Adverse Vascular Remodelling is More Sensitive than Endothelial Dysfunction to Hyperglycaemia in Diabetic Rat Mesenteric Arteries

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Abstract

Increased vascular stiffness and reduced endothelial nitric oxide ($NO^{\bullet}$) bioavailability are characteristic of diabetes. Whether these are evident at a more moderate levels of hyperglycaemia has not been investigated. The objectives of this study were to examine the association between the level of glycaemia and resistance vasculature phenotype, incorporating both arterial stiffness and endothelial function. Diabetes was induced in male Sprague Dawley rats with streptozotocin (STZ; 55mg/kg i.v.) and followed for 8 weeks. One week post STZ, diabetic rats were allocated to either moderate (~20 mM blood glucose, 6-7 U/insulin s.c. daily) or severe hyperglycaemia (~30 mM blood glucose, 1-2 U/insulin s.c. daily as required). At study end, rats were anesthetized, and the mesenteric arcade was collected. Passive mechanical wall properties were assessed by pressure myography. Responses to the endothelium-dependent vasodilator acetylcholine (ACh) were assessed using wire myography. Our results demonstrated for the first time that mesenteric arteries from both moderate and severely hyperglycaemic diabetic rats exhibited outward hypertrophic remodelling and increased axial stiffness compared to arteries from non-diabetic rats. Secondly, mesenteric arteries from severely (~30 mM blood glucose), but not moderately hyperglycaemic (~20 mM blood glucose) rats exhibit a significant reduction to ACh sensitivity compared to their non-diabetic counterparts. This endothelial dysfunction was associated with significant reduction in endothelium-derived hyperpolarisation and endothelium-dependent $NO^{\bullet}$-mediated relaxation. Interestingly, endothelium-derived nitroxyl ($HNO^{\bullet}$)-mediated relaxation was intact. Therefore, moderate hyperglycaemia is sufficient to induce adverse

structural changes in the mesenteric vasculature, but more severe hyperglycaemia is essential to cause endothelial dysfunction.

Key words (5) – endothelial function, arterial wall stiffness, diabetes, differential hyperglycaemia, mesenteric artery

Non-standard abbreviations and acronyms

AGE, advanced-glycation endproducts; BH_4, tetrahydrobiopterin; BK_{Ca}, large conductance calcium-activated potassium channel; COX, cyclooxygenase; EDH, endothelium-derived hyperpolarisation; eNOS, endothelial nitric oxide synthase; GHB, glycated haemoglobin; HNO, nitroxyl; HXC, hydroxocobalamin; IK_{Ca}, intermediate conductance calcium-activated potassium channel; K_V, voltage-gated potassium channel; KcaB, a cocktail of TRAM-34, apamin and iberiotoxin; KPSS, potassium physiological saline solution; L-Cys, L-cysteine; MHG, moderate hyperglycaemia; NG, normal glycaemia; $NO^{\bullet}$, nitric oxide; NOS, nitric oxide synthase; Nox2, NADPH oxidase 2; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one; pEC_{50}, negative log of half-maximal effective concentration; PGI_2, prostacyclin; R_{max}, maximum relaxation; SHG, severe hyperglycaemia; sGC, soluble guanylate cyclase; SK_{Ca}, small conductance calcium-activated potassium channel; SNP, sodium nitroprusside; STZ, streptozotocin; WT, wall thickness

1. **Introduction**

Cardiovascular complications (both micro and macrovascular) are the leading cause of diabetes-related morbidity and mortality (1). These vascular complications, including arterial stiffness (caused by adverse vascular remodelling) and endothelial dysfunction are regarded as critical initiating factors in the development of diabetic vascular complications (2, 3), and may represent surrogate markers of prognosis.

Streptozotocin (STZ)-induced diabetes is a well-established animal model of experimental diabetes (4, 5). STZ causes pancreatic beta cell death due to DNA alkylation (6, 7), resulting in marked hyperglycaemia in rodents (4). Hyperglycaemia-induced oxidative stress causes arterial stiffness and endothelial dysfunction (8). Although these vascular complications are well characterised in STZ-induced hyperglycaemic rats, the majority of studies only address severely hyperglycaemic rats (blood glucose $\geq$30 mM) (9-12); the potential effects of more moderate hyperglycaemia on the vasculature are largely not considered. Therefore, understanding of the relationship between the level of glycaemia and vascular structure and function is incomplete.

Arterial wall stiffness is a common hallmark of diabetes and can increase the risk of further cardiovascular events such as myocardial infarction or stroke (13). This increased stiffness results from adverse arterial remodelling, secondary to changes in the composition and structure of the arterial wall and resultant altered passive mechanical wall properties (14). Diabetes increases the formation of advanced-glycation endproducts (AGEs) (15-17) which, by forming cross-links

with the extracellular matrix protein collagen, renders the artery wall more rigid and less able to stretch (18). Additionally, increased vascular collagen content (19) further exacerbates vascular stiffness (20, 21) .

The key role of the endothelium in regulating normal vascular tone is well-known; the release of the vasoactive factors such as prostacyclin (PGI_2), nitric oxide ($NO^{\bullet}$) and endothelium-derived hyperpolarisation (EDH) all contribute to vascular regulation. Reduced production of these endothelial-derived relaxing factors leads to endothelial dysfunction (2, 22). Emerging evidence suggests that the one-electron-reduced and protonated form of $NO^{\bullet}$, nitroxyl (HNO), is likely produced endogenously in the vasculature (23-25) and contributes to endothelium-dependent relaxation (26, 27). HNO stimulates soluble guanylate cyclase (sGC) in a similar manner to $NO^{\bullet}$ to elicit vasodilation (26, 28, 29). In addition, it has been demonstrated that HNO activates K_V channels in vascular smooth muscle to stimulate hyperpolarisation and subsequent vasorelaxation (26, 29).

In diabetic mesenteric arteries, it is well-established that EDH-type relaxation is impaired (9-12). The residual component of relaxation, after the inhibition of both cyclooxygenase (COX) and calcium-activated potassium channels (K_{Ca}), is likely to be attributed to endothelial nitric oxide synthase (eNOS)-derived $NO^{\bullet}$, which is also reported to be impaired by diabetes (9, 10, 12). Although both $NO^{\bullet}$ and HNO can be produced by eNOS, previous studies have failed to identify which nitrogen oxide is impacted by diabetes (9, 10, 12). Thus it remains unclear whether $NO^{\bullet}$-mediated, HNO-mediated relaxation, or both, are impaired in diabetes-induced endothelial dysfunction in resistance vessels.

The aims of this study were to characterise the differential changes in arterial wall stiffness and endothelial function in the mesenteric artery induced by moderate versus severe hyperglycaemia in a rat model of experimental diabetes. Furthermore, as HNO offers greater resistance than $NO^{\bullet}$ to the detrimental consequences of oxidative stress (30, 31), which is evident in diabetes (30, 32, 33). Thus, we also sought to investigate if endothelium-derived HNO-mediated relaxation remains intact under hyperglycaemic conditions.

2. Methods

2.1 Animals

This investigation complied with the National Health and Medical Research Council (NHMRC) of Australia code of practice for the care and use of animals for scientific purposes. All procedures involved in this project were approved by the Alfred Medical Research Educational Precinct (AMREP) Animal Ethics Committee (approved E/1519/2014/B). Type 1 diabetes was induced in rats as previously described (34). Briefly, adult male outbred Sprague Dawley rats (n= 50) obtained from AMREP animal services (bodyweight ~230g; approximately 6 weeks of age) were randomly assigned to receive STZ to induce diabetes (55mg/kg i.v., n=35) or citrate buffer (normal glucose (NG), n=15) via the tail vein following an overnight fast. One week following STZ, diabetic rats were further assigned to two groups: severe hyperglycaemia (SHG, n=16) and moderate hyperglycaemia (MHG, n=19) using a single daily insulin (Eli Lilly) injection (long-lasting Humulin NPH) to titrate blood glucose levels to >28 mM (1-2 units, s.c. per day) and ~20 mM (6-7 units, s.c. per day) as required, respectively. Blood glucose and body weight were monitored weekly. Following 8 weeks of diabetes or non-diabetic control, rats were euthanised with an intraperitoneal injection of ketamine-xylazine (100 and 12 mg/kg, respectively) and the mesenteric arcade was collected. Blood glucose and glycated haemoglobin (GHB) levels were measured using a one touch glucometer (Roche, Sydney, NSW, Australia) and Cobas HbA1c analyser (Roche, Sydney, NSW, Australia), respectively.

