Dynamic Synchrotron Imaging of Diabetic Rat Coronary Microcirculation In Vivo

Mathew J. Jenkins, Amanda J. Edgley, Takashi Sonobe, Keiji Umetani, Daryl O. Schwenke, Yutaka Fujii, Russell D. Brown, Darren J. Kelly, Mikiyasu Shirai, James T. Pearson

Objective—In diabetes, long-term micro- and macrovascular damage often underlies the functional decline in the cardiovascular system. However, it remains unclear whether early-stage diabetes is associated with in vivo functional impairment in the coronary microvasculature. Synchrotron imaging allows us to detect and quantify regional differences in resistance microvessel caliber in vivo, even under conditions of high heart rate.

Methods and Results—Synchrotron cine-angiograms of the coronary vasculature were recorded using anesthetized Sprague-Dawley rats 3 weeks after treatment with vehicle or streptozotocin (diabetic). In the early diabetic state, in the presence of nitric oxide and prostacyclin, vessel diameters were smaller ($P<0.01$) and endothelium-dependent vessel recruitment was already depressed ($P<0.05$). Endothelium-dependent and -independent vasodilatory responses in individual coronary vessels were not different in vivo. Inhibition of NO and PGI$_2$ production in diabetes uncovered early localized impairment in dilatation. Diabetic animals displayed focal stenoses and segmental constrictions during nitric oxide synthase/cyclooxygenase blockade, which persisted during acetylcholine infusion ($P<0.05$), and a strong trend toward loss of visible microvessels.

Conclusion—Synchrotron imaging provides a novel method to investigate coronary microvascular function in vivo at all levels of the arterial tree. Furthermore, we have shown that early-stage diabetes is associated with localized coronary microvascular endothelial dysfunction. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: endothelium ■ constriction ■ diabetes ■ dysfunction ■ synchrotron

Diabetes is associated with a 2- to 5-fold higher risk of cardiovascular disease relative to nondiabetics.1 This is strongly linked to persistent long-term vascular damage occurring at both micro- and macro-vascular levels.2 The coronary circulation, in particular, is known to be vulnerable to the diabetic milieu and develops endothelial dysfunction, independently of atherosclerosis.3 The exact mechanisms leading to impaired coronary vascular function remain unclear. Previous work suggests that an imbalance develops between vasodilatory factors acting via the endothelium including nitric oxide (NO), prostacyclin (PGI$_2$), and endothelium-derived hyperpolarizing factors (EDHF) and vasoconstrictor factors.2 Prior work indicates that in the later stages of diabetes, chronic hyperglycemia and hyperlipidemia result in increased oxidative stress and inflammation.2 Reactive oxygen species (ROS) acting either directly or indirectly on the endothelium may thus cause impairment in the vasodilatory capacity of the coronary vessels.4,5 Although well-characterized in the later stages of diabetes,6,7 fewer studies have focused on the development of coronary dys-

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tron radiation contrast angiography and (2) to investigate the early origins of vascular dysfunction in individual coronary microvessels using the streptozotocin-induced diabetic rat.

**Methods**

An expanded Methods section appears in the Supplemental Material, available online at http://atvb.ahajournals.org.

**Animals and Experiments at the Synchrotron**

All experiments were conducted at SPring-8, Japan Synchrotron Radiation Research Institute in Hyogo, Japan. Male Sprague Dawley rats (Japan SLC, Kyoto, Japan, 7 weeks old) received either a vehicle injection of sodium citrate (0.1 mol/L, pH 4) (control) or streptozotocin (65 mg/kg ip.) to induce type I diabetes. Three weeks after vehicle or streptozotocin injection all rats underwent terminal experiments.

**Angiograph Protocol**

Iodinated contrast medium (Iomeron 350; Bracco-Eisai Co. Ltd, Tokyo, Japan) was injected intra-arterially as a bolus (0.2–0.5 mL at 0.4 mL/s) into the aorta and cine-angiograms were obtained over 3 s during a temporary cessation of ventilation. A small arterial pressure drop (~10 mmHg) that occurred when ventilation was interrupted was counterbalanced by an increase in pressure during bolus entry (5–10 mmHg). Bolus injection did not cause a significant pressure perturbation during image sequences.

