

Type 2 diabetes alters bone and marrow blood flow and vascular control mechanisms in the ZDF rat

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Abstract

Bone health and cardiovascular function are compromised in individuals with type 2 diabetes mellitus (T2DM). The purpose of this study was to determine whether skeletal vascular control mechanisms are altered during the progression of T2DM in Zucker diabetic fatty (ZDF) rats. Responses of the principal nutrient artery (PNA) of the femur from obese ZDF rats with prediabetes, short-term diabetes, and long-term diabetes to endothelium-dependent (acetylcholine) and -independent (sodium nitroprusside) vasodilation and potassium chloride, norepinephrine (NE), and a myogenic vasoconstrictor were determined *in vitro*. Few changes in the PNA vasomotor responses occurred for the prediabetic and short-term diabetic conditions. Endothelium-dependent and -independent vasodilation were reduced, and NE and myogenic vasoconstriction were increased in obese ZDF rats with long-term diabetes relative to lean age-matched controls. Differences in endothelium-dependent vasodilation of the femoral PNA between ZDF rats and controls were abolished by the nitric oxide synthase inhibitor *N*^G-nitro-L-arginine methyl ester. The passive pressure–diameter response of the femoral PNA was also lower across a range of intraluminal pressures with long-term T2DM. Regional bone and marrow perfusion and vascular conductance, measured *in vivo* using radiolabeled microspheres, were lower in obese ZDF rats with long-term diabetes. These findings indicate that the profound impairment of the bone circulation may contribute to the osteopenia found to occur in long bones during chronic T2DM.

Key Words

- ▶ diabetes
- ▶ bone blood flow
- ▶ endothelium
- ▶ vascular conductance
- ▶ Zucker diabetic fatty rat

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Introduction

The increase in fracture risk observed among patients with type 2 diabetes mellitus (T2DM) has been suggested to be associated with corollaries of the disease, such as peripheral neuropathy, decreased physical fitness, vision loss, and poor balance (Schwartz 2003, Melton *et al.* 2008,

Leslie *et al.* 2012). Emerging results also indicates a causal link between cardiovascular function and indices of bone health (Parfitt 2000, Farhat & Cauley 2008, Lampropoulos *et al.* 2012, Prisby *et al.* 2012). Cardiovascular disease is a leading complication of T2DM (Grundy *et al.* 1999),

including impairment of endothelium-dependent vasodilation of large conduit arteries (Hogikyan *et al.* 1998, Makimattila *et al.* 1999, van de Ree *et al.* 2001, Rossi *et al.* 2005).

Previous findings obtained using obese Zucker diabetic fatty (ZDF) rats (Lesniewski *et al.* 2008), an animal model of T2DM, indicate that an impairment of endothelium-dependent vasodilation through the nitric oxide (NO) signaling mechanism effectively changes the balance of vasomotor control in skeletal muscle arterioles to favor vasoconstriction. Decreases in bone mineral density (BMD) have also been shown to occur in obese ZDF rats with long-term T2DM (Prisby *et al.* 2008) and is one basis for the decrease in mechanical strength of long bones in the hindlimb. It has been proposed that one contributing factor to this decrease in BMD and mechanical strength could be a reduction in bone and marrow blood flow and impairment of coupling mechanisms linking endothelium-dependent vasodilation to bone cell remodeling activity (Prisby *et al.* 2008). For example, reduced blood flow to the bone and marrow of the femur occurs in aged rats (Prisby *et al.* 2007), and this is accompanied by an impairment of the ability of the femoral principal nutrient artery (PNA) to vasodilate by way of a NO signaling mechanism (Prisby *et al.* 2007). In addition, results of previous experiments with young and old exercise-trained rats (Dominguez *et al.* 2010) and ovariectomized rats (Prisby *et al.* 2012) indicate a coupling of endothelium-dependent vasodilation to measures of bone volume. Therefore, the purpose of this study was to determine whether control mechanisms of the skeletal resistance vasculature, including endothelium-dependent vasodilation, are altered during the progression of T2DM when BMD is both increasing and decreasing (Prisby *et al.* 2008). We proposed that endothelium-dependent vasodilation of the femoral PNA would be higher in prediabetic 7-week-old obese ZDF rats when femoral BMD is greater than that in 7-week-old lean ZDF rats, and that endothelium-dependent vasodilation would be impaired in long-term diabetic 20-week-old obese ZDF rats when femoral BMD is reduced. Results from these vascular studies indicated that endothelium-dependent vasodilation of the PNA was diminished with long-term diabetes, along with other vascular mechanisms that serve to regulate bone perfusion. Consequently, a secondary purpose of this study was to determine the effects of frank T2DM on hindlimb bone and marrow blood flow and vascular conductance. We proposed that bone and marrow perfusion would be lower in animals with long-term diabetes relative to that in 20-week-old lean ZDF animals.

Materials and methods

Animals

All experimental procedures were approved by the University of Florida's Institutional Animal Care and Use Committee and conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Eighth edition, 2011). Lean (371:+/?) and obese (370:Fa/Fa) ZDF rats were obtained from Charles River Laboratories (Kingston, NY, USA). Obese ZDF rats are homozygous for the fatty (*Fa*) gene (Truett *et al.* 1991) and when fed the Purina 5008 diet they manifest obesity and hyperlipidemia (Leonard *et al.* 2005). Seven-week-old obese ZDF rats manifest hyperinsulinemia and mild hyperglycemia and have been classified as prediabetic. The strain develops more severe hyperglycemia by 13 weeks of age (short-term diabetes) and becomes normo- or hypoinsulinemic by 20 weeks of age (long-term diabetes) (Peterson *et al.* 1990, Etgen & Oldham 2000). Animals used in this study were provided Purina 5008 diet and water and allowed to eat and drink *ad libitum* maintained on a 12 h light:12 h darkness cycle, and studied at 7 weeks (lean, $n=28$; obese, $n=29$), 13 weeks (lean, $n=28$; obese, $n=28$), and 20 weeks (lean, $n=51$; obese, $n=51$) of age to correspond to the prediabetic, short-term diabetic, and long-term diabetic states in the obese ZDF rats respectively.