2.2 Passive Mechanical Wall Properties ex vivo

Following euthanasia, a section of the mesenteric arcade was immediately placed

in an ice-cold Ca^{2+}-free physiological saline solution (14.9 mM NaCl, 4.7 mM KCl, 1.7 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.7 mM $MgSO_4$, 5 mM glucose, 10 mM HEPES and 2 mM EGTA) and cleared of fat and connective tissue. Third order mesenteric arteries were cleared of fat and connective tissue and leak-free segments were mounted on the cannula of a pressure myograph (Living Systems Instrumentation, Burlington, VT, USA). The lumen was gently flushed to remove any remaining blood, and the distal end occluded. Arteries were incubated at 37°C for 20 minutes before measures of vessel wall parameters (vessel length, outer diameter [OD] and wall thickness [WT] were obtained, in 10 mmHg increments from 5mmHg to 120mmHg). Inner diameter (ID), wall stress and wall strain were calculated as described previously (35). Volume distensibility was calculated as Δ volume/([Δ cross-sectional area × length]× Δ Pressure), where cross-sectional area was calculated as $(\pi \times ID^2)/4$) (35, 36). The % change in length with pressure was calculated using the following equation: %length = [(value at pressure)/(value at baseline)] × 100 (37).

2.3 Vascular Reactivity ex vivo

The remaining mesenteric arcade was immediately placed in an ice-cold Kreb's bicarbonate solution containing (in mmol/l) NaCl 120, KCl 5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11.1, $CaCl_2$ 2.5, EDTA 0.0026. Indomethacin (10 μmol/l), a non-selective cyclooxygenase (COX) inhibitor to inhibit the synthesis of prostanoids, was present in the Kreb's solution at all times. Third order mesenteric arteries were cleared of connective fat and tissue, cut into 2mm rings and mounted on a Mulvany-Halpern wire myograph (model 610M; Danish Myo Technology, Aarhus, Denmark). Arteries were allowed to stabilise at zero tension

before normalisation as described previously (38). All experiments were performed at 37°C and the baths were continuously bubbled with 95% O_2 and 5% CO_2.

Thirty minutes after normalisation, arteries were maximally contracted using K^+ physiological saline solution (KPSS, 100 mmol/l) and the integrity of the endothelium was determined as described previously (39). After further washout, arteries were again precontracted to similar levels (70-80% of KPSS response) using either the combination of phenylephrine (0.1-3 μmol/l) or U46619 (0.1-1 μmol/l) where indicated. In some cases, arteries were precontracted using depolarising solution (30 mmol/l K^+) to globally block all K^+ channels. The impact of MHG and SHG on mesenteric artery function was determined via the construction of cumulative concentration-response curves to the endothelium-dependent vasodilator, acetylcholine (ACh; 0.1 nmol/l-10 μmol/l), NO donor, Diethylamine nonoate (DEANO; 0.1 nmol/l-10 μmol/l), HNO donor, isopropylamine-NONOate (IPANO; 0.1 nmol/l-10 μmol/l) and the endothelium-independent vasodilator, sodium nitroprusside (SNP; 0.1 nmol/l-10 μmol/l). Responses to ACh were also assessed following 20 minute incubations with nitric oxide synthase inhibitor, N^{ω}-Nitro-L-arginine methyl ester (L-NAME; 200 μmol/l) either alone or in combination with, L-cysteine, a selective HNO scavenger (3 mmol/l, added for the final 3 minutes), hydroxocobalamin, a selective $NO^{\bullet}$ scavenger (HXC; 100 μmol/l), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one, a soluble guanylate cyclase inhibitor (ODQ; 10 μmol/l). A cocktail of inhibitors of calcium-activated potassium channels (K_{Ca}), 1-[(2-chlorophenyl)diphenylmethyl]-(1)H-pyrazole (TRAM34; 1 μmol/l), apamin (1

μmol/l) and iberiotoxin (0.1 μmol/l) was also incubated for 20 minutes either alone or in combination as indicated.

2.4 Assessment of Basal nitrogen oxide, NO• and HNO bioavailability ex vivo

In a separate series of experiments, the impact of glycaemic level on basal levels of eNOS-derived nitrogen oxides, as well as basal bioavailability of NO• and HNO were examined through the addition of L-NAME (200 μmol/l), HXC (100 μmol/l) or L-cysteine (3 mmol/l) in endothelium-intact rings submaximally-contracted with phenylephrine (10-100nmol/l to ~20% of KPSS) (40). Under these conditions, a contractile response to L-NAME was considered to reflect the level of basal eNOS-derived nitrogen oxides, which includes both NO• and HNO. The contractile responses to HXC or L-cysteine were considered to reflect the basal level of NO• or HNO, respectively.

2.5 Drugs

All drugs were purchased from Sigma-Aldrich (St Louis, MO, USA) except for iberiotoxin (Tocris Bioscience, Bristol, UK), U46619 and ODQ (Cayman Chemical, Ann Arbor, MI, USA). All drugs were dissolved in distilled water, with the exception of indomethacin, which was dissolved in 0.1 mol/l sodium bicarbonate, ODQ and TRAM34, which was dissolved in 100% DMSO (final concentration less than 0.1% DMSO) and U46619, which was dissolved in 100% ethanol (final concentration less than 0.1% ethanol) as 1 mmol/l stock solution and subsequent dilutions were in distilled water.

2.6 Statistical Analyses

231 Data are expressed as mean ± SEM and n represents the number of animals per
232 group. Concentration-response curves were computer-fitted to a sigmoidal curve
233 using non-linear regression (Prism 5.0; GraphPad Software, San Diego, CA, USA),
234 and the sensitivity of each agonist (pEC_{50}) was calculated. Maximum relaxation
235 (R_{max}) to vasodilators was measured as a percentage of precontraction. Group pEC_{50},
236 R_{max} and systemic characteristics were compared via one-way ANOVA with post-hoc
237 analysis using Dunnett's test or Tukey's test as appropriate. Stress-strain curves and
238 pressurised wall parameters (WT, ID and OD) were analysed with repeated
239 measures two-way ANOVA (treatment vs. strain) with Bonferroni post hoc
240 analysis. Volume distensibility and arterial lengthening were analysed using a
241 two-way ANOVA with Bonferroni post hoc analysis. $P<0.05$ was considered
242 statistically significant.
243

3. Results

3.1 Systemic Characteristics at Endpoint in vivo

Body weight gain after 8 weeks, was significantly lower in the SHG rats compared to both NG and MHG rats (Table 1). Both %GHB and blood glucose levels were significantly increased in the SHG rats compared to NG and MHG rats. Interestingly, although MHG rats exhibited significantly increased %GHB and blood glucose levels compared to non-diabetic NG rats, this was not associated with retarded body weight gain (Table 1).

Table 1. Systemic characteristics at study endpoint *in vivo:* A comparison of body weight gain, endpoint blood glucose and glycated haemoglobin (GHB) levels from normal glucose (NG), moderate hyperglycaemic (MHG) and severe hyperglycaemic (SHG) rats. Results are shown as mean ± SEM. *P<0.05 versus NG, #P<0.05 versus MHG, one-way ANOVA with Tukey's post-hoc analysis. N/A: below the detection limit of the assay (4% for GHB).