**Experimental Protocol**

Endothelium-dependent and -independent vasodilatory responses were recorded in control (n=5) and diabetic (n=6) animals. This was achieved by recording coronary angiograms during infusions of vehicle (lactate 3.0 mL/h); acetylcholine (Ach; 3.0 μg/kg/min), sodium nitroprusside (SNP; 38 μg/kg/min), during lactate infusion 30 minutes after nitric oxide synthase (NOS) inhibition with Nω-nitro-L-arginine methyl ester (L-NAME; 50 mg/kg iv. bolus), and 15 minutes after further cyclooxygenase (COX) inhibition with meclofenamate (3 mg/kg IV bolus). Repeat imaging was performed after combined L-NAME + meclofenamate treatment and a repeat infusion of Ach (3.0 μg/kg/min). At the termination of experiments, rats were killed and their hearts removed and were fixed in 10% formaldehyde.

**Histology and Immunohistochemistry**

Perivascular fibrosis expression was evaluated in coronary arterioles of picrosirius red stained LV sections. Immunohistochemical staining was performed against murine-specific endothelial cell marker isolectin B4 (1:50, Vector Laboratories, Burlingame, CA) to assess capillary density. Automatic quantification was made using pre-defined algorithms on Aperio Imagescope (v11.0.2.725, Aperio Technologies, CA).

**Assessment of Vessel Internal Diameter**

Quantitative analysis of vessel internal diameter (ID) was based on measurements from the middle of discrete vessel segments in individual cine-angiograms using Image-J (v1.41, National Institutes of Health, Bethesda, MD). Angiograms presented in this article underwent median filtering (2 pixel radius) to improve vessel clarity for publication purposes only. Arterial vessels were categorized according to their branching order (root, 1st, 2nd, and 3rd) and their basal vessel ID size class (40–100 μm, 100–200 μm, 200–300 μm, and >300 μm; Supplemental Figure II, available online at http://atvb.ahajournals.org). Reported results for vessel ID and vessel number during drug infusions are expressed as percentage change from baseline (Δ) to account for differences in absolute baseline vessel ID and vessel number between groups. Vessel recruitment was determined as the Δ in vessel number from baseline during each treatment for the same field of view.

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<th>Table. Animal Characteristics in Anaesthetized Control and Diabetic Sprague-Dawley Rats</th>
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<td>MAP (mm Hg)</td>
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<td>Heart rate (bpm)</td>
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Values expressed as mean±SEM. Control, n=5 and diabetic n=6. MAP indicates mean arterial pressure; NS, not significant. P<0.05 determined by t-test.

**Quantification of Focal and Segmental Vasoconstrictions**

Localized focal vasoconstrictions were considered to be present when a small portion of a vessel segment showed a constricted ID less than 50% of upstream nonoccluded segment ID in the case of focal constrictions and less than 70% of baseline vessel ID in the case of segmental constrictions (most of segment length).

**Statistical Analysis**

Data are expressed as mean ± SEM unless otherwise stated. For each individual rat the mean vessel ID or mean Δ vessel ID (%) of each branching order was used for group comparisons. One-way and 2-way ANOVA with Bonferroni correction were carried out to assess within- and between-group differences, followed by a 2-tailed Student t-test to determine statistical significance between groups. The Statistical Package Software System (SPSS v15, SPSS, Chicago, IL) was used for all analysis with values of P<0.05 deemed significant.

**Results**

**Animal Characteristics**

Diabetic rats had significantly higher blood glucose concentration and lower final body weight compared to controls (P<0.001, Table). Mean arterial pressure (MAP), measured prior to imaging, was ~40 mmHg lower in diabetics rats (P<0.05) although heart rate was comparable to controls (Table). Myocardial capillary density (Supplemental Figure IIIA, IIIC, and IIID, available online at http://atvb.ahajournals.org) and perivascular fibrosis (Supplemental Figure IIIIB, IIIE, and IIIF) were not significantly different.

**Baseline Vessel Internal Diameter**

Vessel ID in diabetic animals when categorized by branching orders showed that under basal conditions diabetics had significantly smaller ID in the 1st. (194±15 versus 267±23 μm, P<0.01), 2nd. (110±6 versus 144±9 μm, P<0.01), and 3rd. (73±4 versus 110±17 μm, P<0.01) order vessels compared to controls (Figure 1). The root segment was only visualized in 1 control and 1 diabetic animal was not included in subsequent analysis. The minimum ID visible across both groups was 42 μm.

