptide (CGRP) antibody (1:1000, Cat. No. 1720-9007, Biogenesis Ltd, England, UK). In addition, separate tissues were treated with the pan-neuronal labels rabbit anti-protein gene product 9.5 (PGP9.5; 1:1000, Cat No. RA95101, Ultraclone Limited, England, UK) or mouse anti-\( \beta \)-tubulin III (1:750, Cat. No. MMS-435P, Covance, Princeton, NJ, USA). Tissues were washed with PBS (3 x 10 minutes) and incubated at room temperature for one hour in antibody
diluent containing fluorescent secondary antibodies raised in donkey (Molecular Probes, Inc., OR, USA); Alexa Fluor 564 anti-mouse (1:500), Alexa Fluor 647 anti-sheep (1:500) and Alexa Fluor 488 anti-rabbit (1:1000). Tissues were again washed with PBS (3 x 10 minutes) and coverslipped in fluorescence mounting medium (Dako North America, Inc., CA, USA). Z-stacks of the fluorescent images through the entire adventitial thickness were collected using a Zeiss Pascal confocal microscope system. For these images the laser power was set between 10-15% and the excitation/emission wavelengths were 488 nm/band pass 505-530 nm for Alexa Fluor 488, 561 nm/band pass 575-615 nm for Alexa Fluor 594 and 633 nm/long pass 650 nm for Alexa Fluor 647. Collection of Z-stacks through the full thickness of the adventitia (15-25 μm) took 8-12 minutes and each region of the artery was only imaged once to minimize the effects of bleaching. The specificity of secondary antibodies was tested with omission of the primary antibodies, which always resulted in no immunostaining.

Data analysis

The output from the myographs was recorded and analyzed using a PowerLab data acquisition system and the program Chart (ADInstruments, Bella Vista, NSW, Australia). The amplitudes of contractions to trains of electrical stimuli and to contractile agents were measured and converted to ETP. The EC50 values for the α-adrenoceptor agonists were determined by fitting the concentration response data to the Hill equation using the curve fitting functions in Igor Pro (Wavemetrics Inc., Lake Oswego, OR, USA) and are presented as their negative logarithm (pEC50). All statistics were performed using SPSS 19 (IBM Corp, Armonk, NY, USA). The stimulus frequency response curves were compared by repeated measures ANOVA with a single independent variable for between group comparisons. Other comparisons were made with unpaired t-tests or Mann Whitney U-tests if the variances were not homogeneous (indicate by Levene’s tests). When multiple pairwise comparisons were made in individual tissues, the P values were adjusted using the false
discovery rate procedure (5). Data are presented as mean and SEM or median and interquartile range (IQR) if comparisons were made with Mann Whitney U-tests. $P$ values $<0.05$ were considered to indicate significant differences. Unless otherwise indicated in the text, $P$ values were obtained using unpaired $t$-tests. In all cases, $n$ indicates the number of animals studied.

The density of the immunolabeled axon plexus was quantified using maximum intensity Z-projection images collected with x20 (MAs; pixel size 1.01 $\mu$m$^2$) or x40/x63 objectives (PMAs; pixel size 0.38 $\mu$m$^2$/0.24 $\mu$m$^2$ respectively). This was done by collecting two line profiles at the same points on all images (one placed in the upper half and the other in the lower half of the image) using the program ImageJ (National Institute of Health, Bethesda, MD, USA). After subtracting the mean maximum background value (determined from 10 points on the image where there was no detectable fluorescence), the number (intercept number) and widths (intercept widths) of the regions along the line profiles with pixel values $>0$ (see Figures 5A and B) were determined using an in-house Igor Pro routine. The percentage area of the vessel surface covered by the immunolabeled nerve plexus in each image was also measured using ImageJ. For images of TH-immunoreactivity (TH-IR), the mean value of the peaks along the line profiles was measured to ascertain if there was a difference in the intensity of fluorescence of the intercepts (nerve bundles). The integrated TH-IR fluorescence for the entire vessel surface in each image was also measured after subtraction of the mean background level (determined at 10 points on the image where there was no detectable fluorescence) and this was normalized to 100 $\mu$m$^2$ of vessel surface. For CGRP immunoreactive (CGRP-IR) axons in both PMAs and MAs, the numbers of fluorescent intercepts were determined along 3 lines placed at the same points (one placed in the upper half, one in the middle and the other in the lower half of the image) on images collected with a x20 objective. Measures of intercept number along the line profiles were calculated per 100 $\mu$m. For each measure, one-sample $t$-tests were used to determine if the measures in arteries from STZ-treated animals expressed as a percentage of those in the control tissues collected on the same day (and processed in parallel).
differed significantly from 100%. For each animal and vessel type, the measures were the average obtained from at least three image Z-stacks.

Results

Animals

When assessed 5 - 7 days post injection the STZ-treated rats (STZ rats) had blood glucose levels in excess of 20 mmol/L, and the rats receiving no insulin (STZ-NI) and those receiving a low dose of insulin (STZ-LI) maintained levels >20 mmol/L. In contrast, the STZ rats receiving a high dose of insulin (STZ-HI) maintained blood glucose levels <15 mmol/L. At termination, STZ-NI rats had little net body weight gain whereas STZ-LI and STZ-HI rats had greater body weight gain but this was still smaller than that of their controls (Table 1). However, body weight gain in STZ-HI rats was greater than in STZ-LI rats (Table 1). At termination the blood glucose levels in STZ-NI and STZ-LI rats were greater than those of their controls, but those of STZ-HI rats were more variable and did not differ significantly from their controls (Table 1). However, the % glycated hemoglobin levels were significantly higher in both STZ-LI and STZ-HI rats when compared with their controls, but those of STZ-HI rats were less markedly increased (Table 1).