	NG	MHG	SHG
Body Weight gain (g)	138±6	150±6	18±5*#
Blood glucose (mM)	7.0±0.2	21.6±0.9*	30.1±0.8*#
GHB (%)	N/A	4.33±0.08	7.19±0.17#

3.2 Impact of Diabetes on Vascular Remodelling and Axial Stiffness

Mesenteric arteries from diabetic rats, exhibited a significant ($P<0.0001$) increase in inner diameter (ID), outer diameter (OD) and wall thickness at baseline (5 mmHg) regardless of the degree of hyperglycaemia (Figure 1A-1C). This increase in vessel diameter also persisted over the full range of pressures studied, indicating outward hypertrophic remodelling. Interestingly, this was evident even in arteries isolated from the moderate hyperglycaemic rats, with no significant differences in vessel dimensions between MHG and SHG mesenteric arteries. These findings suggest that vascular remodelling is particularly sensitive to impaired glycaemic control (Figure 1). The stress-strain relationship was not significantly different between the three groups (Figure 2A), indicating that increasing blood glucose levels does not impact on circumferential vascular stiffness. There was however, a significant reduction in volume distensibility in mesenteric arteries from hyperglycaemic compared to non-diabetic rats, over the physiological pressurisation range (50-120 mmHg, $P<0.01$) (Figure 2B, C). Interestingly, this was again evident even in arteries isolated from MHG rats, with no further impact evident in SHG mesenteric arteries. To assess whether the effects of diabetes on volume distensibility were associated with changes in axial stiffness, the % change in arterial length over the full range of pressures studied was determined. In both MHG and SHG, mesenteric arteries exhibited a modest but statistically significant reduced ability to lengthen at physiological to high pressure range (80 – 120 mmHg) compared to NG (Figure 2D, $P<0.05$), again with no further differences in the length-pressure relationship with more marked hyperglycaemia (Figure 2). Together these data suggest that passive mechanical wall properties are particularly sensitive to impaired glycaemic control.

290 Specifically, hyperglycaemia promotes axial stiffening to reduce mesenteric artery
291 distensibility.
292
293 *3.3 Pharmacological characterisation of Hydroxocobalamin and L-Cysteine*
294 The $NO^{\bullet}$ donor, DEANO and the HNO donor, IPANO caused concentration-
295 dependent relaxation in mesenteric arteries from NG, MHG and SHG rats
296 (Supplementary Table 1). Regardless of glycaemic status, the $NO^{\bullet}$ scavenger, HXC
297 (100 µmol/l) or HNO scavenger, L-cysteine (3 mmol/l) significantly decreased the
298 sensitivity but not maximum relaxation to DEANO or IPANO respectively,
299 confirming the specificity of these pharmacological scavengers. Therefore, the
300 HXC-sensitive and L-cysteine-sensitive pathway is likely to be attributed to $NO^{\bullet}$
301 and HNO, respectively. Interestingly, the sensitivity to DEANO but not IPANO was
302 significantly decreased in mesenteric arteries from SHG rats in comparison to
303 either NG or MHG rats (Supplementary Table 1).
304 *3.3 Impact of Diabetes on Basal Nitrogen Oxide/$NO^{\bullet}$/HNO Bioavailability*
305 The contraction evoked by KPSS (100mmol/l) in rat mesenteric arteries was not
306 significantly different between the 3 groups (NG, MHG and SHG; Figure 3A). In
307 contrast, L-NAME and HXC-induced arterial contractions were significantly
308 reduced in mesenteric arteries from both MHG and SHG compared to NG rats
309 (Figure 3B-3C, both $P<0.05$). However, L-cysteine-induced contraction, likely
310 attributed to basal levels of HNO, was not significantly different between the 3
311 groups (Figure 3D). Together these findings suggest that the bioavailability of
312 endogenous eNOS-derived nitrogen oxides, namely $NO^{\bullet}$, is particularly sensitive
313 to impaired glycaemic control, whereas basal HNO bioavailability is unaffected by
314 hyperglycaemia.

Supplementary Table 1. Pharmacological characterisation of NO• and HNO scavengers *ex vivo:* A comparison of sensitivity (pEC_{50}) and maximum relaxation (R_{max}) to DEANO and IPANO in the absence or presence of HXC or L-cysteine in endothelium-intact mesenteric arteries isolated from normal glucose (NG), moderate hyperglycaemic (MHG) and severe hyperglycaemic (SHG) rats. n= the number of experiments. Results are shown as mean ± SEM. *P<0.05 versus control, unpaired Student's t-test. #P<0.05 versus NG or MHG, one-way ANOVA with Tukey's post-hoc analysis. ND: Not determined.

	NG			MHG			SHG		
DEANO	**n**	**pEC_{50}**	**R_{max} (%)**	**n**	**pEC_{50}**	**R_{max} (%)**	**n**	**pEC_{50}**	**R_{max} (%)**
Control	6	7.29±0.08	99±1	6	7.32±0.08	100±1	4	6.87±0.06#	99±1
HXC	6	6.46±0.15*	99±1	6	6.58±0.15*	98±1	4	5.59±0.26*	98±1
IPANO									
Control	5	6.58±0.14	96±1	5	6.72±0.12	99±1	4	6.42±0.11	97±1
L-cysteine	5	6.11±0.04*	93±2	5	6.20±0.16*	98±1	4	6.01±0.06*	96±2

326

327

Figure 1

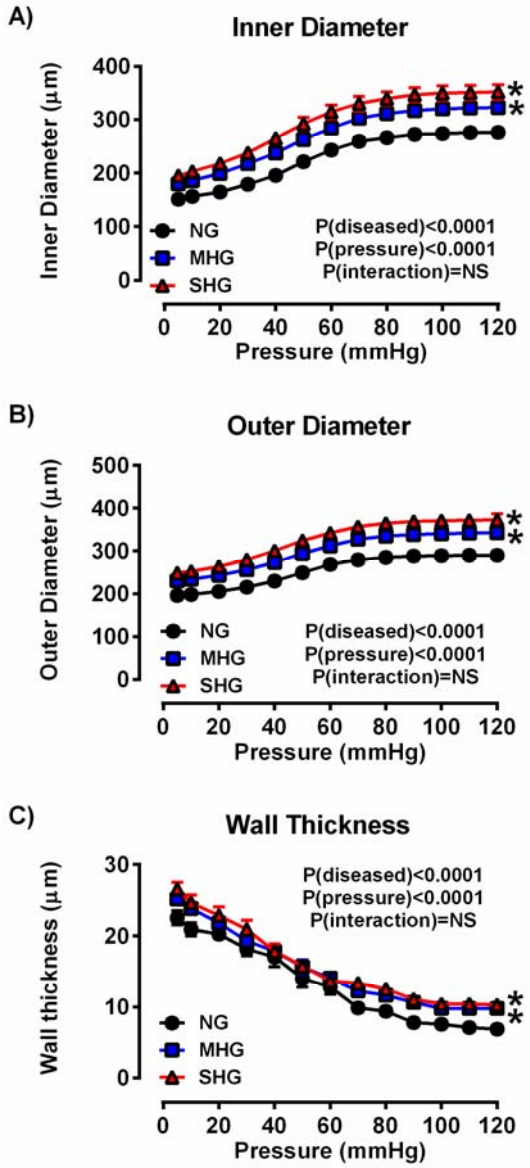


Figure 1. Sensitivity of Passive Mechanical Wall Properties to Hyperglycaemia

Hyperglycaemia significantly increased each of (A) inner diameter (ID); (B) outer diameter (OD) and (C) wall thickness (WT) against intraluminal pressure in the rat mesenteric artery compared to arteries from normoglycaemic (NG) non-diabetic rats. These increases were evident in vessels isolated from both moderate (MHG) and severe (SHG) hyperglycaemic rats. Values are mean ± SEM. n= 7-11 per group *P<0.05 vs NG (2-way repeated measures ANOVA, Bonferroni post hoc analysis).