**Vessel Response to Endothelium-Dependent/-Independent Stimulation**

Basal endothelium-dependent dilatory responses did not differ between control and diabetic animals with infusion of ACh, evoking similar vasodilatory responses in both control and diabetic rats on the basis of branching order (Figure 2A) and vessel class (Supplemental Figure IVA, available online.
Vessel Recruitment During Endothelium Stimulation

Vessel number was significantly lower in diabetic animals during the first ACh infusion (Δ25.8±16.1% versus −19.9±12.4%, P<0.05) compared to controls, but not during SNP (Figure 3B). NOS blockade alone caused no significant difference in visible vessels in either group. However with further blockade of COX, there was a strong trend toward reduced visible microvessels in diabetic rats compared to controls (P=0.059, Figure 3B). Vessel number remained lower in diabetics compared to controls following postblockade infusion of ACh; however this was not significantly different between the 2 groups (Figure 3B).

Localized Vascular Constrictions Assessment

No focal constrictions were observed in any control animals. In contrast focal constrictions in diabetic animals were observed during NOS blockade and their incidence increased after further COX inhibition (Figure 4D; dashed black arrows insert, Figure 5A, and Supplemental Movie I, available online at http://atvb.ahajournals.org). Mean vessel occlusion was 63.8% and 66.2±1.1% of upstream nonconstricted vessel ID during NOS blockade and combined blockade, respectively. Notably, severe focal constrictions were primarily located at branching points of the secondary and tertiary conduits (Supplemental Figure VA, available online at http://atvb.ahajournals.org; asterisk) and were present over the entire cardiac cycle as demonstrated in Supplemental Figure VB. Importantly, these constrictions persisted following ACh administration, as depicted in Figure 5A, although the mean vessel occlusion lessened slightly to 61.7±2.1% of upstream nonoccluded vessel ID (Figure 4F; black arrows).

The percentage of vessels classified as being segmentally constricted relative to baseline was not significantly different in control and diabetic animals during endothelium-dependent and -independent stimulation (Figure 5B). This was also the case during NOS blockade alone. Following combined NOS/COX blockade, however, there was a strong trend toward a greater proportion of segmentally constricted vessels in diabetic compared to control animals (Figure 5B). Segmental constrictions occurred predominately in 2nd-order vessels in both groups (Supplemental Figure VI, available online at http://atvb.ahajournals.org). Subsequent endothelial stimulation postblockade, completely abolished all segmental constrictions in controls but significantly was unable to do so in diabetics (P<0.05, Figure 4F; white asterisk, Figure 5B). Segmental constrictions in diabetics persisted in 1st-order vessels in diabetics with a slight reduction in the proportion of affected 2nd-order vessels. A summary of the branching orders with segmental constrictions can be seen in Supplemental Figure VI.

Discussion

To the best of our knowledge, this is the first in vivo study to characterize early impairment in the diabetic coronary microvasculature in rodents using synchrotron radiation. Furthermore, our results show that EDHF-mediated vasodilation in the coronary resistance vessels is impaired prior to changes in NO and PG12, with 3 lines of evidence to support this conclusion. First, endothelium-dependent and -independent vasodilation responses...
were normal in diabetic rat hearts in the presence of NO and PGI2, but there was a reduction in endothelium-dependent vessel recruitment. Second, we showed an increased presence of focal and segmental constrictions in the conduit and resistance vessels of the coronary circulation of diabetic hearts after NOS and COX blockade. Furthermore, in the absence of NO and PGI2 coronary constrictions were not alleviated by ACh stimulation.

This study investigated early structural changes in the diabetic coronary circulation and found no observable difference in capillary density or perivascular fibrosis, suggesting that a longer diabetic time course is required for impairment in coronary flow and vessel loss to become apparent. Myocardial perfusion and vascular elasticity are thus comparable to controls and would be assumed to be adequate for basal function. In this study the main endothelium-derived vasodilators, NO, PGI2, and EDHF, and their specific contributions were of principal interest. The exact identity of EDHF however is still unknown, with considerable evidence now suggesting that EDHF is not a single entity but rather a collection of factors including epoxyeicosatrienoic acids, H2O2, K+ ions, and/or gap junctions.

