Impaired Myogenic Tone in Isolated Cerebral and Coronary Resistance Arteries from the Goto-Kakizaki Rat Model of Type 2 Diabetes

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Key Words

Myogenic tone • Intracellular calcium • Resistance arteries • Endothelium • Type 2 diabetes

Abstract

Aim: Type 2 diabetes is associated with stroke and cardiac dysfunction. We therefore investigated isolated middle cerebral arteries and coronary septal arteries from the diabetic Goto-Kakizaki (GK) rat model of nonobese type 2 diabetes. **Methods:** Myogenic tone and agonist-induced responses were investigated under isobaric conditions with simultaneous recording of $[Ca^{2+}]_i$. Rho-kinase and NO pathways were investigated using specific pharmacological tools. **Results:** Arteries from GK rats developed less tone at pressures from 20 to 100 mm Hg than arteries from control Wistar (CW) rats while $[Ca^{2+}]_i$ was similar. Blocking the Rho-kinase pathway decreased the pressure-induced development of tone and after blockade no difference in myogenic tone between arteries from GK and CW rats was seen. Cerebral arteries had similar tone to a maximal concentration of U46619 (GK: $35.5 \pm 2\%$ vs. CW: $31.6 \pm 5\%$), while coronary arteries from GK rats developed less tone than arteries from CW rats (12 ± 3 vs. $26.1 \pm 3\%$). Endothelium-dependent vasodilation to A23187 (cerebral) and to acetylcholine (coronary) was not different between arteries from GK and CW rats. **Conclusion:**

Our data suggest that in resistance arteries from the brain and the heart of GK rats the myogenic tone is decreased due to impaired calcium sensitivity likely due to a defective Rho-kinase pathway.

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Introduction

Type 2 diabetes (T2D) is associated with the development of diabetic angiopathy, presenting with microvascular (retinopathy, nephropathy and peripheral neuropathy) and macrovascular (stroke, cardiac dysfunction and peripheral ischemic leg disease) complications [1–3]. The nature of these vascular abnormalities is not fully understood, but it is believed that the vascular abnormalities associated with diabetes partly arise from a disruption in the local control of flow in the resistance arteries [4, 5]. However, it is uncertain whether the same cellular abnormalities lead to the same vascular abnormalities in different vascular beds. The resistance arteries are crucial in controlling that local blood perfusion meets the tissue requirements and the control is exercised by changing the flow in the arteries by contraction of the vascular smooth muscle cells. A change in the contractile state of the vascular smooth muscle cells alters the diameter of the blood

vessels and, as resistance is related to the fourth power of the vessel radius, even small changes in internal diameter cause major changes in flow.

A specific type of smooth muscle contraction dominant in the microvasculature is the pressure-induced contraction, also known as the myogenic response [6]. This response plays a central role in the autoregulation of blood flow and control of capillary pressure [7–9] and recent studies have shown that both the structure [10] and myogenic response [11] are altered in resistance arteries from patients with diabetes. The myogenic response is the initial response to a given change in transmural pressure across the arterial wall. This change is followed by a new level of myogenic tone. [12]. Myogenic tone is considered the backbone upon which vasodilators, such as nitric oxide (NO) and calcium channel blockers, act to lower resistance [6].

The present study was undertaken to compare two vascular beds with significant impact on morbidity and mortality in T2D, namely the heart and the brain. In particular, we have investigated the hypothesis that the myogenic tone and the sensitivity of the contractile apparatus to intracellular calcium are abnormal in a rat model of nonobese T2D, the Goto-Kakizaki (GK) rat, and that the arteries from these rats express endothelial dysfunction.

Accordingly, we investigated the vascular response and myogenic tone in isolated cerebral and coronary septal arteries from GK rats and compared those to the responses of similar arteries from control Wistar (CW) rats. We particularly wanted to address the sensitivity of the contractile apparatus to Ca^{2+} and the potential role of Rho-kinase by using the pharmacologic tools Y-27632 and fasudil, which both have an IC_{50} of approximately 1 μM [13–15].

The GK rat model is derived by repetitive inbreeding of glucose-intolerant CW rats [16], and the GK rat strain presents with glucose intolerance, hyperglycemia, hypoinsulinemia and in some reports mild hypertension. These abnormalities develop spontaneously as early as 4 weeks after birth [17–21], and can to some degree be reversed by treatment with metformine [17]. We chose to use animals at a late stage of diabetes, i.e. 22 weeks, as this would provide a situation where the diabetes would be well manifested and the pathological abnormalities of the arteries would most likely be expressed [17, 22].