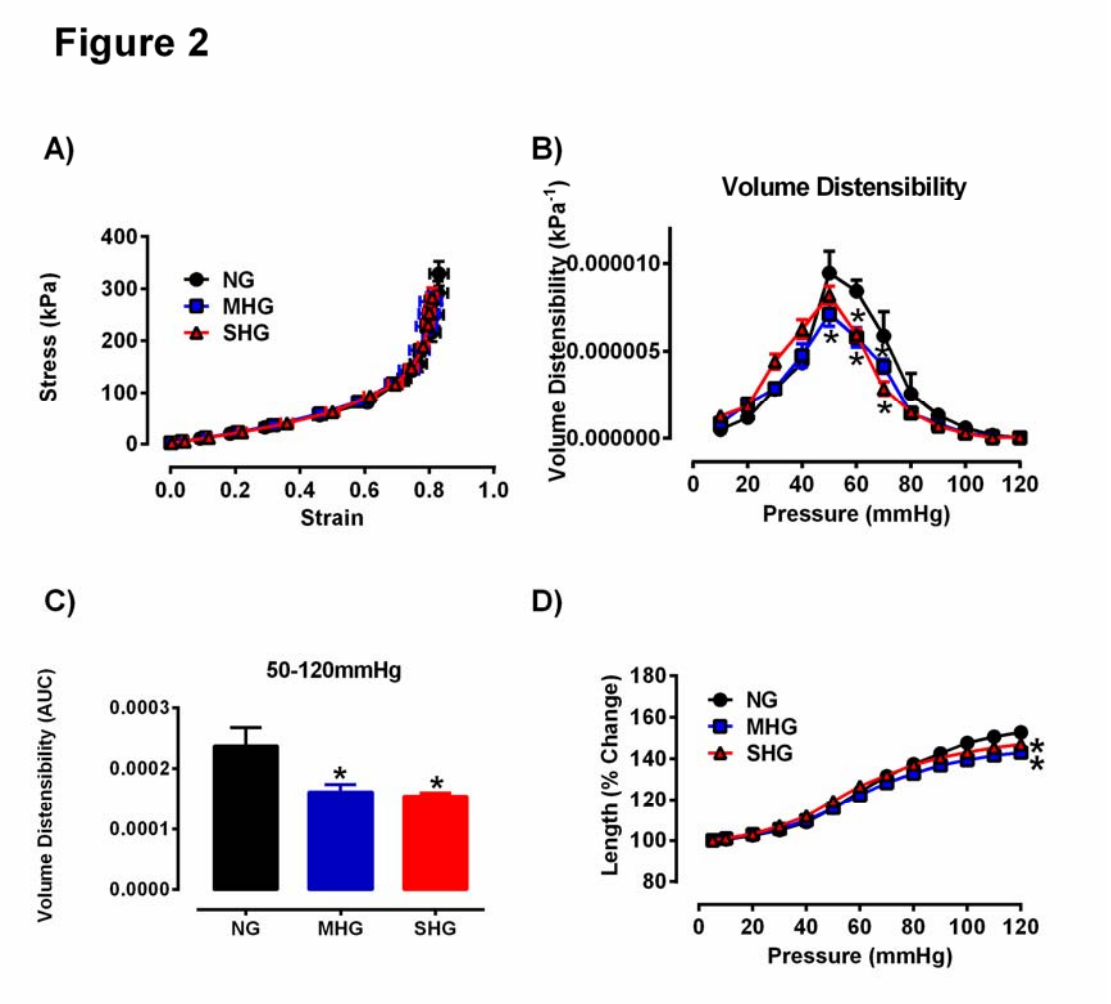


Figure 2. Sensitivity of Vascular Stiffness to Hyperglycaemia

Although chronic hyperglycaemia *in vivo* had no effect on the (A) stress-strain relationship *ex vivo*, hyperglycaemia reduced (B) volume distensibility over the full range of pressures studied (0-120mmHg) and (C) the area-under-curve (AUC) for this pressure-volume relationship over physiological pressure range (50-120mmHg). (D) This was associated with a modest but significant hyperglycaemia-induced reduction in the percentage change in vessel length with increasing pressure in both MHG and SHG mesenteric arteries compared to NG. Values are mean ± SEM. n=7-11 per group *P<0.05 vs NG (2-way repeated measures ANOVA with Bonferroni post hoc analysis).

Figure 3

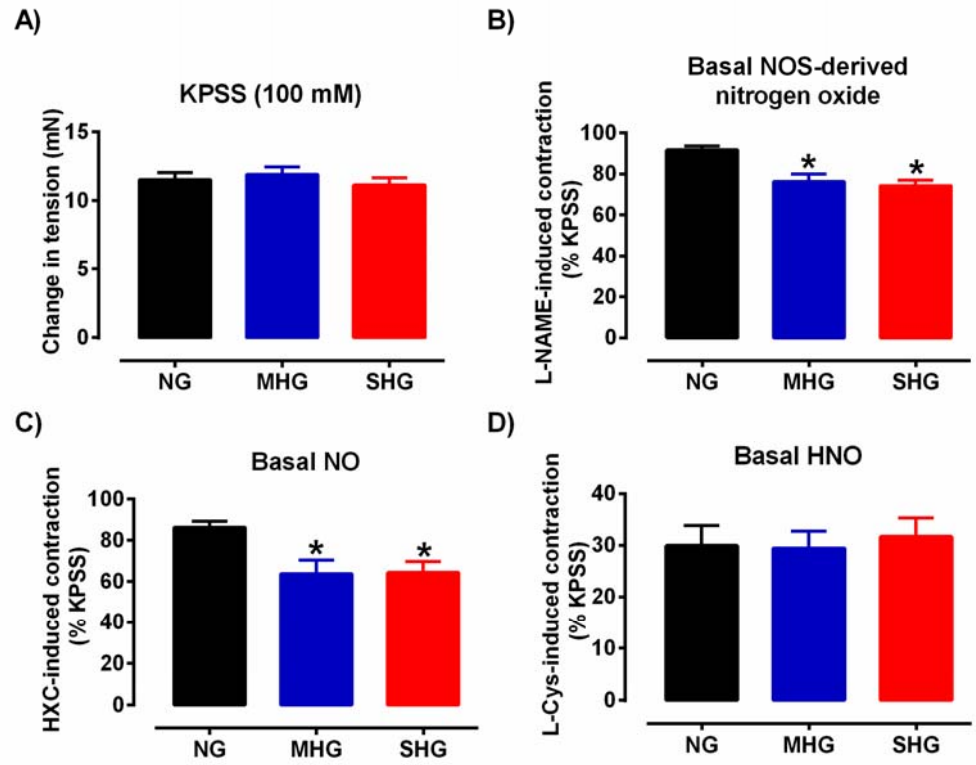


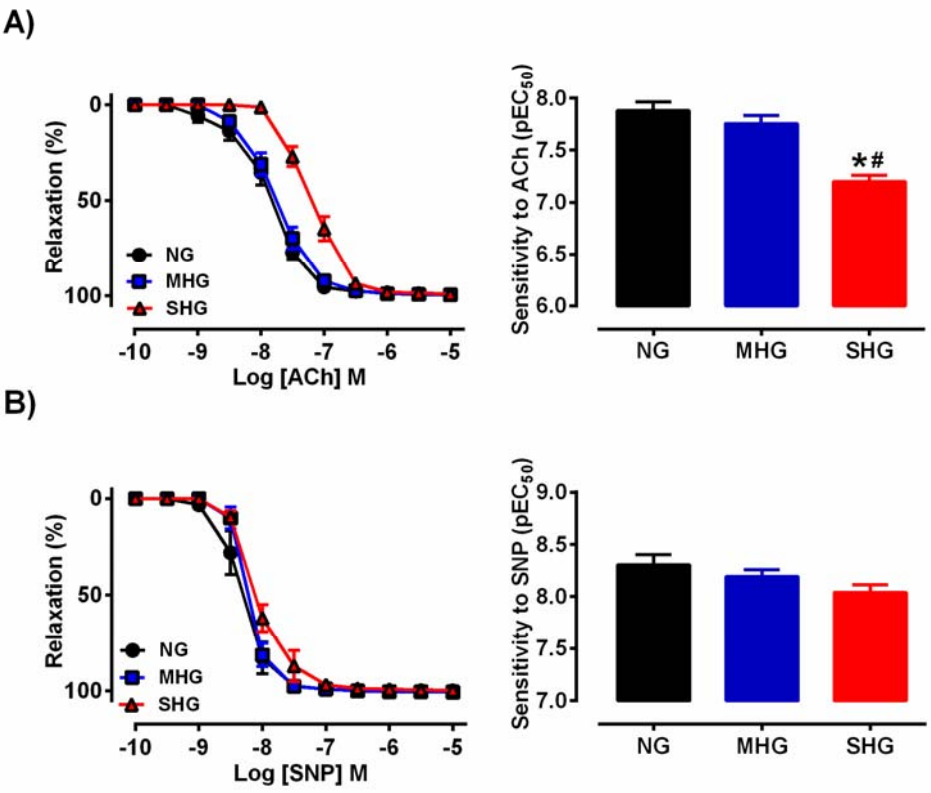
Figure 3. Impact of Hyperglycaemia on the bioavailability of eNOS-derived nitrogen oxides, HNO and NO• in rat mesenteric arteries

Hyperglycaemia did not affect maximum contraction to (A) high-potassium physiological saline solution (KPSS), but was associated with loss in endogenous bioavailability of both (B) eNOS-derived nitrogen oxide, which includes both NO• and HNO and (C) NO•. (D) Basal HNO bioavailability was resistant to hyperglycaemia in rat mesenteric arteries. In each group of experiments, mesenteric arteries were preconstricted with phenylephrine to similar levels, prior to the addition of L-NAME (NOS inhibitor; n = 7-9 per group; B), HXC (NO• scavenger, n = 7-8 per group; C) and L-cysteine (HNO scavenger, n = 6-9 per group; D). Values are mean ± SEM. * P<0.05 vs NG (1-way ANOVA, Tukey's post hoc test).