Isolated microvessels

Animals were anesthetized with isoflurane (2%/O₂ balance), a thoracotomy was performed, and 2 ml of blood was withdrawn via cardiac puncture before excision of the heart. Immediately after killing, the hindlimbs were removed and placed into a dissecting bath containing 4 °C physiological saline solution (PSS). Femoral PNAs, which regulate approximately 70% of the blood supply to the femoral bone marrow and cortex (Bridgeman & Brookes 1996), were isolated, cannulated, and pressurized with PSS at 60 cm H₂O (44 mmHg) as described previously (Prisby *et al.* 2007, Dominguez *et al.* 2010). This pressure was selected on the basis of intravascular arterial pressures measured within similar sized skeletal muscle resistance arteries of 43–46 mmHg (Meininger *et al.* 1984).

Experimental design

Endothelium-dependent vasodilation of femoral PNAs ($n=14$ –15/group) was assessed by the cumulative addition of acetylcholine (ACh, 10^{-9} – 10^{-4} mol/l). To determine

the contribution of the NO signaling pathway to endothelium-dependent vasodilation, PNAs were incubated with the NO synthase (NOS) inhibitor N^G -nitro-L-arginine methyl ester (L-NAME, 10^{-5} mol/l) (Muller-Delp *et al.* 2002, Prisby *et al.* 2007, Dominguez *et al.* 2010), and the ACh dose-response was repeated. To investigate the contribution of prostaglandin signaling to endothelium-dependent vasodilation, PNAs were co-incubated with L-NAME and the cyclooxygenase (COX) inhibitor indomethacin (10^{-5} mol/l) (Prisby *et al.* 2007), and the ACh dose-response was repeated. Endothelium-independent vasodilator responsiveness was assessed via the cumulative addition of the NO donor sodium nitroprusside (SNP, 10^{-9} – 10^{-4} mol/l). Lastly, maximal intraluminal diameter and medial wall thickness were determined after two 15-min incubations in Ca^{2+} -free PSS supplemented with SNP (10^{-4} mol/l) to achieve complete smooth muscle relaxation. Medial wall thickness was recorded as the average of three distinct wall measurements made with a video caliper as previously described (Stabley *et al.* 2013).

Using a separate set of rats ($n=13$ – 14 /group), vasoconstrictor responses of PNAs to increasing concentrations of potassium chloride (KCl, 10–100 mmol/l) were determined to investigate the contribution of voltage-gated Ca^{2+} channels. Concentrations of NaCl and KCl in PSS were balanced such that bath osmolarity was maintained and the desired isotonic K^+ bath concentrations were achieved (Donato *et al.* 2005). Vasoconstrictor responses to the cumulative addition of norepinephrine (NE, 10^{-9} – 10^{-4} mol/l) were recorded to measure the contribution of α adrenoreceptors (Delp 1999). In a separate group of 20-week-old animals (lean, $n=11$; obese, $n=10$), the endothelium of PNAs was removed by passing 3–5 ml of air through the lumen as described previously (Donato *et al.* 2005). Administration of a bolus dose of ACh (10^{-5} mol/l) was used to confirm the successful removal of the endothelium, and measurement of the PNA response to the cumulative addition of NE was determined.

Active myogenic responses to stepwise increases in intraluminal pressure were determined by raising the height of both fluid reservoirs in 15 cm H_2O increments from 0 to 135 cm H_2O . Intraluminal pressure was then decreased in 15 cm H_2O decrements back to 0 cm H_2O . Passive myogenic responses to stepwise increases in intraluminal diameter were determined using the steps employed for the active myogenic response described above except that the vessel bathing solution was replaced with Ca^{2+} -free PSS plus SNP (10^{-4} mol/l) as described previously (Delp 1999).

Peripheral quantitative computed tomography

Tomographic scans were performed *ex vivo* on femoral mid-shafts and distal femora in PBS using a Stratec XCT Research-M device as reported previously (Prisby *et al.* 2008). Reported measurements of cortical BMD were derived from the femoral mid-shaft and reported measurements of total and cancellous BMD were derived from the distal femora. For precision of the measurements, calibration of the Stratec XCT Research-M device was performed before scanning by use of a hydroxyapatite standard cone phantom. The distal metaphysis of the femur was scanned 4, 5, and 6 mm from the proximal plateau and the mid-diaphysis was scanned (on center) 8 mm from the distal end of the lateral epicondyles. For both the distal metaphysis and mid-diaphysis, values from multiple slices were averaged. Scans were performed at 5 mm/s with voxel resolution of $0.07 \times 0.07 \times 0.5$ mm. In addition, analyses were performed using cut and peel modes of 3 and 2. According to the manufacturer's data, machine precision is ± 3.0 mg/cm³ for cancellous bone and ± 9.0 mg/cm³ for cortical bone.