Our data suggest an impaired myogenic tone of both the cerebral and coronary resistance arteries and an attenuation of the calcium sensitivity of the contractile apparatus in both vascular beds, probably due to an attenuation of the Rho-kinase-dependent pathway.

Methods

Twenty-six male GK and CW rats (Wistar-Hannover-GALLAS) were used. All rats were obtained from Taconic Europe (Ry, Denmark) and had free access to drinking water and standard rat chow (Altromin 1324; Lage, Germany). At the age of 16 weeks a subset of rats was weighed and nonfasting blood glucose determined (Precision Xtra™; Abbott Laboratories Medisense Products, Bedford, Mass., USA) along with blood pressure measured by tail plethysmography. These measures were repeated at 22 weeks. Experiments with isolated arteries were conducted at the age of 22 weeks when the rats were anesthetized with gaseous carbon dioxide and decapitated before the heart and brain were removed and the hearts weighed. All animal experiments were conducted according to the Danish ethical guidelines.

Isobaric Diameter Measurement of Resistance Arteries

Myogenic tone in coronary septal arteries and middle cerebral arteries was examined in a pressure myograph system (DMT, Aarhus, Denmark) that allowed us to control the transmural pressure.

After decapitation, the brain and the heart were carefully removed and placed in a physiological salt solution (PSS; for composition see below) at 4°C. After placing the brain in a Petri dish, the proximal part of the middle cerebral artery was dissected free of surrounding connective tissue and a 3-mm branch-free segment between the circle of Willis and the point of bifurcation was harvested. When dissecting the heart, the right ventricular cavity was exposed and the coronary septal artery was carefully dissected free from the cardiac tissue. The 3 most distal millimeters of the coronary septal artery before the point of bifurcation were used. The arterial segments were then moved to the pressure myograph bath which was filled with oxygenated PSS. Care was taken to select segments without branches. The vessels were cannulated at both ends with oxygenated PSS-filled glass micropipettes. The distal pipette was occluded allowing experiments to be made under conditions of no flow. The proximal pipette was connected to a pressure regulator (Mini Pressure Regulator; DMT). The arteries were then emptied of residual blood by gentle flushing through the lumen and finally mounted on the proximal pipette and tied with a single strand of braided 4-0 silk suture. Only vessels without leaks were used for further experiments.

PSS was continuously bubbled with 5% CO_2 in air to maintain pH 7.4, and the temperature was kept at 37°C. The myograph was placed on the stage of an inverted microscope (Leica, Wetzlar, Germany) equipped with a CCD camera (SONY, model XC-75CE; Tokyo, Japan) and the external diameter of the vessels was measured from the video images of the preparation during continuous transillumination with red light (wavelength >600 nm).

Measurement of $[\text{Ca}^{2+}]_i$ in Resistance Arteries

After mounting, the arteries were loaded with 5 μM fura-2 acetoxymethyl ester for 1 h at 37°C. Fura-2 acetoxymethyl ester (50 μg) was dissolved in a 4- μl load mix (10 ml DMSO with 0.2 ml Cremophor EL and 40 mg Pluronic F 127) and the final DMSO concentration in the bath was 0.04%. After loading, the vessels were washed with PSS and rested for 15 min at 20 mm Hg. Fluorescence from the preparation was measured using epifluorescence. The preparation was excited by light alternating between two different wavelengths (340 and 380 nm), generated by feeding

the light from a 75W Xenon lamp into two separate monochromators by a computer-controlled optical chopper (DeltaScan Illuminator; PTI, UK). The transmitted light and fluorescence emission were collected by a 20× objective (NA 0.50; Leica) and directed to a microscope photometer (model D-104B/C; PTI) via a dichroic mirror (>400 nm). In the photometer, the transmitted red light was redirected to the video camera by a dichroic mirror (>580 nm) for measurements of external vessel diameters, and the fluorescent light passed through a 520–560-nm bandpass filter to a photomultiplier tube (PMT, model 710; PTI). All signals were digitized at 10 Hz and stored on computer.