3.4 Severe Hyperglycaemia is Required to Induce Endothelial Dysfunction

Severe hyperglycaemia significantly reduced the sensitivity, but not the maximum relaxation response, to ACh compared to both NG and MHG (Figure 4A), indicating that SHG is required to cause endothelial dysfunction in the mesenteric artery. However, responses to the endothelium-independent agonist, SNP were not significantly different between the 3 groups (Figure 4B), indicating that vascular smooth muscle function was unaffected by diabetes.

Figure 4



377

378 **Figure 4. Sensitivity of Endothelial Dysfunction to Hyperglycaemia in Rat**

379 **Mesenteric Arteries.**

380 Severe hyperglycaemia, but not moderate hyperglycaemia, decreased the

381 sensitivity (pEC_{50}) to (A) ACh, but had no effect on the sensitivity to (B) SNP,

382 indicating endothelial dysfunction. Indomethacin (10 μM) was always present in

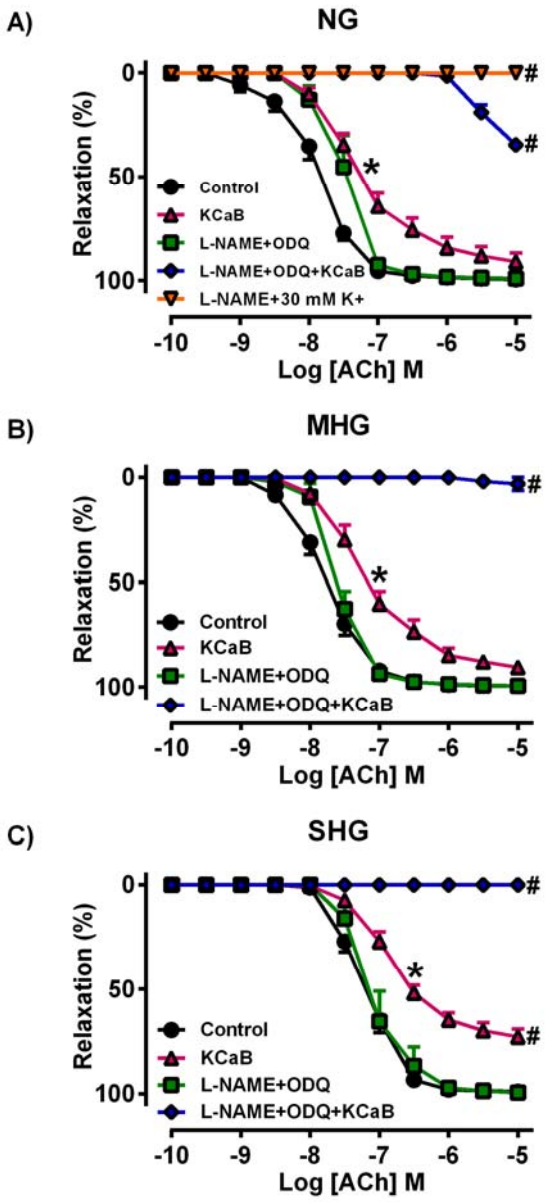
383 the Krebs buffer. Values are mean ± SEM. n= 4-19 per group *P<0.05 vs NG, $^{\#}$

384 P<0.05 vs MHG (1-way ANOVA, Tukey's post-hoc test).

385

386 *3.5 Impact of Hyperglycaemia on the Relative Contribution of EDH to ACh-Evoked*
387 *Relaxation*
388 Vascular reactivity to ACh was further examined in the presence of either the
389 combination of calcium-activated potassium channel blockers (KCaB; iberiotoxin,
390 apamin, and TRAM-34) or the combination of L-NAME and ODQ, to assess the
391 relative contribution of EDH and NOS-derived nitrogen species respectively, to
392 endothelium-dependent relaxation in NG, MHG and SHG rat mesenteric arteries
393 (Figure 5, Table 2). In mesenteric arteries from all groups, ACh-evoked relaxation
394 was significantly inhibited by the presence of KCaB but not by the combination of
395 L-NAME and ODQ, indicating that EDH (rather than NOS-derived nitrogen species)
396 is the predominant endothelium-derived vasodilator (Figure 5A-5C, Table 2). To
397 further examine whether EDH-mediated vascular relaxation was differentially
398 affected by the degree of hyperglycaemia, reactivity to ACh was assessed in the
399 presence of L-NAME + ODQ. We observed that the sensitivity to ACh in the
400 presence of concomitant L-NAME and ODQ, but not R_{max}, was significantly reduced
401 by hyperglycaemia compared to NG but only in SHG mesenteric arteries; MHG
402 arteries studied in the presence of L-NAME + ODQ retained full sensitivity to ACh
403 (Table 2). Furthermore, ACh-induced relaxation was abolished in the presence of
404 the L-NAME+ODQ+KCaB combination, in both MHG and SHG mesenteric arteries
405 (Figure 5B-5C). In contrast, a residual component of relaxation remained in NG
406 arteries (Figure 5A). This residual relaxation response to ACh in the
407 normoglycemic NG arteries was abolished by the combination of L-NAME + 30mM
408 K^+ (Figure 5A).
409

Figure 5



410

411 **Figure 5. Relative Contribution of EDH and NOS-Derived Nitrogen Species to**
412 **Relaxation in Rat Mesenteric Arteries: Impact of Hyperglycaemia**

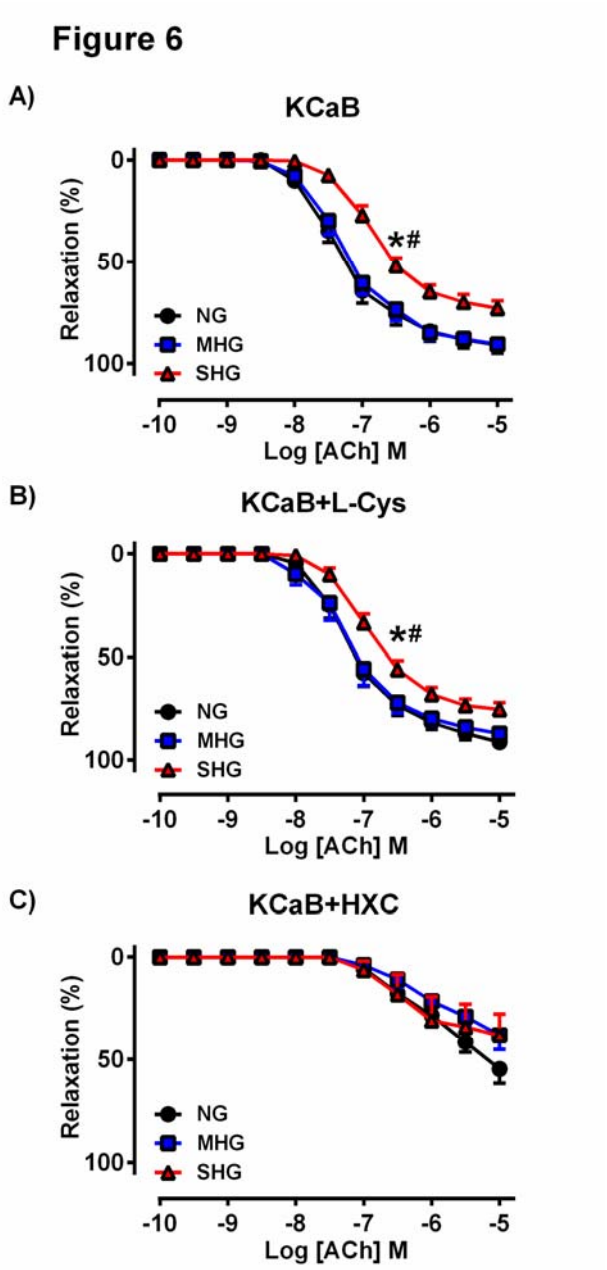
413 Concentration-response curves to ACh in the absence (control) and presence of
414 TRAM-34, apamin and iberiotoxin (KCaB), L-NAME + ODQ, L-NAME, ODQ + KCaB,
415 and L-NAME + 30mM K^+ where any residual response remained. Mesenteric
416 arteries isolated from (A) NG; (B) MHG; and (C) SHG rats. Indomethacin (10 μM)
417 was always present in the Krebs buffer. Values are mean ± SEM. n = 4-12 per group
418 * pEC_{50}, # R_{max} vs control ($P<0.05$, 1-way ANOVA, Dunnett's post-hoc test). See
419 table 2 for pEC_{50} and R_{max} values.