Plantar metatarsal arteries (PMAs)

The dimensions and endothelium-dependent relaxations of PMAs were not change by diabetes, but K⁺-evoked contractions were reduced in vessels from STZ-NI rats

The lumen diameter (estimated from the measured lumen circumference) and the basal ETP after equilibration for PMAs from STZ-NI, STZ-LI and STZ-HI rats did not differ from those of their controls (Table 2). Similarly, the % relaxation of vessels constricted with PE (3 μM) when their endothelium was stimulated with carbachol (0.1 and 1 μM) did not differ between PMAs from each of the groups of STZ rats and their controls (Figure 2A-C). The peak increases in ETP produced by
depolarization of the vascular muscle with 60 mM K⁺ were smaller in the PMAs from STZ-NI rats than in their controls (Figure 2D). In contrast, the peak increases in ETP produced by 60 mM K⁺ in PMAs from STZ-LI or STZ-HI rats did not differ from those of their controls (Figure 2E, F).

Nerve-evoked increases in ETP were reduced in PMAs from STZ-NI rats

Figure 3 shows stimulus frequency contraction data for PMAs from STZ-NI rats (Figure 3A, B), STZ-LI rats (Figure 3C) and STZ-HI rats (Figure 3D) together with those for their controls. At all frequencies of stimulation, the peak increases in ETP for PMAs from STZ-NI rats were smaller than control (Figure 3A, B). In contrast, stimulation frequency-contraction data for PMAs from STZ-LI and STZ-HI rats did not differ significantly from control (ANOVA between groups $P = 0.23$ and 0.36 respectively; Figure 3C, D).

The effects of capsaicin (1 μM) on responses to 10 pulses at 1 Hz were only determined in PMAs from STZ-NI rats ($n = 6$) and their controls ($n = 6$). In both these groups of vessels, the peak amplitudes of the stimulus-evoked contractions measured 3 minutes following the addition of capsaicin were reduced by about 60% (Control 56 ± 11%; STZ-NI 64 ± 16 %). During the 15 minute application of capsaicin the contraction amplitudes returned close to control levels, consistent with this agent activating and desensitizing the peptidergic primary afferent axons. The contractions measured 20 minutes following washout of capsaicin did not differ significantly from those measured prior to its addition (% of pretreatment contractions: control 98 ± 12%; STZ-NI 100 ± 9 %; paired $t$-test $P > 0.8$ for both comparisons), indicating that capsaicin desensitization of the peptidergic primary afferent axons did not change the nerve-evoked contractions.

In all groups of PMAs ($n = 6$ for all STZ and control groups), the contractions to 100 pulses at 1 Hz were reduced by the $\alpha_1$-adrenoceptor antagonist prazosin (10 nM: ~70% blockade) and by the $\alpha_2$-adrenoceptor antagonist idazoxan (0.1 μM; ~30% blockade) and together these agents reduced the contractions by about 85%. The magnitude of the blockades produced by the $\alpha$-
adrenoceptor antagonists did not differ between the arteries from any of the STZ-treated groups and their control groups \( (P > 0.1 \) for all comparisons). The P2X1-purinoceptor antagonist NF449 (10 μM) blocked contractions to 100 pulses at 1 Hz by ~20% in arteries from both STZ-NI \( (19 \pm 8\% , n = 5) \) and control rats \( (18 \pm 6\% , n = 5; P = 0.92) \). The effects of NF449 were not tested in arteries from STZ-LI and STZ-HI rats.

PMAs from STZ-NI rats had reduced sensitivity to α-adrenoceptor agonists

Both in the absence and in the presence of the NET inhibitor, DMI (30 nM), there was a small but significant decrease in the pEC\(_{50}\) for PE in PMAs from STZ-NI rats compared to their controls (Figure 3A, B). This decrease in pEC\(_{50}\) to PE was not observed in PMAs from STZ-LI (Figure 4C, D) or STZ-HI rats \(-DMI: \) control 6.03 ± 0.08, STZ-HI 5.98 ± 0.13, \( P = 0.72\); \(+DMI:\) control 6.58 ± 0.13, STZ-HI 6.52 ± 0.10, \( P = 0.74\)).

The leftward shift in the EC\(_{50}\) for PE produced by blockade of NET with DMI (measured by the EC\(_{50}\) ratio) did not differ between PMAs from any of the STZ-treated groups and their controls \( (P > 0.2 \) for all comparisons). While there was a tendency for the maximum increase in ETP produced by PE to be smaller than control in PMAs from STZ-NI rats (Figure 4A, B) and larger than control in PMAs from both STZ-LI (Figure 4C, D) and STZ-HI rats \(-DMI: \) control 30.1 ± 1.8 mN/mm\(^2\), STZ-HI 33.9 ± 3.1 mN/mm\(^2\), \( P = 0.31\); \(+DMI:\) control 33.9 ± 3.1 mN/mm\(^2\), STZ-HI 36.0 ± 3.2 mN/mm\(^2\), \( P = 0.27\)), these differences did not reach the level of statistical significance.

In comparison with their controls, PMAs from STZ-NI rats had a reduction in their pEC\(_{50}\) for both methoxamine and clonidine but, in addition, these vessels had a reduction in their maximum contraction to both these agents (Table 3). These reductions in reactivity to methoxamine and to clonidine were not observed in PMAs from STZ-LI rats (Table 3). However PMAs from STZ-HI rats had an increase in their pEC50 for clonidine and these vessels also had a trend towards an increased sensitivity to methoxamine (Table 3).
In arteries from STZ-NI rats, the contractions to $\alpha,\beta$-methylene ATP (1 $\mu$M) did not differ significantly from those of their controls (control $11.2 \pm 1.5$ mN/mm$^2$, $n = 5$; STZ-NI $9.9 \pm 0.8$ mN/mm$^2$, $n = 5$; $P = 0.49$). Reactivity to $\alpha,\beta$-methylene ATP was not tested in arteries from STZ-LI or STZ-HI rats.