**Vascular Function in the Presence of Nitric Oxide and Prostacyclin**

Mean arterial blood pressure was significantly lower in diabetic animals consistent with previous studies and may explain the smaller vessel ID in diabetic 1st-, 2nd-, and 3rd-order vessels.

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**Figure 2.** Change in vessel internal diameter (ID) in control and diabetic animals during infusion of vasoactive compounds categorized by branching order. No significant differences were noted between control and diabetic responses to ACh (A), sodium nitroprusside (SNP) (B), Nω-nitro-l-arginine methyl ester (L-NAME) (C), combined L-NAME + meclofenamate (D), and combined + ACh (E). Control, n=5, and diabetic, n=6. Values expressed as mean±SEM. #P<0.01, †P<0.001 vs baseline, †P<0.05 vs control.
observed under basal conditions. Autoregulatory alterations in nervous and vascular tone almost certainly account for the smaller caliber, but a change in the balance of vasodilators and vasoconstrictors might also play a small role.22 Our findings of normal basal function are in agreement with previous studies where 2 to 6 weeks of diabetes was insufficient to impair endothelium-dependent and -independent vascular function.9,23 The exact mechanism by which endothelial function is maintained in the face of hyperglycemia remains unclear, although recent work suggests that even in the presence of increased ROS in early diabetes, NO from non-NOS sources may stimulate soluble guanylate cyclase to open K⁺ channels and thus preserve dilation.23

Although dilatory responses in diabetic animals were normal we did observe a smaller decrease in MAP in response to endothelial stimulation. This reduced responsiveness in mean arterial pressure may stem from impairment in endothelial function in other peripheral vascular beds, even though basal coronary endothelial function is normal.24 There was also a concurrent reduction in endothelium-dependent opening of new vessels. Changes in vessel recruitment as assessed by the presence or absence of vessel contrast are indicative of altered resistance within these vessel segments. Thus, the inability of endothelium-dependent stimulation to evoke opening of vessel segments, suggests that diabetic vessels are unable to sufficiently reduce resistance to allow contrast perfusion. Hence, there appears to be early indications of impaired vessel responsiveness in resistance microvessels within the coronary circulation, even though vessel ID responses to endothelium-dependent stimulation were normal. However, we cannot preclude the possibility that in the diabetics there were no further arterial segments to recruit.

Basal EDHF and Reserve in the Absence of Nitric Oxide and Prostacyclin

There are few studies specifically investigating the contribution of EDHF to the coronary circulation in the early stages of diabetes, although EDHF downregulation in other vascular beds in the later stages of diabetes is well characterized.25,26 Imbalances in the production of endothelium-derived vasoconstrictors including endothelin-127 and COX-dependent
vasoconstrictor factor have been noted in diabetes and it seems possible that even if basal EDHF function was normal, in the presence of increased vasoconstrictor influence, it may be insufficient to maintain vasodilatation. Responses to ACh post-COS/NOX blockade in diabetic vessels were similar to that observed in control rats, suggesting that vasodilation mediated by endothelial stimulation of EDHF was not widely impaired at this early stage of diabetes. Thus when stimulated, global EDHF reserve appears to be sufficient to overcome inherent vasoconstrictor tone in the absence of NO and PGI2 production.

Focal and Segmental Constrictions

This in vivo study is one of the few to explore the presence of vasospasm in the early stages of diabetes when basal endothelial function, as discussed previously, is thought to be normal. Focal constrictions following NOS blockade were only noted in diabetics, whereas their incidence increased further following COX inhibition. In stark contrast, control rats have sufficient vasodilatory reserve to compensate for this loss and maintain vasodilatory capacity. These focal constrictions in diabetic rats may result from localized limitations in the ability of the dysfunctional coronary endothelium to produce vasodilators, including EDHF, as has been previously shown in the systemic circulation. Increased vasoconstrictor influence at highly localized sites within the diabetic coronary vasculature may also contribute. Furthermore, the RhoA/Rho-kinase pathway is known to be upregulated in the diabetic vasculature and has been implicated in the development of coronary artery spasm, warranting further investigation. Endothelial stimulation post-NOS and COX blockade in diabetic rats, resulted in no change in focal constriction incidence, but a slightly reduced severity. Focal constrictions were however entirely absent in control animals. The persistence of focal constrictions during endothelium stimulation, provides further evidence that localized areas of the endothelium, even in the early stages of diabetes, are deficient in EDHF-mediated vasodilatory reserve. Coronary constrictions are most often observed in the human condition as a result of coronary artery disease. It is important to note however that rodents have cholesterol profiles dissimilar to humans and do not develop atherosclerosis. Thus, the presence of these constrictions in diabetic rodents suggests that diabetes itself is the major cause of their development.