Table 2. Pharmacological parameters of endothelial function *ex vivo:* A comparison of sensitivity (pEC_{50}) and maximum relaxation (R_{max}) to ACh in the absence or presence of various inhibitors in endothelium-intact mesenteric arteries isolated from normal glucose (NG), moderate hyperglycaemic (MHG) and severe hyperglycaemic (SHG) rats. All experiments were conducted in the presence of indomethacin (10 μM). n= the number of experiments. Results are shown as mean ± SEM. *P<0.05 versus NG, #P<0.05 versus MHG, one-way ANOVA with Tukey's post-hoc analysis. † P<0.05 versus control within each group, one-way ANOVA with Dunnett's test. ND: Not determined.

	NG			MHG			SHG	
ACh	**n**	**pEC_{50}**	**R_{max} (%)**	**n**	**pEC_{50}**	**R_{max} (%)**	**n**	**pEC_{50}**	**R_{max} (%)**
Control	15	7.88±0.09	99±1	19	7.76±0.08	99±1	17	7.20±0.06*#	99±1
L-NAME+ODQ	8	7.50±0.12	99±1	9	7.61±0.09	99±1	8	7.09±0.14*#	99±1
KCaB	10	7.05±0.18†	91±4	12	7.03±0.13†	90±2	12	6.22±0.12*#†	73±4*#†
KCaB+HXC	5	ND	55±7†	5	ND	38±7†	6	ND	38±10†
KCaB+L-Cys	8	6.96±0.13†	91±2	8	6.95±0.18†	87±3	9	6.38±0.12*#†	75±3*#†
KCaB+HXC+L-Cys	4	ND	0±0	4	ND	14±14†	5	ND	7±6†
L-NAME+KCaB	5	ND	65±13†	5	ND	67±13†	4	ND	0±0*#†
L-NAME+ODQ+KCaB	4	ND	35±3†	5	ND	3±3†	4	ND	0±0†

3.6 Impact of Hyperglycaemia on the Relative Contribution of Endothelium-derived NO˙ and HNO to Mesenteric Relaxation

In the presence of KCaB, the sensitivity and R_{max} to ACh-evoked relaxation were significantly reduced in SHG mesenteric arteries compared to both NG and MHG arteries ($P<0.05$, Figure 6A, Table 2). To determine if the relative contributions of endothelium-derived NO˙ vs HNO in the mesenteric artery were differentially affected by hyperglycaemia, cumulative concentration responses to ACh were performed in the presence of KCaB with either HXC (to scavenge NO˙) or L-cysteine (to scavenge HNO). In the presence of KCaB + L-cysteine, the residual relaxation responses to ACh are mediated by NO˙ but not by HNO (Figure 6B, Table 2). Comparison of the responses to ACh suggests that both the sensitivity and R_{max} to ACh were significantly decreased in SHG mesenteric arteries compared to NG and MHG (Figure 6B, Table 2). Conversely, in the presence of KCaB and NO˙ scavenger HXC, the residual relaxation response to ACh is attributed to HNO. When the contribution of NO˙ was eliminated by HXC (Figure 6C, Table 2), the R_{max} to ACh were not significantly different between all groups, indicating that HNO-mediated relaxation was not affected by hyperglycaemia. Furthermore, the combination of KCaB, HXC and L-cysteine abolished ACh-induced relaxation in mesenteric arteries from NG, MHG and SHG rats (Table 2).



448

449 **Figure 6. Relative Contribution of HNO and NO˙ to ACh-Induced Relaxation in**
450 **Rat Mesenteric Arteries: Impact of Hyperglycemia**

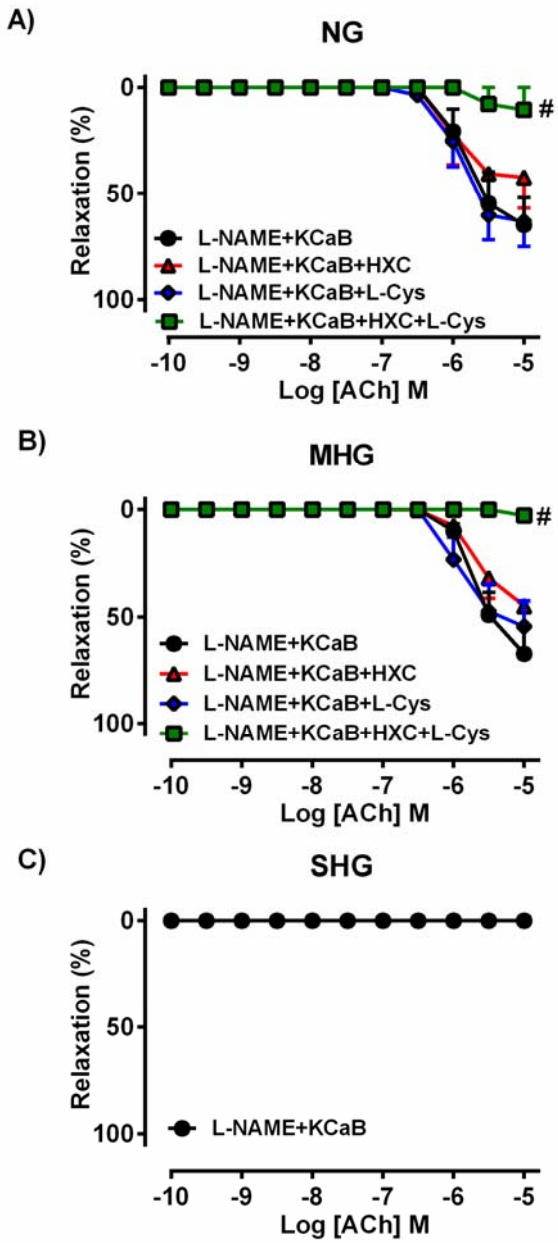
451 Severe hyperglycaemia, but not moderate hyperglycaemia, decreased the
452 sensitivity and maximum relaxation to ACh in the presence of (A) KCaB and (B)
453 KCaB + L-cysteine but not (C) KCaB + HXC, indicating that ACh-evoked relaxation
454 mediated by NO˙, but not HNO,-inducedwas impaired. Indomethacin (10 μM) was
455 always present in the Krebs buffer. Values are mean ± SEM. n = 4-12 per group
456 *P<0.05 vs NG and # P<0.05 vs MHG (1-way ANOVA, Tukey's post hoc test). See
457 table 2 for pEC_{50} and R_{max} values.

458 *3.7 Relative Contribution of Non-NOS Sources of NO•/HNO to Relaxation*

459 The component of ACh-evoked relaxation remaining in the presence of KCaB + L-
460 NAME was evident in both NG and MHG mesenteric arteries but this was abolished
461 in SHG (Figure 7A-C, Table 2). The complete combination of KCaB+L-NAME+L-
462 cysteine + HXC was required to abolish this component of ACh-induced relaxation
463 in both NG (Figure 7A) and MHG (Figure 7B). This suggests that there is a non-
464 NOS source of HNO and NO• that contributes to mesenteric relaxation. There was,
465 however, no significant difference between the combination of KCaB+L-NAME+L-
466 cysteine or the combination of KCaB+L-NAME+HXC compared to KCaB + L-NAME
467 alone (Figure 7A-B).