Surgical procedures

Blood flow was measured using a separate group of 20-week-old lean ($n=12$) and obese ($n=12$) ZDF rats as described previously (Colleran *et al.* 2000, Prisby *et al.* 2007). The animals were anesthetized with isoflourane (2–2.5%/O₂ balance) and a polyethylene catheter filled with heparinized saline solution (100 U/ml) was implanted in the ascending aorta via the right carotid artery. Blood (approximately 300 μ l) was collected from this catheter to determine plasma insulin and glucose concentrations. A separate polyethylene catheter was implanted into the caudal artery of the tail for withdrawal of reference blood samples (Delp & Armstrong 1988). Each animal was allowed a minimum of 3 h of recovery after wound closure and the cessation of anesthesia before experiments to measure tissue blood flow were performed. Results of previous research indicate that cardiovascular dynamics, regional blood flow, and acid-base status recover to normal levels within 3 h after anesthesia (Flaim & Zelis 1980).

Determination of blood flow

Bone blood flow was measured using radiolabeled microspheres while the animals with long-term diabetes and 20-week-old age-matched lean controls were quietly standing as described previously (Colleran *et al.* 2000,

Prisby *et al.* 2007, Stabley *et al.* 2013). After completion of the blood flow experiment, animals were killed with Beuthanasia-D Special (approximately 0.6 ml/kg, Schering-Plough Animal Health, Union, NJ, USA) administered via the carotid artery catheter. Hindlimb bones were removed, cleared of muscle and tendon, and divided into regions as described previously (Colleran *et al.* 2000, Prisby *et al.* 2007, Dominguez *et al.* 2010). Individual tissue and reference blood sample radioactivity were determined and individual tissue blood flow (ml/min per 100 g) was calculated according to the reference sample microsphere method (Ishise *et al.* 1980). Tissue vascular conductance (ml/min per 100 g/mmHg) was calculated by dividing individual tissue blood flow by mean arterial pressure.

Statistical analyses

Vascular responses were calculated and expressed as a percentage of vasoconstriction or vasodilation as follows:

$$\text{Percentage vasoconstriction} = (D_b - D_s)/D_b \times 100$$

$$\text{Percentage vasodilation} = (D_s - D_b)/(D_m - D_b) \times 100$$

where D_b was the initial baseline intraluminal diameter measured before experimental intervention, D_s was the steady-state intraluminal diameter measured after agonist addition, and D_m was the maximal diameter recorded at 60 cm H_2O .

Basal tone was expressed as a percentage of the maximal diameter (D_m) as follows:

$$\text{Percentage basal tone} = (D_m - D_b)/D_m \times 100$$

The significance of differences in body mass and bone tissue mass was determined via Student's unpaired *t*-tests. Pressure-response and concentration-response curves were evaluated by using repeated-measures ANOVA with one within (intraluminal pressure or agonist concentration) and one between (experimental groups) factor. Planned contrasts were conducted at each intraluminal

pressure or concentration level to determine whether differences existed between experimental groups (lean versus obese). Regression analyses were used to individually investigate the relationship between peak-endothelium-dependent vasodilation and cancellous BMD in the distal femur and total BMD and cortical BMD in the femur diaphysis. Differences in hindlimb bone blood flow and vascular conductance between experimental groups were determined by one-tailed independent-samples *t*-tests. An α level of 0.05 delineated significance.

Results

Animal and PNA characteristics

Body mass was greater in obese ZDF rats for the prediabetic, short-term, and long-term diabetic conditions relative to the age-matched lean controls (Table 1). Blood glucose concentration was higher in ZDF rats with short- and long-term diabetes when compared with age-matched lean rats (Table 1), and blood insulin levels were higher in obese ZDF rats under the prediabetic and short-term diabetic conditions (Table 1).

Maximal diameter of the femoral PNA did not differ between lean and obese ZDF rats for the prediabetic or short-term diabetic conditions (Table 2). However, maximal diameter of the PNA was smaller in obese ZDF rats with long-term diabetes than that in age-matched lean controls (Table 2). Basal tone of the PNA did not differ between lean and obese ZDF rats at any age studied (Table 2).

PNA vasodilation

Endothelium-dependent vasodilation of the femoral PNA was greater in prediabetic ZDF rats when compared with age-matched lean ZDF animals (Fig. 1A). This difference was abolished by inhibition of NOS alone and by co-inhibition of NOS and COX (Fig. 1A). There was no difference in endothelium-dependent vasodilation

Table 1 Body mass, blood glucose, insulin concentrations, and femoral bone mineral density (BMD). Values are mean \pm s.e.m.

	7 week control	7 week pre-diabetic	13 week control	13 week short-term	20 week control	20 week long-term
Body mass (g)	128 \pm 5	174 \pm 6 ^a	276 \pm 8	341 \pm 14 ^a	369 \pm 5	402 \pm 13 ^a
Glucose (mg/dl)	86 \pm 4	104 \pm 8	88 \pm 5	219 \pm 17 ^a	94 \pm 4	242 \pm 14 ^a
Insulin (ng/ml)	1.3 \pm 0.2	4.7 \pm 1.0 ^a	1.2 \pm 0.1	5.2 \pm 1.4 ^a	1.5 \pm 0.1	1.6 \pm 0.1
Total BMD (mg/cm ³)	459 \pm 4	471 \pm 6	562 \pm 7	513 \pm 16	617 \pm 11	487 \pm 7 ^a
Cancellous BMD (mg/cm ³)	350 \pm 9	383 \pm 9 ^a	317 \pm 7	309 \pm 20	326 \pm 10	227 \pm 23 ^a
Cortical BMD (mg/cm ³)	1163 \pm 4	1152 \pm 4	1333 \pm 3	1324 \pm 2 ^a	1386 \pm 3	1368 \pm 3 ^a

^aSignificant differences from age-matched lean controls ($P < 0.05$).