The fluorescence ratio was calculated as the ratio of emitted light in response to excitation at 340 and 380 nm (340/380 nm), and taken as an estimate of $[Ca^{2+}]_i$.

At the end of each experiment at a pressure of 100 mm Hg, 40 μ M of ionomycin in a Ca^{2+} -free solution was added to the bath. After obtaining the lowest fluorescence ratio (R_{min}), 2.5 mM Ca^{2+} was added in order to obtain the highest fluorescence ratio (R_{max}). To quench the dye, allowing the background signal to be obtained, 20 μ M of manganese chloride was added to the bath. The background signal was subtracted from all fluorescence measurements. No differences were seen between the two groups of rats in either the cerebral arteries or the coronary septal arteries with respect to R_{min} , R_{max} or the background signal.

Experimental Protocol for Resistance Cerebral Arteries

In all arteries a pressure-diameter curve was obtained by increasing the pressure from 20 to 100 mm Hg in 20-mm Hg increments. The arteries were maintained at each pressure level for 5 min, allowing the vessel to reach a steady-state diameter. One group of arteries was then incubated with 100 μ M N-nitro-L-arginine (L-NAME) for 30 min at a pressure of 20 mm Hg before another pressure-diameter curve was obtained. The pressure was again set to 20 mm Hg and still in the presence of L-NAME the artery was incubated for 15 min with 3 μ M Y-27632 or fasudil before a third pressure-diameter curve was obtained.

In another group of arteries the pressure was set to 60 mm Hg for 15 min, after the pressure-diameter curve had been obtained, before a cumulative concentration-response curve to the thromboxane analog U46619 (0.01, 0.1, 0.3 μ M) or AVP (0.3, 1, 3, 10 μ M) was obtained. After washout the vessels were incubated for 15 min with 3 μ M Y-27632 at 20 mm Hg before a second pressure-diameter curve was obtained. Vessels were then washed and pressure set to 60 mm Hg before substituting PSS with 125 mM K^+ -PSS (see 'Solutions and Chemicals') for depolarization of the artery. A third group of arteries was precontracted with cumulative concentrations of U46619 at 60 mm Hg before being exposed to either increasing concentrations of the endothelium-specific calcium ionophore A23187 (0.1, 1, 10 μ M) or a single high concentration (10 μ M). In 4 experiments cerebral arteries from GK and CW rats were incubated with 100 μ M L-NAME before the application of A23187. At the end of each experiment a pressure-diameter curve was obtained in Ca^{2+} -free PSS containing 5 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid to obtain the passive diameter.

Experimental Protocol for Coronary Resistance Arteries

In all arteries a pressure-diameter curve was obtained as described above. One group of arteries was then incubated with 100 μ M L-NAME for 30 min at a pressure of 20 mm Hg before the

pressure-diameter curve was repeated. The pressure was again set to 20 mm Hg and the arteries were incubated with 3 μ M fasudil for 15 min before a third pressure-diameter curve was obtained. In another group of arteries the pressure was set to 60 mm Hg and after 15 min 3 cumulative concentrations of U46619 were applied (0.01, 0.1, 0.3 μ M) creating a concentration-response curve. After washout the pressure was set to 20 mm Hg and the artery was incubated with 3 μ M Y-27632 for 15 min before a pressure-diameter curve and a concentration-response curve to U46619 at 60 mm Hg were obtained. In a third group of arteries pressure was set to 60 mm Hg and arteries precontracted with 30 μ M U46619 before a cumulative concentration-response curve to ACh was obtained (0.1, 0.3, 1, 10 μ M). ACh was then washed out and the artery was incubated with 100 μ M L-NAME for 30 min before a concentration-response curve to ACh was obtained. ACh was again washed out and the artery exposed to 30 mM K^+ -PSS and 125 mM K^+ -PSS before and after incubation with 10 μ M nifedipine. At the end of each experiment a pressure-diameter curve was obtained under Ca^{2+} -free conditions as described for cerebral arteries.

Calculations

As response to increasing pressure (myogenic response) was assessed at steady state, results are presented as myogenic tone. Both myogenic tone and agonist-induced tone are expressed as relative diameter, which is the diameter as a percentage of the diameter in Ca^{2+} -free PSS [$1 - (D_{PSS}/D_{Ca-free}) \times 100\%$, where D is the diameter at the same pressures]. In the dilation curves 0% represents precontracted diameter to U46619 and 100% represents the diameter in Ca^{2+} -free solution.