468

Figure 7



469

470 **Figure 7. Role of non-NOS source of NO˙ and HNO in Rat Mesenteric Artery**
471 **Relaxation**

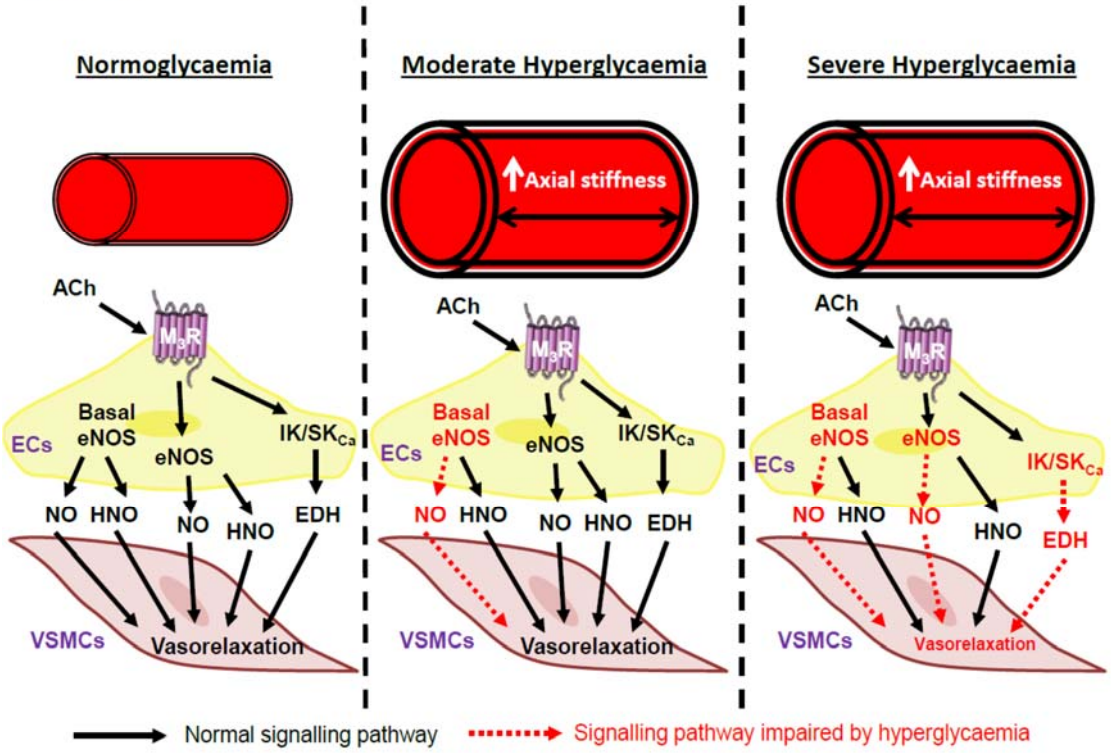
472 Concentration-response curves to ACh in mesenteric arteries isolated from (A)
473 NG, (B) MHG, and (C) SHG rats, obtained in the presence of L-NAME + KCaB, alone
474 or with the further addition of either HXC, L-Cysteine, or HXC + L-Cysteine.
475 Indomethacin (10 μM) was always present in the Krebs buffer. Values are mean ±
476 SEM. n = 4-7 per group # R_{max} vs L-NAME + KCaB ($P<0.05$, 1-way ANOVA,
477 Dunnett's post-hoc test).

478

4. Discussion

The key findings obtained in this study were that a moderate degree of hyperglycaemia is sufficient to elicit diabetes-induced vascular remodelling (outward hypertrophic) and vascular axial stiffness (reduced volume distensibility and ability for the arteries to lengthen). In contrast, endothelial function was only impaired in mesenteric arteries isolated from severely hyperglycaemic but not moderately hyperglycaemic rats, despite the significantly elevated blood glucose and GHB levels compared to non-diabetic rats. These observations reveal that adverse vascular remodelling is more sensitive than endothelial dysfunction to hyperglycaemia in the rat mesenteric vascular bed. Furthermore, this endothelial dysfunction was underpinned by impaired $NO^{\bullet}$-mediated and EDH-type relaxation, but not endothelium-derived HNO-mediated relaxation (Figure 8).

Figure 8



493

494 **Figure 8. Schematic diagram to summarise the impact of differential level of**

495 **glycaemia on mesenteric artery structure and function.**

496 Both moderate and severe hyperglycaemia induces outward hypertrophic

497 vascular remodelling, leading to increase axial wall stiffness of mesenteric

498 arteries. Interestingly, only severely hyperglycaemia, but not moderate

499 hyperglycaemia, is required to cause endothelial dysfunction of mesenteric

500 arteries. Endothelial dysfunction of severe hyperglycaemic mesenteric arteries is

501 underpinned by impaired $NO^{\bullet}$- and EDH-mediated relaxation. However,

502 endothelium-derived HNO-evoked vasorelaxation remain intact regardless of

503 glycaemia levels.

504

4.1 Considerations of the Animal Model Utilised

The characteristics of STZ-induced diabetes are well known. STZ increases blood glucose and GHB levels, with a concomitant retarded body weight gain, particularly in rats (5). The majority of previous studies using STZ to induce diabetes have only assessed the impact on vascular pathology and function in the context of severe glycaemia (~30mM) (9, 10, 40, 41). It is well established that the regimen and route of application as well as the nature of insulin (short or long lasting) plays an important role in regulating blood glucose levels. For example, a previous study has shown that 4-5 U/d of insulin are enough to reduce BG to levels of 200 mg/dL and less (42). Furthermore, daily administration with 1.5-10 U/d of insulin therapy for 1 week completely normalized blood glucose levels and diabetic complications in STZ rats (43). Similarly, continuous infusion of 2.5 U/d of insulin by osmotic minipumps for 2 weeks also completely normalized blood glucose levels and vascular complications in STZ rats (44). In this study, we specifically included a group of rats in which we tightly regulated blood glucose levels with daily injection of long acting insulin (6-7 U/d) to achieve a more moderate degree of hyperglycaemia, in addition to the conventional normal glucose and severely hyperglycaemic (30mM) groups. Interestingly, moderately hyperglycaemic rats exhibited comparable body weight gain compared to non-diabetic rats; both had significantly higher body weights compared to severely hyperglycaemic rats. As the MHG rat cohort exhibited blood glucose and GHB increases part way between the non-diabetic and SHG rats, they were considered to exhibit the general characteristics of a milder degree of diabetes.

4.2 Hyperglycaemia Induces Vascular Stiffness

Increased arterial wall stiffness is an important vascular complication of diabetes and is a risk factor for many adverse cardiovascular events (45). This diabetes-induced increased arterial stiffness is particularly well-characterised in large conduit vessels (46, 47); the impact of a more moderate degree of hyperglycaemia in small resistance arteries was previously largely unknown. This study now reveals that even moderate hyperglycaemia is sufficient to increase both inner and outer vessel diameter, indicating outward hypertrophic remodelling. Furthermore, the increased wall thickness is suggestive of vascular smooth muscle cell hypertrophy. Despite the changes in circumferential measurements (OD, ID and wall thickness), there was no diabetes-induced change in the mesenteric stress-strain relationship, indicating a lack of a diabetes-induced increase in circumferential wall stiffness. Our observations are in contrast to the diabetes-induced increased circumferential wall stiffness observed in rat cremaster arterioles (48) and femoral arteries (12), but are consistent with previous findings specific to mesenteric arteries (12). Hence, diabetes-induced arterial stiffness is likely vascular-region dependent. It is well-documented that this diabetes-induced arterial stiffness results from vascular hypertrophy (20, 21), increased collagen content (19) and an increased in AGEs (15-17). AGEs form cross-links with the extracellular matrix protein collagen, to increase arterial wall stiffness (18). Although lack of tissue availability precluded further morphological analysis (e.g. examination of changes in vascular AGE and/or collagen content), our findings confirm that neither moderate nor severe hyperglycaemia altered circumferential stiffness in rat mesenteric arteries. Arterial remodelling can also be evoked by increased blood flow (49). Diabetes has been reported to increase blood flow to the small intestine (50), associated with outward mesenteric arterial