**PMAs from diabetic rats had an increased level of TH-IR labeling and those from STZ-NI rats had a reduced density of perivascular axon bundles.**

There was no difference between the widths of the wholemount vessel segments from each of the groups of STZ rats and their controls (Table 4). The perivascular plexus of PMAs revealed with pan-neuronal makers (anti-PGP9.5 or $\beta$-tubulin III) was comprised of a dense network of axon bundles (Figure 5A-F). The great majority of fibers within the perivascular plexus were TH-IR (Figure 5G, H). Both the mean peak intensity of the TH-IR fluorescent intercepts along the line profiles and the integrated TH-IR fluorescence were higher than control in vessels from STZ-NI and STZ-LI rats, but not STZ-HI rats (Figure 5L, M). However, as TH-IR did not resolve the finest fibers well (Figure 5G, H), we chose to use the pan-neuronal markers to assess changes in the density of fibers in the perivascular plexus (Figure 5A-F). In comparison with their controls, % area of the PMA surface covered by the axon plexus was reduced in STZ-NI, increased in STZ-LI and unchanged in STZ-HI rats (Figure 5I). There was also a ~25% reduction in the frequency of fluorescent intercepts along the line profiles in PMAs from STZ-NI rats, but this change was not seen in PMAs from STZ-LI and STZ-HI rats (Figure 5J). In PMAs from STZ-NI and STZ-LI rats, there was an increase in the widths of the fluorescent intercepts along the line profiles (Figure 5K).

In addition to TH-IR fibers, a small number of CGRP-IR fibers were also observed within the perivascular plexus (Figure 6A, B) but the frequency of CGRP-IR intercepts along the line profiles in vessels from each of the STZ-treated groups did not differ significantly from those in their control vessels (Figure 6C).
Mesenteric arteries (MAs)

MAs from diabetic rats had increased lumen diameters and impaired endothelial vasodilator function but no change in their responses to capsaicin or $K^+$

We only studied MAs from STZ-NI rats. After the normalization procedure the lumen diameter was about 20% larger for MAs isolated from the STZ-NI rats compared to their controls (control $382 \pm 17 \mu m, n=8$; STZ-NI $459 \pm 19 \mu m, n=8$; $P<0.01$). However, after equilibration, the basal ETP did not differ significantly between these two groups (control $7.1 \pm 0.3 \text{mN/mm}^2$; STZ $6.4 \pm 0.4 \text{mN/mm}^2$; $P=0.15$). In MAs from STZ-NI rats, the % relaxation of vessels constricted with PE (3 $\mu M$) when the endothelium was stimulated with carbachol (0.1 and 1 $\mu M$) was significantly smaller than in control MAs (Figure 7A). In contrast, the % relaxation produced when the peptidergic sensory axons were activated with capsaicin (0.01 and 0.1 $\mu M$) did not differ between STZ-NI and control MAs (Figure 7B). The peak increase in ETP produced by application of physiological saline containing 60 mM $K^+$ also did not differ between these groups of vessels (Figure 7C).

Nerve-evoked increases in ETP were reduced in MAs from diabetic rats

Over the range of stimulation frequencies studied, the nerve-evoked increases in ETP in MAs from STZ-NI rats were smaller than those in MAs from control rats (Figure 8A, B; ANOVA between groups $P<0.05$). However, post hoc comparisons between the responses of these groups of vessels at each stimulation frequency did not reach the level of statistical significance. As the increased dimensions of the MAs from STZ-NI rats might cause the reductions in nerve-evoked increases in ETP, Figure 8C displays the same data plotted as increases in wall tension (force/2 x vessel segment length, see (23). When expressed in this manner the nerve-evoked responses did not differ between
MAs from STZ-NI and control rats (ANOVA between groups $P = 0.65$), indicating that the absolute increase in force generated by nerve stimulation did not differ between these groups of vessels. In MAs from STZ-NI rats, there was a small but significant decrease in the % blockade of contractions to 20 pulses at 10 Hz produced by prazosin (10 nM: control 89 ± 2%, $n = 8$; STZ-NI 76 ± 5%, $n = 8$; $P < 0.05$). However there was no difference in the % blockade produced by the P2-purinoceptor antagonist suramin (0.1 mM: control 36%, IQR 31 - 38%, $n = 8$; STZ-NI 43%, IQR 28 - 66%, $n = 8$; Mann Whitney U-test $P = 0.63$) or by the combination of prazosin and suramin (control 99 ± 1%, $n = 8$; STZ 97 ± 2%, $n = 8$, $P = 0.28$) between MAs from STZ-NI and control rats.

**MAs from diabetic rats had no change in their sensitivity to phenylephrine**

Both in the absence and in the presence of DMI (30 nM), the pEC$_{50}$ and maximum increase in ETP to PE did not differ between MAs from STZ-NI and control rats (Figure 9A, B). The magnitude of the change in the pEC$_{50}$ for PE produced by DMI (measured by the EC$_{50}$ ratio) also did not differ between these groups of vessels ($P = 0.73$).

**MAs from diabetic rats had no change in their responses to $\alpha,\beta$-methylene ATP**

The peak increase in ETP to $\alpha,\beta$-methylene ATP (0.5 μM) did not differ between MAs from STZ-NI and control rats (control 12.4 ± 1.7 mN/mm$^2$, $n = 8$; STZ-NI 11.3 ± 1.1 mN/mm$^2$, $n = 8$; $P = 0.59$).