Table 2 Principal nutrient artery (PNA) maximal diameter and basal tone. Values are mean \pm S.E.M.

	7 week control	7 week pre-diabetic	13 week control	13 week short-term	20 week control	20 week long-term
PNA maximal diameter (μ m)	164 \pm 3	165 \pm 3	178 \pm 5	183 \pm 3	190 \pm 8	163 \pm 8 ^a
PNA basal tone (%)	50 \pm 4	44 \pm 5	41 \pm 5	33 \pm 5	37 \pm 3	35 \pm 4

^aSignificant differences from age-matched lean controls ($P < 0.05$).

between short-term diabetic animals and lean control ZDF rats (Fig. 1B). Endothelium-dependent vasodilation of the PNA in ZDF rats with long-term diabetes was lower than that of lean controls (Fig. 1C), and this difference was abolished by NOS inhibition alone and in combination with COX inhibition.

Endothelium-independent vasodilation in response to SNP did not differ between lean ZDF rats and obese ZDF animals for the prediabetic and short-term diabetic conditions (Fig. 2). However, SNP-mediated vasodilation was attenuated in PNAs from long-term obese ZDF rats relative to lean controls (Fig. 2).

PNA vasoconstriction

Vasoconstrictor responsiveness to KCl was greater in prediabetic animals relative to lean ZDF rats (Supplementary Figure 1, see section on supplementary data given at the end of this article). However, there were no differences in vasoconstrictor responses to KCl in PNAs from animals with short-term or long-term diabetes.

NE-induced vasoconstriction of the PNA was not different in animals with prediabetes or short-term diabetes relative to age-matched lean controls (Supplementary Figure 2, see section on supplementary data given at the end of this article). However, NE-induced vasoconstriction was greater in animals with long-term diabetes at concentrations between 10^{-7} and 10^{-6} M (Fig. 3A). When the PNAs were denuded of the endothelium, differences in NE-mediated vasoconstriction between obese and lean ZDF rats were abolished (Fig. 3B).

Active myogenic and passive pressure–diameter responses were not different between animals with prediabetes or short-term diabetes and their lean counterparts (Supplementary Figure 3, see section on supplementary data given at the end of this article). However, active myogenic vasoconstriction was greater in animals with long-term diabetes relative to lean control rats (Fig. 4A), and the passive pressure–diameter responses were lower in long-term diabetic rats compared with lean controls (Fig. 4B).

Femoral BMD

Total and cortical BMD were the same in prediabetic rats versus lean controls, whereas cancellous BMD of the distal femur was greater for the prediabetic condition (Table 1). For animals with short-term diabetes, total and cancellous BMD were not different between obese and lean ZDF rats, while cortical BMD was lower in obese ZDF rats (Table 1). Rats in both of these age groups still exhibit skeletal growth, therefore the effects of factors present in the prediabetic and short-term diabetic states may occur as a result of skeletal growth. In long-term obese ZDF rats, where the animals are more skeletally mature, total, cancellous, and cortical BMD were lower than that in lean ZDF animals (Table 1).

Relationship between endothelium-dependent vasodilation and femoral BMD

Cancellous BMD of the femur was positively associated with peak endothelium-dependent vasodilation of the PNA during prediabetes, short-term diabetes, and long-term diabetes (Fig. 5A, B and C). In addition, endothelium-dependent vasodilation was positively correlated with total BMD (Fig. 6A) and cortical BMD (Fig. 6B) of the femur for animals with long-term diabetes.

Blood flow, arterial pressure, and vascular conductance

Blood flow was lower in all regions of the femur (Fig. 7A) and in all other skeletal regions of the hindlimb (Supplementary Figure 4, see section on supplementary data given at the end of this article) in 20-week-old ZDF rats with long-term diabetes. Mean arterial pressure was not different between ZDF rats with long-term diabetes (160 ± 6 mmHg) and lean controls (160 ± 4 mmHg). Consequently, vascular conductance was lower in the femur (Fig. 7B) and all regions of the hindlimb skeleton of 20-week-old obese ZDF rats with long-term diabetes (Supplementary Tables 1 and 2, see section on supplementary data given at the end of this article).

