Increased myogenic tone in skeletal muscle arterioles of diabetic rats. Possible role of increased activity of smooth muscle Ca\(^{2+}\) channels and protein kinase C

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Abstract

Objective: The diabetes mellitus-induced microangiopathy is still not clearly characterized. In this study we aimed to elucidate the effect of streptozotocin (STZ)-induced diabetes on myogenic response of isolated rat skeletal muscle arterioles and the mechanisms responsible for its alterations. Methods: Male rats were divided into two groups: (1) control rats (C, plasma glucose: 6.4±0.5 mmol/l, n=40) 2) diabetic rats (DM, 65 mg/kg STZ iv, plasma glucose: 25.7±0.7 mmol/l, n=40). Changes in diameter of isolated, cannulated gracilis skeletal muscle arterioles (~130 \(\mu\)m in diameter) were measured by video-microscopy. Results: Step increases in perfusion pressure (PP; from 10 to 140 mmHg) elicited significantly greater constrictions in DM than in C gracilis arterioles, in the presence of the endothelium (E). Also, a step increase in PP (from 40 to 100 mmHg) elicited greater and faster constrictions in DM vs. C arterioles. There were no significant differences in the pressure-passive diameter (in Ca-free solution) curves of arterioles. Dilations to acetylcholine were impaired in arterioles of DM as compared to those of C rats (EC50, C: 4.0±0.9×10\(^{-5}\) mol/l, DM: 4.8±2.0×10\(^{-5}\) mol/l (p<0.01), and unaffected by inhibition of nitric oxide synthesis with L-NNA (10\(^{-4}\) mol/l). Arteriolar constrictions to norepinephrine (NE) were significantly greater in DM compared to those of C rats (EC50, C: 6.2±0.6×10\(^{-7}\) mol/l, DM: 8.0±2.0×10\(^{-8}\) mol/l, p<0.01) both in the presence and absence of E. In the absence of the E, constrictions to increases in pressure, or Ca\(^{2+}\) (0.25–7.5 mmol/l), or the voltage-dependent Ca\(^{2+}\)-channel agonist Bay K 8644 (EC50, DM: 4.2±1.5×10\(^{-10}\) mol/l, C: 1.7±0.8×10\(^{-9}\) mol/l, p<0.05) or the protein kinase C activator phorbol 12-myristate 13-acetate (PMA, EC50, DM: 6±2×10\(^{-9}\) mol/l, C: 2±1×10\(^{-8}\) mol/l, p<0.05) were significantly greater in arterioles of DM compared to those of C rats. Conclusion: The novel findings of our study are that in diabetes mellitus the myogenic response of rat skeletal muscle arterioles is enhanced, which seems to be independent from the impaired endothelial function present simultaneously, and likely due to the increased activity of voltage-dependent Ca\(^{2+}\) channels and/or upregulation of protein kinase C in arteriolar smooth muscle. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The microangiopathy described in diabetes mellitus is still not well characterized. Studies in both human and experimental diabetes mellitus have demonstrated changes in blood vessel function which are thought to underlie retinopathy, nephropathy, neuropathy and increase the risk of hypertension, myocardial infarction and stroke [1–3]. Although the multifactorial effects of diabetes on the various regulatory mechanisms governing the diameter of blood vessels are not well understood, it is likely that alterations in vascular reactivity precede the structural changes seen in the established phase of diabetic microangiopathy. Alterations in the local regulation of skeletal muscle arteriolar tone [4–6] can lead to microvascular...
disturbances contributing to the development of diabetic foot syndrome described in diabetes mellitus [1–3].

One of the important mechanisms that regulates vascular resistance is the myogenic response which operates primarily in microvessels [7,8]. There are few studies, however, which have investigated the possible alteration of the myogenic response in diabetes, especially in skeletal muscle microcirculation. In vivo studies of cremasteric arterioles of streptozotocin (STZ)-induced diabetic rats demonstrated an impaired myogenic constrictor response [9]. In isolated cremaster arterioles of rats, Hill and Ege [10] showed that both passive and active characteristics of pressure-induced responses of arterioles have been changed in STZ-induced diabetes, and concluded that myogenic response is impaired.