Solutions and Chemicals

The composition of PSS was (in mM) 119 NaCl, 4.7 KCl, 1.18 KH_2PO_4 , 1.17 $MgSO_4$, 25 $NaHCO_3$, 1.6 $CaCl_2$, 0.026 EDTA and 5.5 glucose. K^+ -PSS was PSS in which NaCl was substituted by an equimolar concentration of KCl to reach 30 mM or 125 mM K^+ -PSS. The Ca^{2+} -free solution was PSS with no $CaCl_2$ and 5 mM EGTA. All chemicals were bought from Sigma Chemical (St. Louis, Mo., USA).

Statistics

All data are presented as mean $\pm$ SEM with a significance level of $p < 0.05$; n is the number of animals. Animal characteristics and maximal relaxation were compared by Student's t test. Differences between groups to increasing pressures were tested by 2-way ANOVA for repeated measures with Bonferroni's posttest. Effect of pharmacological treatment was tested by 2-way ANOVA for repeated measures (within groups). Statistical analyses were made with GraphPad Prism 4.02 (GraphPad Software, La Jolla, Calif., USA).

Results

Animal Parameters

The blood glucose levels were significantly higher in GK rats than in CW rats both at 16 and 22 weeks of age. The GK rats had a lower body weight at 16 and 22 weeks of age than the CW rats. At the age of 22 weeks, the hearts

Table 1. Characteristics of the animals of all experimental groups

	16 weeks		22 weeks	
	CW rats	GK rats	CW rats	GK rats
Blood glucose, 10^{-3} M	5.3 ± 0.1	$10.6 \pm 0.4^*$	5.7 ± 0.2	$9.2 \pm 1.9^*$
Body weight, g	375 ± 4	$351 \pm 5^*$	452 ± 13	$419 \pm 7^*$
Heart weight, g	NA	NA	1.09 ± 0.04	$1.39 \pm 0.06^*$
Heart weight/body weight	NA	NA	$2.4 \times 10^{-3} \pm 8 \times 10^{-5}$	$3.3 \times 10^{-3} \pm 11 \times 10^{-5}^*$
Blood pressure, mm Hg	116 ± 1	118 ± 1	122 ± 5	127 ± 4

Values are mean $\pm$ SEM and were analyzed by Student's t test; n = 8–20 rats per group. * p < 0.05, GK rats vs. age-matched CW rats. NA = Not assessed.

of the GK rats were larger than the hearts of the CW rats both in absolute terms and in relation to body weight despite a similar blood pressure at the respective ages (table 1).

Isobaric Diameter of Middle Cerebral Arteries

The passive diameter of the cerebral arteries in Ca^{2+} -free conditions (at 100 mm Hg) was similar in the two groups: 215 ± 10 versus $199 \pm 8 \mu\text{m}$ (nonsignificant, NS; n = 20, 26) GK and CW rats, respectively. Under control conditions arteries from GK rats developed significantly less tone at all intraluminal pressures (20, 40, 60, 80 and 100 mm Hg) than CW arteries ($p < 0.05$; n = 10, 16; fig. 1a, 2a). Incubation with L-NAME did not affect myogenic tone (fig. 3a) or $[\text{Ca}^{2+}]_i$ (not shown) indicating that the mechanisms responsible for the difference in tone were NO independent. Incubation with the Rho-kinase inhibitor fasudil substantially reduced the myogenic tone in CW arteries while no effect was seen in GK arteries, and in the presence of Rho-kinase inhibition no difference was seen between the arteries from GK and CW rats (fig. 3b). Using Y-27632 – another Rho-kinase inhibitor – also eliminated the difference between GK and CW arteries (data not shown).

Activation with increasing concentrations of the thromboxane analog U46619 revealed that cerebral arteries from GK and CW rats developed the same tone at the maximal concentration used, i.e. 32 ± 4 versus $38 \pm 2\%$ (NS; n = 8, 10) GK and CW rats, respectively (fig. 4a). Incubation with Y-27632 had little effect on tone in GK arteries but substantially decreased maximal tone in CW arteries and in the presence of Y-27632 the tone at the maximal concentration of U46619 was significantly larger in arteries from GK than in arteries from CW rats.