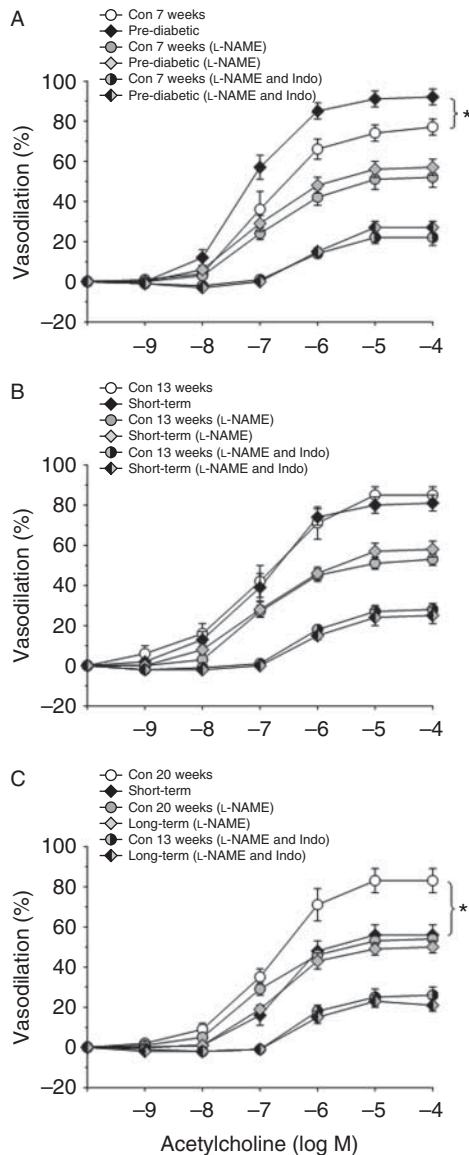


Figure 1
Effects of prediabetes (A), short-term diabetes (B), and long-term diabetes (C) on endothelium-dependent vasodilation of the femoral principal nutrient artery alone, in the presence of the nitric oxide synthase inhibitor L-NAME, and in the presence of L-NAME and the cyclooxygenase inhibitor indomethacin (Indo). Values are means \pm s.e.m., $n = 14$ – 15 /group. *Mean is different from that for age-matched nondiabetic control animals ($P < 0.05$).

Discussion

Previous work has shown a deterioration of both bone health and general cardiovascular function during the progression of T2DM (Grundy *et al.* 1999, Prisby *et al.* 2008, Leslie *et al.* 2012). However, little is known regarding the effects of T2DM on the bone circulation. Thus, the primary purpose of this study was to determine whether

the progression of T2DM through a prediabetic, short-term diabetic, and more chronic diabetic state alters mechanisms of vascular control in bone resistance arteries. The results indicate few adverse effects of the prediabetic and short-term diabetic conditions on the vasodilator or vasoconstrictor properties of the femoral PNA. However, with long-term diabetes the PNA shifts toward a more pro-vasoconstrictor phenotype. This is evidenced by a decrement in endothelium-dependent (Fig. 1C) and -independent (Fig. 2) vasodilation, an enhanced noradrenergic (Fig. 3A) and myogenic (Fig. 4A) vasoconstrictor responsiveness, as well as a decrease in the passive mechanical distensibility (Fig. 4B) of the PNA. Consequently, a secondary purpose of this study was to determine whether this shift toward a more pro-vasoconstrictor phenotype in the bone resistance vasculature diminishes bone and marrow perfusion and vascular conductance *in vivo*. The results indicate an impairment of bone and marrow blood flow (Fig. 7A) and vascular conductance (Fig. 7B) with long-term T2DM. Given the potential coupling of bone vascular signaling and blood flow with skeletal remodeling (Parfitt 2000, Farhat & Cauley 2008, Lampropoulos *et al.* 2012, Prisby *et al.* 2012), the present results indicate that the profound impairment of the bone circulation could contribute to the osteopenia found to occur in long bones with chronic T2DM.

T2DM is a physiologically devastating disease whose deleterious effects on bone and the cardiovascular system are multifaceted. For example, the hyperglycemia and hyperlipidemia associated with T2DM can stimulate mitochondrial free radical production and alter redox balance to promote endothelial dysfunction in the

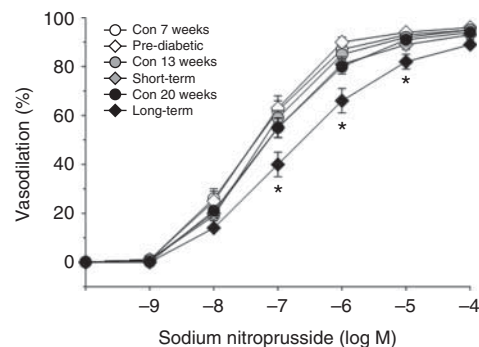


Figure 2
Effects of prediabetes, short-term diabetes, and long-term diabetes on sodium nitroprusside-induced vasodilation of the femoral principal nutrient artery (PNA). Values are means \pm s.e.m., $n = 8$ – 10 /group. *Mean PNA response of 20-week-old obese ZDF rats is different from that of 20-week-old lean ZDF rats ($P < 0.05$).

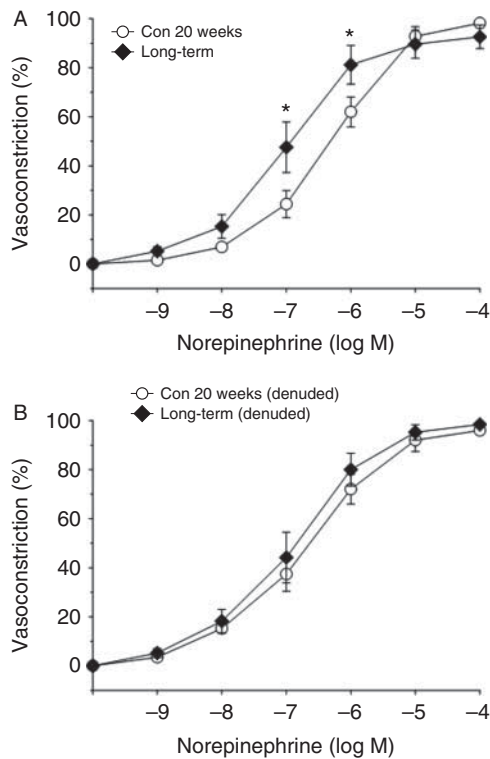


Figure 3 Effects of long-term diabetes on norepinephrine-induced vasoconstriction of the femoral principal nutrient artery (PNA) with the endothelium intact (A) and with the endothelium removed (B). Values are means \pm s.e.m., $n=10-14$ /group. *Mean PNA response of 20-week-old obese ZDF rats is different from that of 20-week-old lean ZDF rats ($P<0.05$).