In addition to focal constrictions, there was a strong trend toward increased segmental constrictions (>30% vessel ID constriction from baseline) in diabetic rats following NOS and COX inhibition. Although nonsignificant, this trend does support impaired diabetic basal EDHF function. More importantly however, endothelium stimulation post-NOS/COX blockade was unable to alleviate segmentally constricted vessels in diabetic animals, contrasting with the complete restoration of all previously constricted vessels in controls. This significant difference mirrors the response of diabetic focal constrictions and again implies that EDHF reserve in at risk diabetic coronary vessels is insufficient. This EDHF impairment may predispose the diabetic circulation toward reduced maximal perfusion and/or impose a greater cardiac work requirement for the maintenance of myocardial blood flow. Furthermore, the importance of this reduced vasodilatory capacity becomes more apparent as the risk of myocardial infarction and stroke increases with diabetes progression.

Possible Mechanisms of Early Vascular Dysfunction

Our data provides functional insights into the role of endothelium-derived vasodilators in diabetes. Increased ROS resulting from hyperglycemia however, seems a prime unifying precursor as it has been shown to activate multiple pathways including increased COX-2 expression, increased vasoconstrictor tone, and activation of RhoA/ROCK all discussed previously. Furthermore it is well-accepted that
hyperglycemia causes vascular endothelial dysfunction, possibly through increased nonenzymatic glycosylation, increased oxidative stress stemming from reactive oxygen and nitrogen species, and activation of protein kinase C and AMPK pathways.40

Synchrotron Radiation Imaging Benefits and Experimental Limitations
There have been a number of previous in vivo studies investigating changes in the coronary vasculature using synchrotron radiation;31–43 however, this technique has not been used to investigate early changes in the diabetic coronary circulation. Coronary microangiography using synchrotron radiation permits the assessment of microvascular function in vivo, in real time even during conditions of high heart rate (>500 bpm) due to its ability to be performed in time orders as low as 1 ms shutter open-times.14 Furthermore, compared to conventional imaging methods, including CT and MRI, synchrotron radiation allows the assessment of microvascular ID and spasms <100 μm, which would normally require the hearts to be arrested prior to imaging.44 Synchrotron radiation also enables within-animal assessment of individual arteries, as only minimal contrast boluses are needed due to high photon flux and collimation resulting from its “point source” emission.14 On the other hand, currently direct application of this technology to imaging patients is limited by facility access. The number of medical imaging beam lines at synchrotron radiation facilities is increasing, but access is limited by competitive merit-based proposal systems. Hence, routine diagnostic imaging of diabetic patients at these facilities is highly unlikely. However, these authors are aware of at least 2 international research efforts in Europe and Japan to produce synchrotron radiation sources small enough to fit within clinics for medical imaging and radiotherapy purposes. Improved X-ray flux and collimation could conceivably improve the diagnostic detection of early diabetic vascular dysfunction as reported in this study.

Summary
In summary, synchrotron radiation microangiography provides a novel in vivo method for investigating the progression of coronary microvascular disease. In the early diabetic state our results suggest that baseline vessel response is relatively normal although the depressed endothelium-dependent vessel recruitment suggests that this is already rapidly changing. Furthermore, using this within-segment analysis technique we have uncovered localized focal and segmental constric- tions during vasodilator inhibition in the early diabetic microvasculature suggesting specific impairment in EDHF function. These findings provide a strong basis for further investigation into the early pathophysiological changes that occur in this increasingly prevalent disease state.

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Disclosures
None.