In certain conditions the function of the endothelium can modify [8,11] the myogenic responses of skeletal muscle arterioles. Previous studies have shown, that diabetes impairs endothelial functions resulting in reduced NO-mediated dilation to ACh [10,13–18] and constriction to increases in perfusate flow [12]. Furthermore, Zimmermann et al. [19] have demonstrated that impaired endothelial NO synthesis elicits an increased myogenic constriction of cerebral arteries isolated from STZ-induced diabetic female rats. The differences between the impaired [10] and enhanced [19] myogenic responses could be due to the fact that the characteristics of the myogenic response are different in various microvascular beds [7,8,19,20] and that diabetes-induced alterations in myogenic response is organ specific due to the redistribution of blood flow in diabetes [4]. Furthermore recent studies indicate a greater role for endothelium in modifying the myogenic response of arterioles from female rats compared to that of males [21,22] which may also explain the different alterations of the myogenic response in the above-mentioned studies.

There are several reports demonstrating alterations of Ca$^{2+}$ signaling in the smooth muscle of large vessels eliciting enhanced contractile response to agonists in diabetes [23–25]. Some of these pathways are known to be involved in the mechanotransduction of intravascular pressure changes, yet the possible effect of these alterations on the myogenic response of skeletal muscle arterioles have not been clearly elucidated. On the basis of these findings and our preliminary observations indicating an enhanced response of skeletal muscle arterioles to increases in intraluminal pressure we hypothesized that these alterations would rather enhance than impair the myogenic response of skeletal muscle arterioles in diabetes mellitus.

Thus, first we aimed to characterize the alterations of myogenic response in arterioles of gracilis skeletal muscle of STZ-diabetic rats. The second aim of this study was to elucidate the mechanisms responsible for the alteration in myogenic responsiveness of these arterioles in diabetes, by assessing the synthesis/release of endothelium-derived nitric oxide, and characterizing the function of voltage-dependent Ca$^{2+}$ channels and protein kinase C (PKC) in smooth muscle known to be involved in the development of the myogenic response.

2. Methods

Diabetes was induced in male Wistar rats (n=40, weighing ~120 g) by a single caudal intravenous injection of streptozotocin (STZ, 65 mg/kg) dissolved in 0.1 mol/l citrate buffer. Non-diabetic control animals (C, n=40) were injected with an equivalent volume of vehicle. All rats were allowed to drink a 10% glucose solution in the first 24 h after injection of STZ. Animals were housed separately, fed standard rat chow, and had free access to drinking water. Blood glucose concentration was measured by glucose oxidase method once a week. Rats were considered as diabetic when blood glucose levels were >20 mmol/l and glycosuria was present. All experiments were performed in accordance with the Guiding Principles in the Care and Use of Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.1. Isolation of arterioles

On the 12th week of diabetes, experiments were conducted on isolated first-order arterioles (approximately 130 μm diameter) of rat gracilis muscle as previously described [8]. Rats were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg) and the gracilis muscle was exposed by an incision of the skin and isolated from surrounding tissues. The muscle was then dissected out and placed in a silicone-lined Petri dish containing cold (0–4°C) physiological salt (PS) solution composed of (mmol/l): 110 NaCl, 5.0 KCl, 2.5 CaCl$_2$, 1.0 MgSO$_4$, 1.0 KH$_2$PO$_4$, 10.0 dextrose and 24.0 NaHCO$_3$ and was equilibrated with a gas mixture of 10% O$_2$ and 5% CO$_2$, balanced with nitrogen, at pH 7.4. Using microsurgery instruments and an operating microscope, a segment ~1.5 mm in length, of the first-order arteriole running intramuscularly was isolated, and transferred in an organ chamber containing two glass micropipettes filled with PS solution. From a reservoir the vessel chamber (15 ml) was continuously supplied with PS solution at a rate of 40 ml/min. After the vessel had been mounted on the proximal micropipette and was secured with sutures, the perfusion pressure was raised to 20 mmHg to clear the clotted blood from the lumen. Then the other end of the vessel was mounted on the distal pipette. Both micropipettes were connected with silicone tubing to an adjustable reservoir. Pressure on both sides were measured by an electromanometer. The perfusion pressure was slowly (approximately over 1 min) increased to 80 mmHg. The temperature was set at 37°C by a temperature controller.
(Grant Instruments, Great Britain) and the vessel was allowed to equilibrate for approximately 1 h.