vasculature (Schalkwijk & Stehouwer 2005, van den Oever *et al.* 2010). Impaired endothelium-dependent vasodilation of conduit arteries is present in adolescents (Naylor *et al.* 2011) and adults (Hogikyan *et al.* 1998, Makimattila *et al.* 1999, Bruno *et al.* 2012, Kotb *et al.* 2012) with T2DM, and has been shown to be associated with elevated oxidative stress and the uncoupling of endothelial NOS (Pannirselvam *et al.* 2002), resulting in diminished bioavailability of NO to induce vascular smooth muscle cell relaxation. Although T2DM-induced decreases in conduit artery endothelium-dependent vasodilation (Makimattila *et al.* 1999, Rossi *et al.* 2005) do not necessarily reflect changes in the microvasculature (Meyer *et al.* 2008), results from this study also demonstrate endothelial dysfunction in the bone microcirculation in T2DM (Fig. 1C). Furthermore, the results indicate that this is due to an impairment of endothelial NO signaling, as indicated by the elimination of differences in endothelium-dependent vasodilation between PNAs from obese ZDF rats and age-matched lean controls when NOS inhibition is present.

The enhanced adrenergic vasoconstriction of the femur resistance vasculature detected in the present study (Fig. 3A) coincides with observations of greater adrenergic vasoconstriction in the forearms of humans with T2DM (Hogikyan *et al.* 1999). Such changes in vasoconstrictor responsiveness could be mediated through the α -adrenergic receptor signaling pathway in smooth muscle cells that elicit vasoconstriction, or the modulatory influence of α -adrenergic receptor signaling in endothelial cells that promote vasodilation. Results for bone resistance arteries indicate the latter effect, because removal of the endothelium abolished differences in PNA responses to NE (Fig. 3B). Results of previous research with skeletal muscle resistance arteries have indicated a similar endothelium-dependent effect on NE-mediated vasoconstriction with long-term diabetes (Lesniewski *et al.* 2008). Together, the results of endothelium-dependent vasodilation and NE-mediated vasoconstriction are indicative of a critical role for the

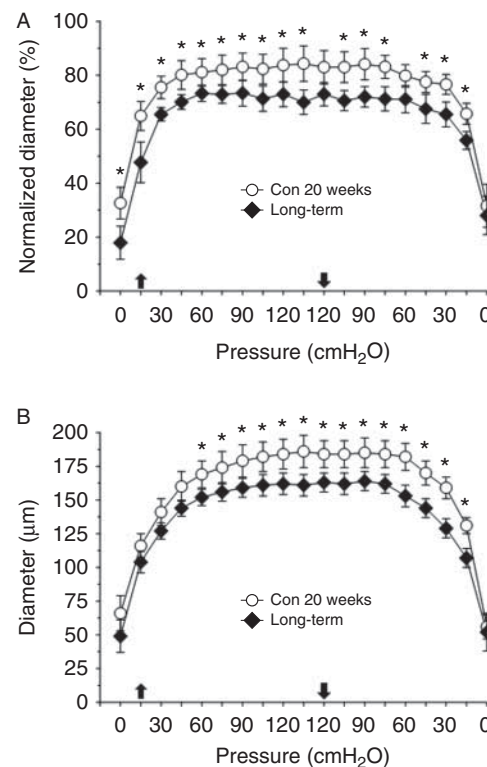
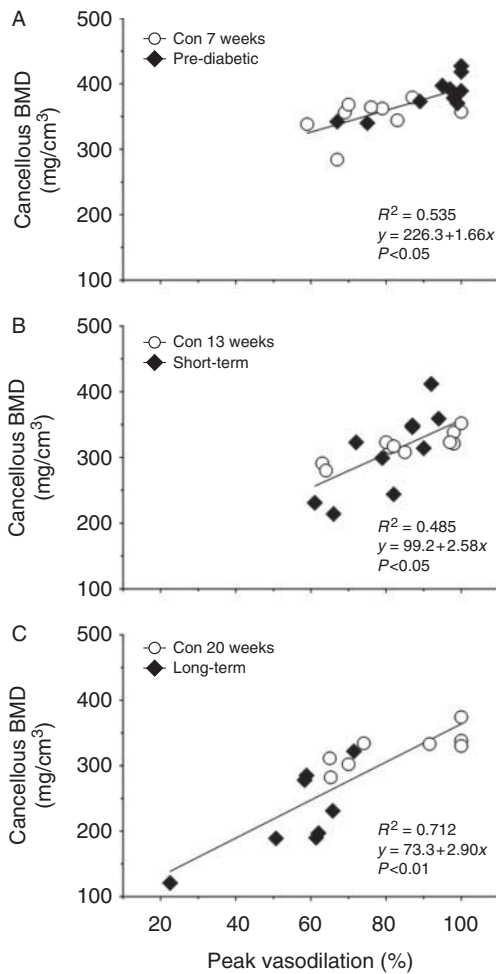


Figure 4 Effects of long-term diabetes on active myogenic vasoconstriction (A) and the passive pressure-diameter relation (B) of the femoral principal nutrient artery. Upwards-pointing arrows indicate where pressure began to be increased; downward-pointing arrows indicate where pressure began to be lowered. Values are means \pm s.e.m., $n=13-14$ /group. *Mean PNA response of 20-week-old obese ZDF rats is different from that of 20-week-old lean ZDF rats ($P<0.05$).