References


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Supplement Material

Dynamic synchrotron imaging of diabetic rat coronary microcirculation in vivo

Jenkins - Endothelial dysfunction in coronary circulation

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Extended Methods

Animals and Experiments at the Synchrotron

All experiments were conducted at the Beamline 28B2 of SPring-8, Japan Synchrotron Radiation Research Institute, Hyogo, Japan with approval from the Animal Experiment Review Committee (Proposals 2009A1333, 2010A1207) in accordance with the guidelines of the Physiological Society of Japan. Male Sprague Dawley rats (Japan SLC, Kyoto, Japan, 7 wks old) received either a vehicle injection of sodium citrate (0.1M, pH 4) (control) or streptozotocin (STZ; 65 mg/kg i.p.) to induce type I diabetes. All rats were on a 12 h light/dark cycle at 18-25°C and were provided with food and water ad libitum. Three weeks after vehicle or STZ injection all rats underwent terminal experiments at Beamline 28B2 (BL28B2). Fasted conscious blood glucose was measured using a One-Touch Ultra Glucometer (Medisense Products, VIC, Australia) via the tail vein 2 days prior to imaging.

Experimental Preparation

Under sodium pentobarbital anesthesia (50 mg/kg i.p.) rats were intubated, artificially ventilated (40% oxygen) and the right carotid artery cannulated with a radiopaque 20-gauge BD Angiocath catheter (Becton Dickinson, Inc., Sandy, Utah, USA), placing the tip at the entrance of the aortic valve. Body temperature was maintained at 37°C using a rectal thermistor coupled with a thermostatically controlled heating pad. Anesthesia level was maintained via additional intraperitoneal boluses of pentobarbital (25 mg/kg/h). Sodium lactate (Sigma Aldrich, NSW, Australia) was administered intravenously via the right jugular vein to maintain body fluids (3.0 ml/hr). A catheter filled with heparinized saline (12 units/ml), was inserted into the right femoral artery to record arterial pressure via a disposable pressure transducer (MLT0699, AD Instruments, NSW, Australia). The analogue arterial
pressure signal was digitized at 1000 Hz and recorded using CHART software (v5.4.1, AD Instruments, NSW, Australia) to determine mean arterial pressure and heart rate, simultaneous with recordings of the camera trigger over the cardiac cycle.

**Angiography Protocol**

Each rat was then moved into the X-ray hutch for contrast angiograms, with the heart placed in line with the horizontal X-ray beam and SATICON detector system (Hitachi Densi Techno-system, Ltd., Tokyo, Japan and Hamamatsu Photonics, Shizuoka, Japan), described previously.

Pancuronium bromide (Mioblock; 2mg/kg, Sankyo, Tokyo, Japan,) was administered for neuromuscular blockade to prevent spontaneous breathing when artificial ventilation was stopped during imaging. Iodinated contrast medium (Iomeron 350; Bracco-Eisai Co. Ltd, Tokyo, Japan) was injected intrarterially as a bolus (0.2 – 0.5ml at 0.4ml/s) into the aorta with a clinical autoinjector (Nemoto Kyorindo, Tokyo, Japan) at the start of image recording scans. The ventilator was turned off for around 2-3 seconds for each cine-scan at end-inspiration. A small arterial pressure drop (~10mmHg) that occurred when ventilation was interrupted was counterbalanced by an increase in pressure during bolus entry (5-10mmHg). Bolus injection did not cause a significant pressure perturbation during image sequences. At least 10 minutes was allowed for renal clearance of contrast between imaging scans.

During each cine-scan, monochromatic X-rays at 33.2 keV (energy bandwidth 20-30 eV) and a flux $\sim 10^{10}$ photons/mm$^2$/s passed through the rat’s chest and were recorded on the X-ray detector (Hitachi Densi Techno-System, Ltd., Tokyo, Japan) at 30 frames/s at 10-bit resolution for ~3 s intervals. For each cine-scan 100 frames were recorded with a shutter open time of 1.5-2.0 ms/frame. The detector features a 9.5µm equivalent pixel size that captures a
9.5×9.5µm input field with images stored in 1024x1024 pixel format. Periodic inspection between angiograms confirmed that the coronary vasculature cleared of contrast agent during this time.

**Experimental Protocol**

Endothelium-dependent and –independent vasodilatory responses were recorded in control (n=5) and diabetic (n=6) animals. This was achieved by recording coronary angiograms at the end of 5 minute infusions of vehicle (lactate 3.0 ml/hr), ACh (3.0 µg/kg/min) and sodium nitroprusside (SNP 3.0 µg/kg/min). Vascular responses were also assessed during lactate infusion 30 minutes after nitric oxide synthase (NOS) inhibition with Nω-nitro-l-arginine methyl ester (L-NAME, 50 mg/kg iv. bolus). Angiograms were also recorded 15 minutes after further cyclooxygenase (COX) inhibition with sodium meclofenamate (3 mg/kg iv. bolus). For simplicity Combined treatment refers to L-NAME + meclofenamate treatment together. Endothelium-dependent vasodilation was then assessed during Combined treatment (NOS and COX inhibition) with a repeat infusion of ACh (3.0 µg/kg/min). At the termination of experiments rats were killed and their hearts removed, fixed in 10% formaldehyde, paraffin embedded and sectioned to 4µm thickness for histology and immunohistochemistry.