Also, during stimulation with $10 \mu\text{M}$ AVP (which is a supramaximal concentration) arteries from GK and CW rats had the same tone, i.e. 38 ± 5 versus $34 \pm 5\%$ (NS; n = 3, 3) GK and CW rats, respectively, which was also the case when the arteries were depolarized by 125 mM K^+ -PSS: 43 ± 3 versus $40 \pm 3\%$ (NS; n = 5, 5) GK and CW rats, respectively.

To test the endothelial function, the cerebral arteries were precontracted with $0.3 \mu\text{M}$ U46619 before exposing them to either increasing concentrations of the calcium ionophore A23187 (fig. 5a) or a single high concentration of $10 \mu\text{M}$ A23187 (fig. 5b). A stepwise increase in concentrations revealed a concentration dependency of the response, with a maximal relaxation of 61 ± 10 and $74 \pm 4\%$ (NS; n = 3, 3) at the highest concentration of A23187 ($10 \mu\text{M}$), in GK and CW rats, respectively (fig. 5a). A single high concentration resulted in an almost complete dilation of arteries from both groups, i.e. 89.8 ± 4.0 versus $91.4 \pm 3.0\%$ (NS; n = 6, 9), in GK and CW rats, respectively. Following incubation with L-NAME the relaxation to A23187 was significantly reduced to 42 ± 11 and $48 \pm 7\%$ ($p < 0.05$; n = 3, 3), in GK and CW rats, respectively (fig. 5b).

Despite the observation that arteries from GK rats had less myogenic tone compared to arteries from CW rats, there were no differences in $[\text{Ca}^{2+}]_i$ between the two groups throughout the entire pressure range (fig. 1a, 6a). After incubation with fasudil we still observed the same levels of $[\text{Ca}^{2+}]_i$ in the two groups throughout the entire pressure range (fig. 6a). Furthermore, in the absence of extracellular Ca^{2+} there was no difference in $[\text{Ca}^{2+}]_i$ between arteries from the two groups of rats, i.e. fluorescence ratio 1.1 ± 0.2 versus 1.1 ± 0.1 at 60 mm Hg (NS; n = 9, 10), in GK and CW rats, respectively.

Fig. 1. Original traces of diameter and intracellular calcium concentrations (ratio of fura-2 fluorescence with excitation at 340 and 380 nm) of middle cerebral arteries (**a**) and coronary septal arteries (**b**) from CW and GK rats, as indicated. Transmural pressure was raised from 20 to 100 mm Hg in 20-mm Hg increments, as indicated by black bars. Arteries from GK rats show less myogenic tone than CW arteries. Fura-2 Ca^{2+} -dependent fluorescence is similar in both middle cerebral arteries and coronary septal arteries when comparing arteries from CW to GK rats.

In 4 time control experiments with 3 repeated pressure-diameter curves no significant change in tone was seen, and repeating the U46619 concentration-response curve did not reveal any time-dependent change of response.

Isobaric Diameter of Coronary Septal Arteries

There was no difference in the average diameter of the coronary arteries at 100 mm Hg in Ca^{2+} -free conditions (292 ± 9 vs. 272 ± 11 μm , GK and CW rats, respectively, NS; $n = 19, 18$). In PSS, the myogenic tone was signifi-

cantly lower in the coronary septal arteries from GK rats compared to arteries from CW rats ($p < 0.05$; $n = 7, 8$; fig. 1b, 2b). This was also true after blocking of the NO synthesis as L-NAME had no significant effect on arteries from either GK or CW rats (fig. 3c). In the presence of the Rho-kinase inhibitors fasudil (fig. 3d) or Y-27632 (not shown) there was no difference in myogenic tone between arteries from GK and CW rats.

Increasing U46619 concentration at 60 mm Hg increased the tone of the coronary arteries from both GK and CW rats (fig. 4b). The tones at the highest concen-

Fig. 2. Pressure-diameter curves of middle cerebral arteries (**a**) and septal coronary arteries (**b**) from CW and GK rats. $n = 5-12$; * $p < 0.05$ and *** $p < 0.001$, GK vs. CW (2-way ANOVA repeated measures).

Fig. 3. Pressure-diameter curves of middle cerebral arteries (**a, b**) and septal coronary arteries (**c, d**) from CW and GK rats made under control conditions. **a, c** Effect of incubation with the L-NAME. **b, d** Effect of incubation with fasudil. $n = 5-12$; * $p < 0.05$ and *** $p < 0.0001$, treated vs. untreated (2-way ANOVA repeated measures).