**Figure 5**

Scattergrams showing the relationship between cancellous bone mineral density (BMD) in the distal femur and peak endothelium-dependent vasodilation of the femoral PNA of lean and obese ZDF rats during prediabetes (A), short-term diabetes (B), and long-term diabetes (C). A significant linear relationship ($P < 0.05$) exists between cancellous BMD and peak PNA vasodilation for each condition.

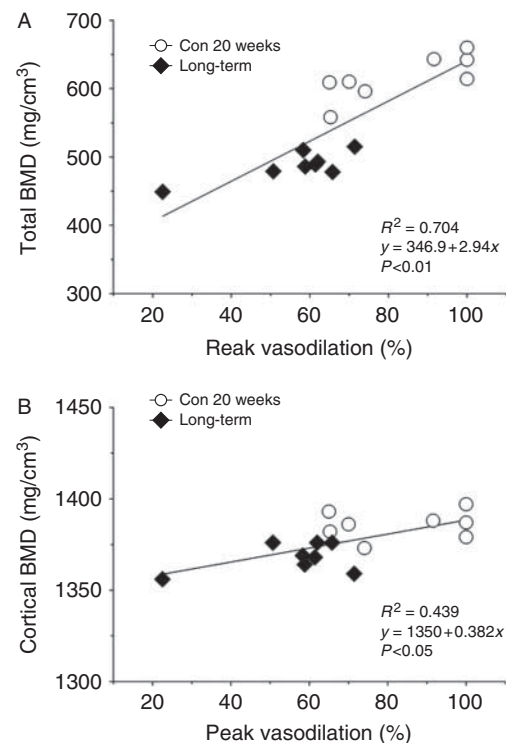
endothelium in the transition of the bone resistance arteries to a more pro-vasoconstrictor phenotype with T2DM.

However, alterations in the bone resistance vasculature with T2DM are not limited to changes in endothelial cell signaling. Endothelium-independent vasodilation to SNP is impaired (Fig. 2), active myogenic vasoconstriction is enhanced (Fig. 4A), and the passive distensibility of the PNA is decreased with long-term diabetes (Fig. 4B). Each of these changes would also contribute to greater vasoconstrictor responsiveness, which correspond to reductions in vascular conductance *in vivo* (Fig. 7B).

The decrements in SNP-mediated vasodilation (Fig. 2) of the PNA indicates stiffer bone arteries are associated with

long-term diabetes. One potential mechanism for increased stiffness is an increase in vascular calcification. Results of previous work have indicated that there is an increased risk of arterial calcification in T2DM (Kreines *et al.* 1985), and that vascular calcification in diabetes is associated with decreased muscle blood flow (Christensen 1968). Results from several studies also indicate positive associations between vascular calcification and osteoporosis or low BMD (Hak *et al.* 2000, Reddy *et al.* 2008, Adragao *et al.* 2009, Choi *et al.* 2009, Hyder *et al.* 2009, Bandeira *et al.* 2012). In particular, iliac artery vascular calcification is positively associated with lumbar spine and femoral neck osteoporosis in men with T2DM (Bandeira *et al.* 2012).

The association between vascular alterations and bone loss are probably affected by other factors beyond changes in arterial calcification. Evidence indicates an active role of the vasculature in coupling vascular signaling and bone remodeling (Colleran *et al.* 2000, Dominguez *et al.* 2010, Prisby *et al.* 2012). For instance, the basic multicellular unit (BMU) that drives bone remodeling activity may be

**Figure 6**

Scattergrams showing the relationship between total bone mineral density (BMD) of the distal femur (A) and cortical BMD of the femoral mid-shaft (B) with peak endothelium-dependent vasodilation of the femoral PNA for lean and obese ZDF rats with long-term diabetes. A significant linear relationship ($P < 0.05$) exists between total and cortical BMD and peak PNA vasodilation.

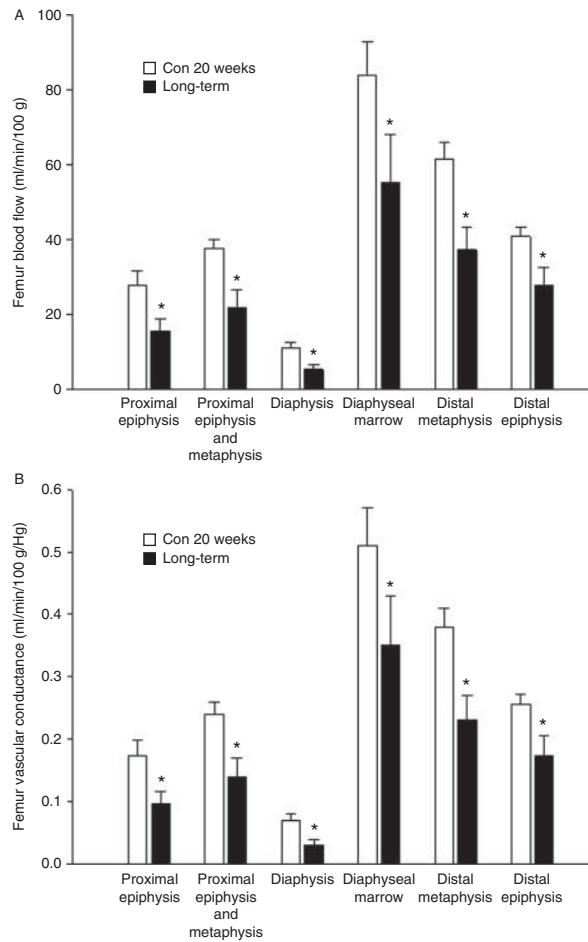


Figure 7
Effects of long-term diabetes on regional femoral blood flow (A) and vascular conductance (B). Values are means \pm S.E.M., $n = 12$ /group. *Mean is different from that for age-matched, nondiabetic control animals ($P < 0.05$).