**MAs from diabetic rats had a reduced density of TH-IR perivascular axon bundles but this change was associated with an increase in vascular dimensions**

The widths of the wholemount MAs from STZ-NI rats were about ~25 % larger than those of their controls (control 322 ± 1 μm, $n = 7$, STZ-NI 407 ± 10 μm, $n = 7$, $P < 0.01$). In MAs from both
STZ-NI and control rats, the perivascular plexus was composed of TH-IR axon bundles of fairly uniform thickness that formed a network pattern across the medial surface (Figure 10A, C). In addition, the perivascular plexus of MAs contained a higher density of CGRP-IR fibers than did that of PMAs (Figure 10B, D; cf. Figure 6A, B). Both the % area of the vessel surface covered by the axon plexus and the frequency of TH-IR intercepts along the line profiles in MAs from STZ-NI rats were ~12% lower than in controls (Figure 10F), but the widths of the intercepts were similar in both groups of vessels (Figure 10F). There was also a reduction in the integrated TH-IR fluorescence and tendency for the mean peak intensity of the TH-IR fluorescent intercepts along the line profiles to be less in MAs from STZ-NI rats (Figure 10F). Relative to control tissues, the frequency of CGPR-IR intercepts along the line profiles in MAs from STZ rats was not significantly different (91 ± 12% of control; one-sample t-test P = 0.48).

Discussion

This study provides the first evidence that neurovascular function is differentially affected in arteries supplying blood to skin of the hind paw digits (PMAs) and to the intestine (MAs). In STZ-NI rats, nerve-evoked increases in ETP were reduced in both PMAs and MAs. The change in neurovascular transmission in PMAs was associated with reduced reactivity to $\alpha$-adrenoceptor agonists and to high [K+] , whereas no change in responsiveness to exogenously applied agents was detected in MAs. PMAs from STZ-LI and STZ-HI rats had no change in their nerve-evoked contractions and their reactivity to exogenously applied agents was not reduced, confirming that the changes observed in PMAs from STZ-NI rats were not the result of a direct toxic action of STZ. The frequency of nerve bundles was reduced in the perivascular nerve plexus of PMAs and MAs from STZ-NI rats, and in PMAs both the intensity of TH-IR and thickness of the nerve bundles (intercept widths) were increased. While the density of perivascular nerve bundles was not
significantly changed in PMAs from STZ-LI rats, the intensity of TH-IR and the thickness of
bundles were increased. These changes were not detected in PMAs from STZ-HI rats.

Previous studies investigating changes in sympathetic neurovascular function in rats with
STZ-induced diabetes have not provided any insulin support. As reported by others (e.g. (8),
treatment of the STZ-treated rats with a low dose of insulin that leaves them markedly
hyperglycemic, improved the overall health of the animals in comparison with those receiving no
insulin support as indicated by the increase in body weight gain. In comparison with STZ-LI rats,
the terminal glycated hemoglobin levels in STZ-HI rats were reduced by about 50% indicating
effective long-term glycemic control. However, in comparison with control rats, the STZ-HI rats at
termination still had elevated levels of glycated hemoglobin and reduced body weight gain.

The effects of STZ-induced diabetes on PMAs have not previously been reported, but they
have been described for small MAs. As we observed, there are several reports that diabetes impairs
endothelium-mediated vasodilation of MAs (e.g. (12, 36, 44). Also as we observed, Wigg et al.
(43) found that diabetes increased the lumen diameters of MAs and these investigators
demonstrated that this change occurred without alterations in the thickness or the mechanical
properties of the vascular wall. A chronic increase in blood flow can increase the lumen diameter
of MAs (10), so a possible explanation for change in vessel dimensions observed in STZ-treated
rats is an increase in intestinal perfusion to meet the demands produced by diabetes-induced
hyperphagia (43). The reported effects of STZ-induced diabetes on the reactivity of MAs to \(\alpha_1\)-
adrenoceptor agonists are variable, with sensitivity to these agents being either increased (36) or
unchanged (12, 44).

With passive (i.e. unconstricted) diameters of \(\sim 400 \mu m\), the size of second order MAs in
control rats is at the upper end of the arterial vessels that produce resistance to blood flow (see (3).
However, as sympathetic nerve activation of MAs \textit{in vivo} can reduce their internal diameter by 50-
70\% (9) it is suggested that arterial vessels of this size play a more important role in neural
regulation of peripheral resistance. Nerve-evoked pressor responses of the perfused isolated mesenteric arterial bed were reduced by 12 weeks of diabetes (27). As the basal perfusion pressure was also decreased by diabetes (27), the reduced nerve-evoked pressor responses are possibly explained by the increased lumen dimensions of the resistance arteries in this vascular bed rather than a change in neurovascular transmission. This appears to be the case in the present study because, while the nerve-evoked increases in ETP were reduced in the MAs from STZ rats, the absolute increases in force produced by nerve stimulation in these vessels did not differ from those in control MAs. These findings contrast with those for the increases in ETP produced by 60 mM K⁺, PE and α,β-methylene ATP, which did not differ between MAs from STZ and control rats and therefore appear to scale with the change in vascular dimensions.

Like second order MAs, the PMAs had passive diameters of ~400 µm and it is therefore likely that constriction of these vessels contributes to the neural regulation of blood flow in the hindpaw digits. The dimensions of PMAs from STZ-NI rats were similar to those of their controls and in these vessels the increases in absolute force produced by nerve stimulation (not shown) were reduced to a similar extent as the nerve-evoked increases in ETP. Therefore neurovascular transmission was reduced in PMAs from STZ-NI rats. The reduction in neurovascular transmission appears to be accounted for, at least in part, by postjunctural changes in the vascular muscle because these vessels also had reduced sensitivity to α-adrenoceptor agonists with decreases in their pEC50’s for PE, methoxamine and clonidine and smaller maximum contractions to methoxamine and clonidine. As the contractions to 60 mM K⁺ were also reduced in PMAs from STZ-NI rats, there appears to be a generalized decrease in the reactivity of these vessels to contractile agents. However, we cannot exclude the possibility that the expression of α₁- and α₂-adrenoceptors in the vascular muscle of PMAs is reduced by diabetes. Contractions to 1 µM α,β-methylene ATP were not reduced in PMAs from STZ-NI rats, but these responses were considerably smaller than those
produced by highest concentrations of $\alpha$-adrenoceptor agonists tested or by 60 mM $K^+$ and perhaps
we would have seen a reduction had we used a higher concentration of this agent.