Fig. 4. Concentration response curves to U46619 in middle cerebral arteries (a) and septal coronary arteries (b) from CW and GK rats under control conditions and after incubation with the Rho-kinase inhibitor Y-27632. n = 8–10; * p < 0.05 and ** p < 0.01, GK vs. CW arteries (2-way ANOVA repeated measures).

Fig. 5. Endothelium-mediated vasodilation in middle cerebral arteries (a, b) and coronary septal arteries (c). a The relaxation to cumulative concentrations of A23817 in middle cerebral arteries from CW and GK rats. b The relaxation of middle cerebral arteries to a single concentration (10 μM) of A23187 before and after incubation with 100 μM L-NAME. c The relaxation of coronary septal arteries to increasing concentrations of ACh in septal coronary arteries from CW and GK rats, before L-NAME and in the presence of L-NAME. n = 4–9; ** p < 0.001, treated vs. untreated (Student's t test).

Fig. 6. Pressure-fluorescence ratio $[Ca^{2+}]_i$ curves of middle cerebral arteries (a) and coronary septal arteries (b) from CW rats and GK rats made under control conditions and after incubation with the Rho-kinase inhibitor fasudil. $n = 4-10$. GK vs. CW arteries (2-way ANOVA repeated measures).

tration of U46619 were 12 ± 3 and $26 \pm 3\%$ ($p < 0.05$; $n = 10, 10$), in GK and CW rats, respectively. Incubation with Y-27632 decreased the response in both groups, and the tones in the presence of the highest concentration of U46619 were 2.6 ± 0.6 and $12.2 \pm 3.2\%$ ($p < 0.05$; $n = 10, 10$), in GK and CW rats, respectively, (fig. 4b).

Depolarization of the smooth muscle cells by 30 and 125 mM K^+ -PSS caused tone development and the tone during depolarization was similar in both groups (fig. 7). In a subset of animals endothelial function of the coronary arteries was investigated by obtaining cumulative concentration-response curves to ACh on arteries pre-constricted with U46619. These curves showed no difference in the sensitivity or the maximal relaxing response (66 ± 9 vs. $62 \pm 10\%$, in GK and CW rats, respectively, NS; $n = 9, 8$; fig. 5c). The ACh-mediated vasodilation was completely abolished in the presence of L-NAME (fig. 5c). In 3 experiments 2 consecutive relaxation curves to ACh on the same vessel without L-NAME were obtained. These time control experiments showed that ACh could elicit full relaxations both times. Despite the differences in tone between the two groups we observed no differences in $[Ca^{2+}]_i$ over the entire pressure range (2-way ANOVA, NS) under control conditions, and after incubation with fasudil there were still no differences in $[Ca^{2+}]_i$ (fig. 1b, 6b), nor were there any differences in the absence of extracellular Ca^{2+} between the two groups in $[Ca^{2+}]_i$ (fluorescence ratio 1.4 ± 0.1 vs. 1.2 ± 0.2 at 60 mm Hg, in GK and CW rats, respectively, NS; $n = 9, 10$). Since

Fig. 7. Contraction of coronary arteries to 125 mM K^+ (K^+ -PSS) before and after inhibition with nifedipine. $n = 5$. * $p < 0.001$, 30 mM K^+ -PSS vs. 125 mM K^+ -PSS.

there was little myogenic tone and no pressure-induced increase of $[Ca^{2+}]_i$ in the arteries, we measured the effect of the L-type Ca^{2+} channel blocker nifedipine on the contraction in arteries depolarized by 30 and 125 mM K^+ -PSS. Nifedipine decreased the tone at both 30 and 125 mM

K⁺-PSS (fig. 7). In 4 time control experiments with 3 repeated pressure-diameter curves no significant change in tone was seen.

Discussion

The vascular abnormalities seen in diabetes are far from being fully characterized, possibly because they not only differ with the duration, severity and type of diabetes, but also may differ within the different vascular beds from the same individual [23, 24]. The mechanism underlying diabetes-induced alterations of myogenic responses are also unclear. Some studies point towards a diabetes-induced increased myogenic tone, possibly via decreased responsiveness of the smooth muscle K-ATP-sensitive potassium channels [25], hyperglycemia-mediated activation of ET-1 and ET_A receptors leading to vascular remodeling [26] or reduced endothelial function [27–29], while other studies point at reduced myogenic tone [30].