555 remodelling (51, 52). It is possible that hyperglycaemia-induced vascular
556 remodelling observed here occurred secondary to altered intestinal blood flow.
557 Potential mechanisms of diabetes-induced vascular remodelling were however
558 not investigated.
559
560 Despite the absence of diabetes-induced changes in circumferential wall stiffness,
561 even moderate hyperglycaemia was sufficient to impair the ability of the
562 mesenteric artery to lengthen, reducing volume distensibility. Decreased vascular
563 distensibility is a well-established prognostic indicator, associated with increased
564 risk of further adverse cardiovascular events (53). Impaired arterial compliance
565 and distensibility are evident in diabetic patients (54, 55) and in the STZ diabetic
566 aorta (47). However, the effect of differential levels of hyperglycaemia on arterial
567 distensibility specifically in resistance vessels (such as mesenteric arteries)
568 remains unknown. Our data show for the first time that not only do mesenteric
569 arteries exhibit reduced volume distensibility and ability to lengthen in response
570 to diabetes, indicative of increased axial stiffness, but that even a more moderate
571 degree of hyperglycaemia was sufficient to cause this remodelling.
572
573 *4.3 Hyperglycaemia Induces Endothelial Dysfunction*
574 Endothelial dysfunction is a well-known hallmark of diabetes (2), and is
575 particularly well-characterised in the STZ-induced model of experimental
576 diabetes (10, 11, 40, 56). In the present study, mesenteric arteries isolated from
577 hyperglycaemic rats exhibited reduced sensitivity to the endothelium-dependent
578 agonist ACh, but a more severe degree of hyperglycaemia (~30 mM blood glucose)
579 was required to elicit this dysfunction. Responses to the endothelium-

580 independent vasodilator, SNP, were however not affected by STZ-induced
581 diabetes, indicating that endothelial function was selectively impaired. There are
582 various reports on the duration required following STZ treatment for endothelial
583 dysfunction to be evident (57-59). For example, STZ-induced endothelial
584 dysfunction might not be evident after 6 weeks (58, 59), but longer periods such
585 as 8-10 weeks after STZ treatment are sufficient (10, 11, 32, 40, 56) . In the present
586 study, despite significant vascular remodelling, no impairments in the
587 mechanisms of endothelium-dependent relaxation were apparent in mesenteric
588 arteries after 8 weeks of a more moderate level of hyperglycaemia (~20 mM).
589 Adverse vascular remodelling is thus more sensitive than (and may precede)
590 vascular dysfunction to hyperglycaemia-induced impairments, at least in the
591 mesenteric artery.
592
593 *4.4 Mechanisms of Hyperglycaemia-Induced Endothelial Dysfunction*
594 In resistance arteries such as the mesenteric artery, EDH is the main contributor
595 to ACh-evoked relaxation (26, 60). We observed here that hyperglycaemic
596 mesenteric arteries exhibited reduced responses to ACh in the presence of L-
597 NAME+ODQ+indomethacin compared to normoglycaemic mesenteric arteries,
598 indicating that EDH-type relaxation was impaired. The mechanism underlying the
599 impairment of EDH-type relaxation in the diabetic mesenteric arteries is well-
600 characterised, which may include oxidative stress-mediated inhibition of KCa
601 channels, leading to diminished KCa channel activity (9, 10, 56). Furthermore, the
602 combination of KCaB+L-NAME abolished endothelium-dependent relaxation in
603 mesenteric arteries from hyperglycaemic rats, but had a lesser effect on
604 endothelium-dependent relaxation in normoglycaemic and moderately

hyperglycaemic arteries. This suggests that there is a component of non-classical EDH-type relaxation in normoglycaemic and moderate hyperglycaemic arteries (61) but lost in the hyperglycaemia group, which is consistent with a previous report (9).

Traditionally, the vasodilator actions of endothelium-derived nitrogen species have been solely attributed to $NO^{\bullet}$. It has however now become apparent that the redox sibling of $NO^{\bullet}$, HNO, is produced endogenously as well and contributes to the control of vascular tone (26, 27). In the present study, we observed a significant reduction in basal nitrogen oxide and $NO^{\bullet}$ bioavailability as a result of hyperglycaemia, even in moderate hyperglycaemic mesenteric arteries. In contrast, there was no significant difference in basal HNO level between all three groups, highlighting the preservation of the HNO contribution to basal tone in conditions of hyperglycaemia. Although previous studies have reported inconsistent findings regarding the impact of STZ-induced diabetes on NOS-dependent relaxation (9, 10, 41), none of these sought differentiation between potential contributions of $NO^{\bullet}$ vs HNO to endothelium-dependent relaxation. In the presence of KCaB+indomethacin, residual vascular relaxation was attributed to NOS-derived nitrogen species such as $NO^{\bullet}$ and HNO. We specifically incorporated the ability to distinguish between $NO^{\bullet}$ and HNO-mediated relaxation into our study design, by utilising well-established pharmacological tools, the selective scavenger for $NO^{\bullet}$ (HXC) and the selective scavenger for HNO (L-cysteine) (26, 62, 63). In the presence of KCaB+L-cysteine, responses to ACh were significantly reduced in arteries isolated from severely hyperglycaemic animals compared to non-diabetic and moderately hyperglycaemic rats, specifically

suggesting that NO•-mediated relaxation was impaired by severe hyperglycaemia. Conversely, there were no differences in the responses to ACh between all 3 groups in the presence of KCaB+HXC, excitingly revealing that HNO-mediated relaxation was resistant to hyperglycaemia.

There are extensive reports to demonstrate that diabetes increases vascular superoxide production and impairs NO•-mediated relaxation, with putative contributing mechanisms, including eNOS uncoupling and/or reduced NO• bioavailability, central to diabetes-induced endothelial dysfunction (64, 65). In contrast to NO•, HNO is resistant to scavenging by superoxide (30, 31) and in further contrast to NO• there is also evidence suggesting potential HNO generation from uncoupled eNOS (66). Therefore, it is possible that neither endogenous HNO production nor HNO vasodilator actions are affected in conditions associated with oxidative stress. Indeed, previous studies have shown that the vasodilator actions of HNO are preserved in diabetic aortae (30), hypertension (67) and hypercholesterolemia (68), all diseases associated with oxidative stress. Furthermore, HNO reduces Nox2 (NADPH oxidase 2) expression and suppresses superoxide production in cardiomyocytes (69, 70) and cerebral arteries (71), whilst also preserving myocardial function in diabetes (72).

Despite the exciting findings obtained regarding the impact of diabetes on HNO and NO-mediated relaxation in the mesenteric arteries, there were some limitations to this conclusion. The conclusion that endothelium-derived HNO-mediated relaxation is not affected by diabetes was relied on the use of only 1 pharmacological inhibitor, L-cysteine and HXC. Both L-cysteine and HXC may have

potential non-selective effects such as interaction with S-glutathionylated enzymes and/or reactive oxygen species respectively. Furthermore, the endogenous production of HNO in intact microvessels will not be proven conclusively until direct detection methods for HNO production are available (73). Although direct and indirect measurement for NO production in intact blood vessels are possible, these experiments are not performed due to limited animal tissues. Nonetheless, future studies relying on pharmacological tools to characterize NO or HNO-mediated relaxation should use at least 2 different pharmacological inhibitors.

4.5 Conclusion

We now demonstrate, for the first time, that moderate hyperglycaemia is sufficient to induce vascular remodelling, and subsequent increased axial stiffness, in diabetic rat mesenteric arteries. Interestingly, although these morphological changes are evident after 8 weeks of moderate hyperglycaemia (~20mM), a more severe hyperglycaemia (~30mM) is required to induce endothelial dysfunction in rat mesenteric arteries. This is attributed to impaired $NO^{\bullet}$ and EDH mechanisms. In contrast, HNO mechanisms of vasorelaxation remain intact. Taken together, our findings suggest that adverse arterial remodelling precedes endothelial dysfunction in the context of experimental diabetes.

Additional Information

Competing Interests

The authors have no competing interests to declare.

Author Contributions

N.K, R.H.R, C.H.Q, H.H.N and C.H.L designed the research, N.K, J.A, E.J, H.H.N and C.H.L collected and analysed the data, N.K, R.H.R, L.J.P, B.K.K-H, C.H.Q, H.H.N, M.J and C.H.L interpreted the data. N.K, C.H.Q, R.H.R and C.H.L drafted the manuscript. All authors approved the final version of the manuscript.

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