PMAs are likely to be under strong thermoregulatory control like the rat tail artery that
supplies blood to the skin of the tail (25). In rat tail artery, 8 weeks of STZ-induced diabetes has
been reported to produce a small decrease in nerve-evoked contractions (11) whereas another study
found no change in nerve-evoked contractions after 12 weeks of diabetes (32). In both these
studies, the sensitivity to norepinephrine was assessed with the former study reporting no changes
(11) and the latter reporting an increased sensitivity to this agent (32). In the study of Speirs et al.
(32), the tail arteries from diabetic rats were studied in physiological saline containing a high
concentration of glucose (25 mM) and in control vessels bathed in the same solution the sensitivity
to norepinephrine was similarly increased. In preliminary studies we assessed the effects of
increasing the glucose to 25 mM on reactivity of PMAs to nerve stimulation and to PE,
methoxamine and clonidine and observed no changes. Speirs et al. (32) also reported an increased
contribution of purinoceptors to nerve-evoked constrictions of tail arteries from diabetic rats. The
relative contribution of P2X1-purinoceptors to neurovascular transmission (assessed with NF449) in
PMAs from STZ-NI rats was not changed. Furthermore, blockade of $\alpha_1$- and $\alpha_2$-adrenoceptors with
prazosin and idazoxan, respectively, reduced nerve-evoked contractions to a similar extent in PMAs
from all the STZ and control groups of rats. In MAs, the blockade of nerve-evoked contractions
produced by the P2-purinoceptor antagonist suramin was not changed by diabetes.

PMAs from STZ-LI and STZ-HI rats did not have reduced nerve-evoked contractions and
their sensitivity to $\alpha$-adrenoceptor agonists was also not reduced. The findings in PMAs from STZ-
LI rats, which remained markedly hyperglycemic, may be explained by the improved health of
these animals and it is possible that we would have observed changes had a longer period of
diabetes been studied. However, it is also possible that some of the changes observed in the PMAs
from STZ-NI rats are the result of the marked reduction in insulin levels rather than hyperglycemia
and that these are prevented in the STZ-LI rats. In PMAs from STZ-HI rats, we observed an increase in sensitivity to clonidine and a trend towards an increase in sensitivity to methoxamine. These effects may also be explained by a direct effect of insulin treatment, which has been reported to increase vascular reactivity to α-adrenoceptor agonists (15).

The effects of STZ-induced diabetes on the sympathetic innervation of arteries have rarely been investigated and where they have been examined no changes have been detected (tail artery (32); cerebral arteries (16)). In MAs from STZ rats, we observed a ~12% decrease in both the frequency of TH-IR intercepts along the line profiles of the perivascular plexus and the % area of the vessel surface covered by the immunolabeled nerve plexus. This reduction in nerve fiber density cannot be attributed to a difference in fixation-induced shrinkage because, as with the estimated lumen diameters of the myograph mounted vessels, the widths of the wholemount MAs from STZ rats were 20-25% larger than those of control MAs. However, as the surface area of the vessels was increased, the total number of nerve bundles supplying a segment of artery was not reduced. Instead, it appears that the number of axon bundles in the perivascular plexus did not increase proportionately with the increase in vascular dimensions. This possibility may explain why the absolute increase in force produced by nerve stimulation did not differ between MAs from STZ and control rats.

In PMAs from STZ-NI rats, where the vascular dimensions were not changed, the ~25% reduction in the frequency of fluorescent intercepts along the line profiles indicates that the total number of nerve bundles supplying a segment of artery was reduced. In rat tail arteries, a reduction in innervation density due to axon loss is associated with a decrease in the activity of neuronal NET (39). As the effects of blocking NET on sensitivity to PE did not differ between PMAs from STZ-NI and control rats, perhaps this indicates that the changes in the perivascular plexus are produced by rearrangement of the terminal axons rather than loss of axons. In PMAs from both STZ-NI and STZ-LI rats, the fluorescent intercept widths along the line profiles were larger indicating thicker
axon bundles. As a result, the % area of the vessel surface covered by the immunolabeled nerve plexus was only reduced by ~12% in STZ-NI rats, whereas it was increase by ~21% in STZ-LI rats. The cause of thickening of the axon bundles was not further investigated but it has been reported that STZ-induced diabetes causes swelling of sympathetic axons supplying the intestine (4), corpus cavernosum (22), seminal vesicle (21) and pineal gland (40). In addition to the changed morphology of the perivascular nerve plexus, both the mean peak intensity of the TH-IR fluorescent intercepts along the line profiles and the integrated TH-IR fluorescence were increased in PMAs from both STZ-NI and STZ-LI rats, suggesting an increased content of TH. An increase in the intensity of TH-IR fluorescence has been reported for sympathetic axons supplying the corpus cavernosum (22) and seminal vesicle (21) in STZ rats. The changes in the structure of the axon plexus and in TH-IR suggest that plastic changes are taking place in the sympathetic innervation of PMAs. No changes were detected in the perivascular axon plexus of PMAs from STZ-HI rats, indicating that the effects seen in PMAs from both STZ-NI and STZ LI rats are most likely explained by hyperglycemia.

Sensory nerve-mediated vasodilation of the perfused isolated mesenteric arterial bed has been reported to be reduced in rats eight weeks following induction of diabetes with STZ (28). However, in the present study, we observed no changes in the peptidergic innervation of either MAs or PMAs. Furthermore, we did not detect changes in the inhibitory effects of activating the perivascular peptidergic axons with capsaicin on PE constricted MAs, or on nerve-evoked contractions of PMAs, from STZ-NI rats.