In the present study we found a reduced myogenic tone in isolated middle cerebral arteries and coronary septal arteries from the spontaneously diabetic GK rat, a model of nonobese T2D. The reduced myogenic tone seems to be caused by decreased Ca²⁺ sensitivity, probably due to a defective Rho-kinase pathway.

The vascular bed investigated is of key importance in cardiovascular pathology and is considered to be of importance for the consequences of microvascular angiopathies [1–3]. To ensure comparability we aimed at developing comparable protocols for the two vascular beds. However, as the cerebral arteries surprisingly did not respond to acetylcholine or bradykinin (unpubl. observation), A23187 was used as an endothelium-dependent vasodilator in these arteries. The 22-week-old rats used in this study were hyperglycemic but not hypertensive either at the age of 16 or 22 weeks. Chronic hypertension can therefore not explain the observed enlargement of the hearts. However, it is well established that GK rats develop features of diabetic cardiomyopathy [31], and this possibly explains the larger relative heart weight found in GK rats in this study.

The reduced myogenic tone in cerebral and coronary arteries from GK rats found in this study is in accordance with, and further extends, previous studies showing decreased myogenic tone in cerebral arteries from 18-week-old GK rats [17] and from human retinal and subcutaneous arteries [11, 30], but contradicts results on cerebral arteries from rats with streptozotocin-induced diabetes showing increased myogenic tone [25]. As there was no

difference in response between arteries (both cerebral and coronary) from GK and CW rats to full depolarization with 125 mM K⁺-PSS, it was apparent that the difference in development of myogenic tone was not due to a defective contractile apparatus.

We therefore investigated intracellular calcium and found similar fura-2 fluorescent ratios in response to increasing pressures in both the GK and CW rat vascular preparations. This suggests that the attenuation of myogenic tone is due to a decreased Ca²⁺ sensitivity. In the fura-2 measurements the R_{min}, R_{max} and the background signal were identical between the two groups of animals, supporting the theory that the fluorescence ratio is reflecting [Ca²⁺]_i similarly in both groups. The data therefore strongly suggest that Ca²⁺ handling is not different in the arteries from the two groups.

To provide information on the background for the decreased Ca²⁺ sensitivity, which explained the decreased myogenic response, we used the Rho-kinase inhibitors Y-27632 and fasudil. Both compounds inhibit the phosphorylation of the myosin light chain, preventing the myosin cross-bridge to bind to the actin filaments and causing relaxation of the smooth muscle cells [32]. Y-27632 is considered a specific inhibitor of the Rho-kinase pathway [33], whereas fasudil is known to also affect the PKA and possibly the PKC pathways [34].

In the presence of the Rho-kinase inhibitors little myogenic tone was seen in either group and there was no difference between the groups. This suggests that a difference in Rho-kinase activity could explain the reduced Ca²⁺ sensitivity which underlies the reduced myogenic tone in both cerebral and coronary arteries, as previously shown for other diabetic animal models [35].

When stimulated with increasing concentrations of the vasoconstrictor U46619, both cerebral and coronary arteries showed a concentration-dependent increase in tone. In the cerebral arteries we did not observe a difference in tone to the highest concentration of U46619 used. Consistent with this, the tone in the presence of a supra-maximal concentration of AVP was also similar between the groups. This suggests that in cerebral arteries agonist-induced responses are not different between arteries from GK and CW rats. However, this interpretation is complicated by the fact that the agonist-induced tone is developed on top of a myogenic tone which is different in the two groups.

The complexity of the agonist-induced responses was further underlined by the finding that cerebral arteries from GK rats developed more agonist-induced tone after inhibition of the Rho-kinase than the arteries from the

CW rats. Although this finding is consistent with reduced signaling through the Rho-kinase pathway, it also suggests that a Rho-kinase-independent pathway for tone production is more prominent in cerebral arteries from GK rats compared to arteries from CW rats.