regulated via signals from core capillary endothelial cells to constituent osteoblasts and osteoclasts as the BMU advances through bone (Parfitt 2000). The present results indicate that femur BMD is positively associated with endothelium-dependent vasodilation of the femoral PNA in the prediabetic (Fig. 5A), short-term diabetic (Fig. 5B), and long-term diabetic (Figs 5A and 6) states. The association between endothelium-dependent vasodilation of the femoral PNA and bone volume has been previously demonstrated among young and old sedentary and exercise-trained rats (Dominguez *et al.* 2010), as well as among young and old ovariectomized and estrogen-replaced animals (Prisby *et al.* 2012). Important endothelium-derived signaling molecules involved in local vascular control, including NO, prostacyclin (PGI₂), and prostaglandin E₂ (PGE₂), have also been shown to be important in bone biology (Pead & Lanyon 1989, MacIntyre *et al.* 1991). For example, NO is a

potent inhibitor of osteoclast-driven bone resorption (MacIntyre *et al.* 1991) and has a positive effect on osteoblastic differentiation (Hikiji *et al.* 1997), while PGI₂ has been shown to be a powerful inhibitor of osteoclastic bone resorption (Chambers & Ali 1983) and PGE₂ has been reported to increase osteoblast and decrease osteoclast numbers (Jee *et al.* 1987). Results from this study indicate that NO signaling from the vasculature is the more important molecule in the apparent coupling of endothelium-dependent vasodilation to BMD, given that differences in endothelium-dependent vasodilation in PNAs from obese and lean ZDF rats were abolished by NOS inhibition alone (Fig. 1A and C).

An additional factor affecting the relationship between the vasculature and bone health is bone perfusion. Bone blood flow facilitates the generation of differences in hydrostatic pressure between the medullary cavity and bone capillary efferents that drive centrifugal interstitial fluid flow (Montgomery *et al.* 1988). The resultant transcortical fluid flow stimulates osteocytes and osteoblasts (Knothe Tate 2003) and subsequent bone formation (Turner 1999, Riddle & Donahue 2009). Thus, the decreases in bone and marrow blood flow found in this study (Fig. 7A) could also compromise important flow and pressure stimuli that affect bone formation.

The selection of an animal model of T2DM is important because no single model will permit the investigation of all disease-related questions (Peterson *et al.* 1990). For example, the Goto-Kakizaki rat is a valuable model for investigating the effects of T2DM in the absence of a corresponding increase in adiposity (Akash *et al.* 2013). Therein, the model fails to replicate the typical clinical T2DM phenotype that includes obesity. The Zucker obese rat is a valuable model of the metabolic syndrome (Kurtz *et al.* 1989) with impaired glucose tolerance and insulin resistance, but it fails to demonstrate hyperglycemia (Peterson *et al.* 1990). The ZDF rat used in this study was originally derived from the Zucker obese rat strain via selection and inbreeding of animals that spontaneously demonstrated unusually high blood glucose levels (Peterson *et al.* 1990). The ZDF rat displays a well-defined T2DM disease progression (Kawaguchi *et al.* 1999, Etgen & Oldham 2000) with deleterious changes in obesity and blood lipid profiles (Leonard *et al.* 2005) that correspond with human T2DM. As glucose and insulin levels have important effects on bone health (Inzerillo & Epstein 2004) and resistance artery function (Lesniewski *et al.* 2008), this study was designed to leverage known changes in ZDF rat T2DM disease progression to investigate their effects on potential bone-vascular interactions.

In conclusion, the results of this study indicate that few vasomotor alterations occur in the bone resistance vasculature in the prediabetic and short-term diabetic states. However, the femoral PNA shifts toward a more pro-vasoconstrictor phenotype with long-term diabetes, as indicated by decreases in endothelium-dependent (Fig. 1C) and -independent (Fig. 2) vasodilation, enhanced noradrenergic (Fig. 3A) and myogenic (Fig. 4A) vasoconstrictor responsiveness, and a decrease in the passive mechanical distensibility (Fig. 4B). The enhanced vasoconstrictor responsiveness of the bone resistance vasculature was evident *in vivo* from decreases in bone and marrow blood flow (Fig. 7A) and vascular conductance (Fig. 7B) with long-term T2DM. Given the potential coupling of bone vascular signaling molecules (Figs 5 and 6) and bone fluid dynamics with skeletal remodeling (Parfitt 2000, Farhat & Cauley 2008, Lampropoulos *et al.* 2012, Prisby *et al.* 2012), the present results indicate that the impairment of the bone circulation could contribute to the bone loss found to occur during the progression of T2DM.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-14-0514>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

The authors have made the following declarations about their contributions: J N S, R D P, and M D D designed the study. J N S, R D P, and B J B conducted the study. J N S, R D P, and B J B were involved in data collection. J N S, R D P, and M D D carried out data analysis. J N S, R D P, B J B, and M D D were involved in data interpretation. J N S and M D D helped in drafting the manuscript. J N S, R D P, B J B, and M D D revised manuscript content. J N S, R D P, B J B, and M D D helped in approving the final version of the manuscript. M D D takes responsibility for the integrity of the data analysis.

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