Together these findings suggest that sympathetic neurons supplying the PMAs are more markedly affected by diabetes than those supplying the mesenteric arteries. For somatic sensory and motor neurons, it is known that neurons with long axons (i.e. supplying the limbs) are most susceptible to diabetes-induced damage (1). Little is known about the length dependence of the effects of diabetes on autonomic neurons. However, as deficits in sympathetic nerve-mediated
vasoconstriction of arteries supplying skin of the hands and feet can be an early sign of diabetic autonomic neuropathy (13, 35), sympathetic neurons with long axons may also be particularly susceptible to the effects of diabetes. In rat tail artery, STZ-induced diabetes is reported to produce a length dependent increase in the neuronal content of biogenic amines (norepinephrine, adrenaline, serotonin and dopamine), with the most marked changes occurring in the most distal region of this vessel (20). This length dependence raises the possibility that axonal transport is affected. Previous studies indicated that diabetes affects both anterograde and retrograde axonal transport in the sciatic nerve (37), so in the nerve terminal axons there may be changes in the turnover of proteins required for the normal processing of these biogenic amines (e.g. enzymes and transporters). A change in axonal transport may also explain the increased level of TH-IR detected in PMAs from STZ-NI and STZ-LI rats.

In conclusion, this study demonstrates that the effects of diabetes differ between PMAs and MAs. While in both vessels there were reductions in nerve-evoked responses, only in PMAs did diabetes appear to affect the sympathetic nerve terminals. In MAs, the observed reduction in innervation density produced by diabetes can be explained by the increased size of these vessels. In PMAs, the reduction in nerve-evoked contractions may also be explained by a decrease in the reactivity of their vascular muscle to α-adrenoceptor agonists. Importantly, in PMAs all effects of STZ-induced diabetes observed in this study were prevented in the rats receiving a high dose of insulin, demonstrating their dependence on hyperglycemia and/or loss of insulin signaling. Therefore, PMAs provide a suitable model to investigate the effects of diabetes on sympathetic vasoconstrictor neurons and neurovascular transmission, and to assess the efficacy of neuroprotective treatments.
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Disclosures: The authors have no conflicts of interest regarding the work presented in this article.
References


Figure legends

**Figure 1** The relationship between the internal circumference and the increase in wall tension produced by raising the K⁺ concentration to 60 mM in plantar metatarsal arteries (PMAs). To construct this relationship, PMAs from 4 animals were set up as described in the Methods and, after equilibration for 30 minutes, the level of basal effective transmural pressure was sequentially adjusted to 2.7, 5.3, 8.0, 10.6 and 13.3 mN/mm² (i.e. 20, 40, 60, 80 and 100 mmHg), with responses to 60 mM K⁺ determined at each pressure. On the X-axis the internal circumference at each pressure is expressed as a proportion of that at 13.3 mN/mm² (IC/IC₃₃). Each point represents the mean of both internal circumference and wall tension measures with X- and Y-SEM bars, respectively. In these experiments, the α-adrenoceptor antagonists, prazosin (10 nM) and idaxozan (0.1 µM), were applied to the tissues to prevent the contractile effects of norepinephrine released from the nerve terminals by K⁺-induced depolarization.

**Figure 2** In plantar metatarsal arteries (PMAs), diabetes did not impair endothelium-dependent relaxation but it did reduce contractions evoked by 60 mM K⁺. (A-C) The % relaxations produced by carbachol (0.1 and 1 µM) in phenylephrine (3 µM) constricted PMAs from STZ-treated rats receiving no insulin (A; STZ-NI, n = 10), a low dose of insulin (B; STZ-LI, n = 6) or a high dose of insulin (C; STZ-HI, n = 6) and their controls (n = 10 for STZ-NI and 6 for STZ-LI and STZ-HI). (D-F) Peak increases in effective transmural pressure evoked by application of physiological saline containing 60 mM K⁺ in PMAs from STZ-NI (D), STZ-LI (E) and STZ-HI (F) and their controls. The data are presented as mean and SEM. In D, * indicates a significant difference between responses of PMAs from STZ-NI and control rats (P < 0.01, unpaired t-test).

**Figure 3** In plantar metatarsal arteries (PMAs), diabetes reduced nerve-evoked contractions. (A) Representative traces showing contractions evoked by trains of 25 pulses at 0.1 – 1 Hz in segments of PMA from a control rat (upper) and a STZ-treated rat receiving no insulin (lower; STZ-NI). (B-D) The peak increases in effective transmural pressure produced by these stimuli in PMAs from
STZ-NI rats (B; n = 10), and STZ-treated rats receiving a low dose of insulin (C; STZ-LI, n = 6) or a high dose of insulin (D; STZ-HI, n = 6) and their controls (n = 10 for STZ-NI and 6 for STZ-LI and STZ-HI). The data are presented as mean and SEM. In B, * indicates significant differences between responses of PMAs from STZ-NI and control rats (P < 0.05; unpaired t-test with P values adjusted for multiple comparisons using the false discovery procedure).

**Figure 4** In plantar metatarsal arteries (PMAs), diabetes reduced sensitivity to phenylephrine (PE). (A-D) Concentration response curves and pEC$_{50}$s for PE in PMAs from STZ-treated rats receiving no insulin (A, B; STZ-NI, n = 10) or a low dose of insulin (C, D; STZ-LI, n = 6) and their controls (n = 10 for STZ-NI and 6 for STZ-LI) in the absence (A, C) or in the presence of the norepinephrine transporter (NET) inhibitor desmethyliimipramine (DMI)(B, D). The data are presented as mean and SEM. In A and B, * indicate that the pEC$_{50}$ values were significantly smaller than control in the PMAs from STZ-NI rats both with and without NET blockade (P < 0.05; unpaired t-tests).