**Histology and Immunohistochemistry**

Perivascular fibrosis expression was evaluated in coronary arterioles of picrosirus red stained LV sections. All non-round vessels were excluded from analysis. Perivascular fibrosis was determined as the ratio of the area of fibrosis immediately surrounding the intramyocardial blood vessel walls to the total area of the vessel\(^2\). Immunohistochemical staining was performed against murine-specific endothelial cell marker isolectin B4 (1:50, Vector Laboratories, Inc, Burlingame, CA, USA) to assess capillary density\(^3\). Capillary density was
evaluated by histological examination of the myocardium to detect positively stained endothelial cells. Briefly, after washing with PBS, nonspecific protein binding was blocked with 1:5 dilution of normal goat serum (Dako, Golsstrup, Denmark) Sections were then incubated with biotinylated isolectin B4 at 4°C over-night, followed by avidin-biotin horseradish peroxidase (Vector Laboratories, Inc, Burlingame, CA, USA) and diaminobenzidine (Vector Laboratories, Inc, Burlingame, CA, USA) as described previously. The Aperio ScanScope XT Slide Scanner (Aperio Technologies Inc., CA, USA) system was used to capture digital images with a 20×objective. Quantitative analysis was performed automatically with Aperio Imagescope (v11.0.2.725, Aperio Technologies) using the Positive Pixel Count v9 algorithm (Aperio Technologies) for perivascular fibrosis and capillary density.

**Assessment of Vessel Internal Diameter**

Vessel internal diameter (ID) was measured from the middle of the discrete vessel segments in individual cine-radiograms using Image-J (v1.41, NIH, Bethesda, USA). Individual raw frames (Supplemental Figure IA,C,E) were assessed and the region of interest containing the vessel under analysis (red rectangle) was selected and enlarged (Supplemental Figure IB,D,F). Using Image-J a line of 1 pixel width was manually drawn across the vessel mid-segment, from edge to edge, perpendicular to the vessel direction. The pixel count was then recorded and this process was repeated for the same vessel segment over at least 10 consecutive frames where the vessel was visible. Using the vessel ID from individual frames a mean vessel ID was calculated for each vessel segment. This process was repeated for all visible vessels during subsequent drug treatments. A tungsten wire, with diameter of 50µm in diameter is visible in top left corner of images and was used for calibration to calculate absolute vessel ID. Angiographic images presented in this paper, unless otherwise stated,
underwent median filtering (2 pixel radius) to improve vessel clarity for publication purposes only.

Total visible vessel branches were counted and recorded. A defined field of view was designated for each angiogram with vessels outside this field of view excluded if the angiogram perspective shifted during subsequent treatments. Vessel counts were achieved by tracing vessel branching over all recorded frames thus ensuring transient vessels were identified. Mean total visible vessel branches per animal in the field of view was 13-18 vessels across all treatments in controls and 12-22 vessels in diabetics. Arterial vessels were categorized according to their basal vessel ID size class (40–100µm, 100–200µm, 200–300µm and >300µm) and their branching order (root, 1st, 2nd and 3rd; Supplemental Figure II). Vessel branching order was assumed to have changed if a segment divided approximately equally into 2 daughter segments. The coronary root was defined if the rats had a common branch for the left descending artery and circumflex. Reported results for vessel ID and vessel number during drug infusions are expressed as percentage change from baseline (Δ) to account for differences in absolute baseline vessel ID and vessel number between groups. Vessel recruitment was determined as the Δ in vessel number from baseline during each treatment for the same field of view.