For the coronary arteries the situation was different. With the highest concentration of U46619 used the tone was significantly reduced in arteries from GK rats compared to arteries from CW rats both under control conditions and during Rho-kinase inhibition. This suggests the presence of a defect in the agonist-induced signaling in the coronary arteries which is not mediated by the Rho-kinase pathway. Possible candidates for these defects could include diabetes-induced alterations in the PKA [36] and the PKC [37, 38] pathways as recently shown for isolated glomeruli, but not for brain and liver microvessels, in 9-month-old GK rats [24]. These findings could possibly support our finding of similar agonist-induced responses in cerebral arteries from GK and CW rats.

The link between diabetes and increased activation of the PKC pathway is believed to involve the state of chronic hyperglycemia which leads to an increased level of circulating advanced glycation end products. The circulating advanced glycation end products bind to the endothelial-bound signal-transduction receptor RAGE, which in turn leads to activation of the smooth muscle cell PKC and thus oxidative stress [39, 40]. It would therefore also be interesting in future studies to take into account the effects of glucose-induced PKC activation in hyperglycemic versus normoglycemic diabetes models [41].

The complexity of the vascular responses in the GK rats is further underlined by the observation that in mesenteric small arteries the myogenic tone was increased compared to mesenteric arteries from CW rats [26]. This finding was supported under isometric conditions by our finding of an increased tension development in mesenteric small arteries from GK rats [24] and further stresses that the vascular dysfunction in diabetes may vary between different vascular beds. In this respect it is also interesting that Sandu et al. [42] found evidence of increased basal and thrombin-induced Rho-kinase activity in primary cultured smooth muscle cells from GK rat aortas and concluded that this would lead to enhanced vascular tone. This contrasts with the reduced tone seen in the present study and the evidence of a defective Rho-kinase activity. Whether this difference is due to differences between the aorta and the cerebral and coronary resistance arteries or to the different experimental approaches is difficult to know.

The ACh concentration-dependent relaxation of coronary arteries and the A23187 concentration-dependent relaxation of cerebral arteries were almost complete and without differences between the CW and GK rat arteries. This suggests that no endothelial dysfunction was present in either of these vascular beds. This is in accordance with what we and others have previously shown in small mesenteric arteries [43] and aortic rings [32] from this rat model, although in the basilar artery from GK rats the ACh-induced isometric relaxation has been reported to be reduced [22]. The observation that the relaxation we report is inhibited by L-NAME indicates that the relaxation in the two vascular beds is dependent on the NO pathway, and that NO pathway is not impaired in either of the investigated vascular beds from the GK rat. Inhibition of NO production had no effect on either myogenic tone or $[Ca^{2+}]_i$ in the cerebral arteries or in the coronary septal arteries, indicating that NO has no explanatory potential in relation to the reduced myogenic tone in the GK rats. It is surprising that the inhibition of NO production did not affect myogenic tone in these arteries, as the presence of NO and constrictor prostaglandins have been shown to be essential for the normal development of the myogenic response in rat coronary arteries [44, 45]. However, since inhibition of NO production with L-NAME did inhibit the agonist-induced endothelial-mediated relaxation, we believe that the lack of effect of L-NAME on myogenic tone reflects a lack of NO-mediated contribution to myogenic tone.

The lack of endothelial dysfunction found in this study is somewhat surprising as diabetes is often associated with endothelial dysfunction, both in different animal models of T2D and in humans [30, 46–48]. One explanation may be that the GK rat is only moderately hyperglycemic when compared to other models of T2D, in particular as seen in models of obese T2D, e.g. the Zucker diabetic fatty rat [49–51]. Our findings that blood glucose was increased at the age of both 16 and 22 weeks indicate that at least in situations with mild chronic hyperglycemia the effect of NO is not altered. Also, our data suggest that mild hyperglycemia alone may not be sufficient to induce endothelial dysfunction, which has also been concluded by Bohlen and Lash [52] in the Zucker diabetic fatty rat, where there is no endothelial dysfunction present as long as hyperglycemia is associated with hyperinsulinemia.

In conclusion, in the GK rat model of lean T2D a decreased myogenic tone in cerebral and coronary resistance arteries occurs due to decreased calcium sensitivity. This is most likely explained by a defective Rho-kinase

pathway. During agonist stimulation complex changes of signaling, which appear different in cerebral and coronary arteries, were present. It is of further interest that this model of diabetes appears without dysfunction of the endothelium-mediated NO-dependent relaxation despite hyperglycemia. These findings stress the complexity of the vascular dysfunction associated with diabetes.

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