**Figure 5** In plantar metatarsal arteries (PMAs), diabetes reduced the density of the perivascular nerve plexus and increased the intensity of tyrosine hydroxylase immunoreactivity (TH-IR). (A-F) The perivascular plexus revealed with a pan-neuronal marker (A, B anti-PGP9.5, C-F anti-β-tubulin III) imaged with a x63 objective in a PMA segment from STZ-treated rats receiving no insulin (B; STZ-NI), a low dose of insulin (D; STZ-LI) or a high dose of insulin (F; STZ-HI) and their controls (A, C and E respectively). Above A and B are shown line profiles measured along the blue lines on the images. (G, H) The TH-IR perivascular plexus imaged with a x40 objective in a PMA segment from a control and an STZ-NI rat. (I-K) The % area of the vessel surface covered by the immunolabeled nerve plexus (I) and the fluorescent intercept frequency (J) and width (K) along line profiles for artery segments label with a pan-neuronal marker from STZ-NI (n = 7), STZ-LI (n = 10) and STZ-HI (n = 10) rats expressed as a % of these measures in paired control tissues. (L, M) The mean peak value for the TH-IR fluorescent intercepts (L; Peak TH) along the line profiles, and
the integrated TH-IR fluorescence/100 µm² of vessel surface (M; Integrated TH) for artery
segments from STZ-NI (n = 7), STZ-LI (n = 10) and STZ-HI (n = 10) expressed as a % of these
measures in paired control tissues. The data are presented as mean and SEM. Statistical
assessments were made with one-sample t-tests (* P < 0.05, ** P < 0.01). The scale bars in A and
G indicate 50 µm and also apply, respectively, in B-F and H.

Figure 6 In plantar metatarsal arteries (PMAs), diabetes did not detectably change their
innervation by calcitonin gene-related peptide immunoreactive (CGRP-IR) fibers. (A, B) CGRP-IR
perivascular fibers imaged with a x20 objective in a PMA segment from an STZ-treated rat
receiving no insulin (B; STZ-NI) and a control rat (A). (C) Measures of CGRP-IR intercept
frequency along line profiles in PMAs from STZ-NI rats (n = 7) and STZ-treated rats receiving a
low dose of insulin (STZ-LI, n = 10) or a high dose of insulin (STZ-HI, n = 10) expressed as a % of
measures in paired control tissues. The data are presented as mean and SEM. The scale bar in A
indicates 100 µm and also applies in B.

Figure 7 In mesenteric arteries (MAs), diabetes impaired endothelium-dependent relaxation to
carbachol but did not change relaxation produced by activation of the peptidergic sensory nerves
with capsaicin or contractions evoked by depolarizing the vascular muscle with 60 mM K⁺. (A)
The % relaxation produced by carbachol (0.1 and 1 µM) in phenylephrine (3 µM) constricted MAs
from control (n = 8) and STZ-treated rats (n = 8). (B) The % relaxation produced by capsaicin
(0.01 and 0.1 µM) in phenylephrine (3 µM) constricted MAs from control and STZ-treated rats.
(C) The peak increases in effective transmural pressure produced by application of physiological
saline containing 60 mM K⁺ in MAs from control and STZ-treated rats. The data are presented as
mean and SEM. In A, * indicates significant differences between responses of MAs from control
and STZ-treated rats (P < 0.05; unpaired t-tests).
Figure 8 In mesenteric arteries (MAs), diabetes reduced nerve-evoked increases in effective transmural pressure but did not significantly change these responses when expressed as increases in wall tension. (A) Representative traces showing contractions evoked by trains of 100 pulses at 1 – 10 Hz in a segment of MA from a control rat (upper) and an STZ-treated rat (lower). (B, C) The peak increases in effective transmural pressure (B) or wall tension (C) produced by these stimuli in MAs from control (n = 8) and STZ-treated rats (n = 8). The data are presented as mean and SEM. In B and C, the ANOVA P values are for between group comparisons. While the ANOVA indicated that nerve-evoked increases in effective transmural pressure in MAs from STZ-treated rats were smaller than those of their controls, comparisons between the responses of these groups of vessels to each frequency of stimulation did not reach the level of statistical significance.

Figure 9 In mesenteric arteries (MAs), diabetes did not change sensitivity to phenylephrine (PE). Concentration response curves and pEC₅₀s for phenylephrine in MAs from control (n = 8) and STZ-treated rats (n = 8) in the absence (A) or in the presence of the neuronal norepinephrine transporter inhibitor desmethylimipramine (B). The data are presented as mean and SEM.

Figure 10 In mesenteric arteries (MAs), diabetes reduced the density of tyrosine hydroxylase immunoreactive (TH-IR) perivascular fibers but this change was associated with an increase in vascular dimensions. (A-D) The TH-IR (A, C) and calcitonin gene-related peptide immunoreactive (B, D; CGRP-IR) nerve plexus imaged with a x20 objective in a MA segment from a control (A, B) and an STZ-treated (C, D) rat. (E) The % area of the vessel surface covered by the TH-IR nerve plexus, the frequency, width and mean peak value of the TH-IR fluorescent intercepts (Peak TH) along line profiles and the integrated TH-IR fluorescence/100 µm² of vessel surface (Integrated TH) for MA segments from STZ-treated rats (n = 7) expressed as a % of these measures in paired control tissues. The data are presented as mean and SEM. Statistical assessments were made with one-sample t-tests and * indicates a significant difference (P < 0.05). The scale bar in A indicates 100 µm and also applies in C to D.
Table 1 Measures of net body weight gain, blood glucose and % glycated hemoglobin at termination in control and STZ-treated rats.