Quantification of Focal and Segmental Vasoconstrictions

Assessment of the angiograms identified a number of instances of localized constrictions, either focal or segmental (Supplemental Movie I). Localized focal vasoconstrictions were considered to be present when a small portion of a vessel segment showed a constricted ID less than 50% of upstream non-occluded segment ID in the case of focal constrictions and less than 70% of baseline vessel ID in the case of segmental constrictions. Focal constrictions
were not included in overall vessel order and vessel class analysis as they did not occur mid-
segment. The number of segmental constrictions are presented as a percentage of the visible
vessel segments. Furthermore constrictions were required to persist over the entire cardiac
cycle (~6-8 frames) to exclude instances of transient vessel pinching. Vasocostricctions were
arbitrarily classified as focal constrictions if the occlusion segment length was <100µm or
otherwise as a segmental constriction.

**Statistical Analysis**

Data is expressed as mean ± SEM unless otherwise stated. The mean vessel ID and the
change in ID (%) of each branching order or vessel size class in individual rats in each
treatment period were pooled for group comparisons. One-way and two-way ANOVA with
Bonferroni correction for repeated measures were carried out to assess within and between
group differences resulting from drug infusions versus baseline. Following ANOVA, all
between group comparisons were made using a 2-tailed Student t-test, to determine statistical
significance between control and diabetic animals at baseline and during subsequent drug
treatment periods. The Statistical Package Software System (SPSS v15, SPSS Inc, Chicago,
USA) was used for all analysis with values of $P < 0.05$ deemed significant.
Supplemental References


Supplemental Figures

(Supplemental Figures I to VI are cited in main text)
Supplemental Figure I. Representative consecutive, single frames depicting the determination of internal vessel diameter during baseline lactate infusion. Individual original frames (A, C, E) were assessed and the region of interest (red rectangle) was enlarged (B, D, F). A line of 1 pixel in width (B, D, F) was drawn perpendicular to the vessel direction and the vessel ID recorded. w is tungsten wire, with diameter of 50±9µm. Ao is the aortic root.
Supplemental Figure II. Schematic of left coronary arteries in lateral view depicting the coronary branching nomenclature used in this study. A change in order was assumed when a segment branched into equal daughter branches. In some hearts no common root was visible and the CX main arteries appeared to originate independently from aortic sinus. LAD: Left anterior descending artery, CX: circumflex artery.
Supplemental Figure III. Myocardial capillary density and perivascular fibrosis score in control and diabetic rat hearts. C, Capillary density assessed via isolectin B4 staining in control (A) and diabetic (B) rats (40x objective). F, Perivascular collagen expression was assessed via picrosirius red staining in control (D) and diabetic (E) rats (20x objective). Control, n=4 and diabetic n=6. Values expressed as mean±SEM.
**Supplemental Figure IV.** Change in vessel ID in control and diabetic animals during infusion of vasoactive compounds categorized by vessel class ACh (A), SNP (B), L-NAME (C), Combined; L-NAME + meclofenamate (D) and Combined + ACh (E). Control, n=5 and diabetic n=6. Values expressed as mean±SEM. #P<0.01, ^P<0.001 vs. baseline; †P<0.05 vs. control.
Supplemental Figure V. Representatives synchrotron radiation cine-angiograms from the same diabetic rat depicting focal constriction after NOS and COX blockade. A, baseline vehicle infusion where no focal constriction is present and B, focal constriction (black arrows) persisting over the entire cardiac cycle following L-NAME and meclofenamate treatment. *Shows position of tertiary branching point upstream of focal constriction site (frame 14 in A).
Supplemental Figure VI. The occurrence of segmental constrictions classified by branching order.

Segmental constrictions predominately occurred in 2nd order vessels in control animals. In diabetic animals 2nd order vessels were also preferentially affected, but with combined blockade further segmental constrictions appeared in 1st order branching vessels. Segmental constrictions are expressed as a proportion of total vessels visible.
A video file created from the first 30 frames of a cine-angiogram recording.

**Supplemental Movie I.** Representative cine-angiogram recording depicting focal and segmental constrictions in diabetic coronary microvasculature following NOS and COX inhibition. Focal constrictions can be observed at two sites during the angiogram, slightly downstream from major branching points. An obvious segmental constriction can be observed distal to the focal constrictions. Cine-recording of the same heart presented in Figure 4 and Supplemental Figure V. For the purpose of presentation the recording is replayed at 7 frames/sec (approximately 1/5 actual recording speed). Field of view is 9.5 x 9.5 mm and a 50 µm tungsten wire (bottom right) was included for calibration.