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<th>Body weight gain (g)</th>
<th>Blood glucose concentration (mmol/L)</th>
<th>% Glycated hemoglobin</th>
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<td>156 ± 8</td>
<td>6.5 ± 0.3</td>
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<td>STZ-NI</td>
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<td>(4.7 - 5.8)</td>
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<td>(5.2 - 7.8)</td>
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<td></td>
<td>(4.8 - 12.0)</td>
<td>(7.2 - 8.7)</td>
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P <0.001* <0.001* <0.001* <0.01* 0.22 <0.01*

Data are presented as means ± SEMs or medians and interquartile ranges (in parentheses).

Statistical comparisons between control and STZ groups were made with Student’s unpaired t-tests or Mann Whitney U-tests as appropriate and significant differences are indicated by *.
Table 2 The estimated lumen diameter and effective transmural pressure of plantar metatarsal arteries under basal conditions.

<table>
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<th>Diameter (μm)</th>
<th>Basal transmural pressure (mN/mm²)</th>
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<td>STZ-NI</td>
<td>10</td>
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<td>10.6 ± 0.2</td>
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<td>393 ± 6</td>
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<td>STZ-HI</td>
<td>6</td>
<td>394 ± 8</td>
<td>10.4 ± 0.2</td>
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Data are presented as means ± SEMs. Statistical comparisons between control and STZ groups of arteries were made with Student’s unpaired t-tests.
Table 3 Effects of diabetes on concentration-response curves for methoxamine and clonidine in plantar metatarsal arteries.

<table>
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<th>Clonidine</th>
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<td></td>
<td></td>
<td>pEC$_{50}$</td>
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<td>pEC$_{50}$</td>
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<td></td>
<td>(mN/mm$^2$)</td>
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<tr>
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<td>7.32 ± 0.07</td>
<td>15.2 ± 1.3</td>
</tr>
<tr>
<td>STZ-NI</td>
<td>10</td>
<td>6.11 ± 0.06</td>
<td>18.9 ± 1.3</td>
<td>6.99 ± 0.12</td>
<td>12.0 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;0.05*</td>
<td>&lt;0.05*</td>
<td>&lt;0.05*</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>6.11 ± 0.07</td>
<td>26.4 ± 4.8</td>
<td>7.00 ± 0.12</td>
<td>18.4 ± 1.5</td>
</tr>
<tr>
<td>STZ-LI</td>
<td>6</td>
<td>6.16 ± 0.03</td>
<td>25.7 ± 5.5</td>
<td>6.97 ± 0.08</td>
<td>18.3 ± 1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.50</td>
<td>0.74</td>
<td>0.81</td>
<td>0.96</td>
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<tr>
<td>Control</td>
<td>6</td>
<td>6.14 ± 0.10</td>
<td>27.5 ± 2.7</td>
<td>6.95 ± 0.13</td>
<td>16.2 ± 3.1</td>
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<tr>
<td>STZ-HI</td>
<td>6</td>
<td>6.36 ± 0.05</td>
<td>33.4 ± 3.1</td>
<td>7.34 ± 0.06</td>
<td>22.2 ± 2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.08</td>
<td>0.18</td>
<td>&lt;0.05*</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEMs. Statistical comparisons between control and STZ groups of arteries were made with Student’s unpaired $t$-tests and significant differences are indicated by *.
Table 4 Widths of wholemount planter metatarsal artery segments.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Vessel width</th>
<th>μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>284 ± 8</td>
<td></td>
</tr>
<tr>
<td>STZ-NI</td>
<td>7</td>
<td>289 ± 12</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>248 ± 20</td>
<td></td>
</tr>
<tr>
<td>STZ-LI</td>
<td>10</td>
<td>250 ± 17</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>267 ± 15</td>
<td></td>
</tr>
<tr>
<td>STZ-HI</td>
<td>10</td>
<td>280 ± 11</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as means ± SEMs.
Figure 1
Figure 2

A

B

C

D

E

F

Δ Effective transmural pressure (mN/mm²)

% Relaxation

[Carbachol]

60 mM K⁺ 60 mM K⁺

Control STZ-NI

Control STZ-LI

Control STZ-HI

ABC DE F

Control STZ-NI

Control STZ-LI

Control STZ-HI

0.1 μM 1 μM

0.1 μM 1 μM

0.1 μM 1 μM
Figure 3

A

Control

STZ-NI

0.1 Hz 0.3 Hz 0.5 Hz 1 Hz

B

Control

STZ-NI

Δ Effective transmural pressure (mN/mm²)

Frequency (Hz)

C

Control

STZ-LI

Δ Effective transmural pressure (mN/mm²)

Frequency (Hz)

D

Control

STZ-HI

Δ Effective transmural pressure (mN/mm²)

Frequency (Hz)
Figure 4

A. PE

B. PE + DMI

C. Control

D. Control

Δ Effective transmural pressure (mN/mm²)

[Phenylephrine](μM)
Figure 7

A. % Relaxation

- Control
- STZ

[Carbachol] 0.1 μM, 1 μM

B. % Relaxation

- Control
- STZ

[Capsaicin] 0.01 μM, 0.1 μM

C. Δ Effective transmural pressure (mN/mm²)

- Control
- STZ

60 mM K⁺
Figure 8

A

Control

STZ

B

ANOVA $P < 0.05$

ANOVA $P = 0.65$

C

Δ Effective transmural pressure (mN/mm²)

Δ Wall tension (mN/mm)

Stimulation frequency (Hz)

Stimulation frequency (Hz)
Figure 9

A

PE

Δ Effective transmural pressure (mN/mm²)

[Phenylephrine] (µM)

Control

STZ

pEC₅₀

Control 5.8 ± 0.1

STZ 5.9 ± 0.2

B

PE + DMI

Δ Effective transmural pressure (mN/mm²)

[Phenylephrine] (µM)

Control

STZ

pEC₅₀

Control 6.3 ± 0.1

STZ 6.2 ± 0.1