2.2. Experimental protocols

Only those vessels which developed spontaneous tone in response to increases of perfusion pressure were used and thus no vasoactive agent was added to the PS solution to establish arteriolar tone. After the equilibration period changes in the diameter of arterioles in response to increases in perfusion pressure (from 10 to 140 mmHg, in 10 mmHg steps) were measured under zero-flow conditions. The pressure was maintained for 5–10 min at each pressure step to allow the vessel to reach a steady-state diameter. At the conclusion of each experiment, the suffusion solution was changed to a Ca$^{2+}$-free PS solution, which contained sodium nitroprusside (SNP, 10$^{-4}$ mol/l) and EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 10$^{-3}$ mol/l). The vessel was incubated for 10 min, and the pressure steps were repeated to obtain the maximum passive diameter at each pressure value (pressure-passive diameter relationship). Measurements of internal diameter at the midpoint of the arteriolar segment were made using a high resolution CCD video camera (Sony SSC M-370-CE) connected to a microscope (Carl Zeiss, Jena) and a video calliper calibrated using a stage micrometer [8]. The diameter was recorded with a chart recorder (Cole-Palmer).

In other experiments, pressure was increased from 40 mmHg to 100 mmHg in one step, and the time course of the development of myogenic constriction was recorded for 5 min. Then the suffusion solution was changed to a Ca$^{2+}$-free PS solution, which contained EGTA (10$^{-3}$ mol/l), the vessel was incubated for 10 min, and the pressure step (from 40 to 100 mmHg) was repeated. Diameters were normalized to the passive diameter at 100 mmHg pressure. In order to characterize the dynamics of the myogenic response a mono-exponential curve was fitted to the time-diameter data according to the following formula of Sipkema et al. [26] $y = a + d e^{-(t/r)}$ where ‘a’ is the amplitude of the myogenic constriction, ‘d’ is the plateau diameter (as a percentage of the PD$_{100}$) and ‘r’ is the time constant indicating the speed of the myogenic constriction.

In a second series of experiments the myogenic tone of arterioles from control and diabetic rats was compared in the absence of the endothelium. The endothelium of the cumulative dose–response curves of vasoactive agents, the pressure was maintained for 5–10 min at each pressure value (pressure-passive diameter relationship). The pressure was then increased to 80 mmHg for 30 min to establish a stable tone. The efficacy of endothelial denudation was ascertained by arteriolar responses to acetylcholine (ACh; 10$^{-7}$ mol/l), and sodium nitroprusside (SNP; 10$^{-7}$ mol/l) before and after the administration of the air bolus. The infusion of air resulted in loss of function of the endothelium, as indicated by the absence of dilation to ACh, whereas dilation to SNP remained intact. Moreover, the vasoactive function of endothelium and smooth muscle was assessed by peak responses of arterioles to cumulative doses of ACh (10$^{-10}$ to 10$^{-6}$ mol/l; an endothelium-dependent agent), SNP (10$^{-10}$ to 10$^{-6}$ mol/l; an endothelium-independent agent) and norepinephrine (NE, from 10$^{-10}$ to 10$^{-5}$ mol/l). All drugs were added to the vessel chamber, and final concentrations are reported. After responses to each drug subsided the system was flushed with PS solution. The vessel was then incubated with N$^\omega$-nitro-l-arginine (L-NNA; 10$^{-4}$ mol/l) for 30 min and responses to ACh and SNP were reassessed. In a further series of experiments, constrictor responses of arterioles of control and diabetic rats to NE were compared before and after removal of the endothelium.

All salts and chemicals were obtained from Sigma-Aldrich and were prepared on the day of the experiment. Bay K 8644 was dissolved in ethanol and protected from light; experiments were carried out in the dark. PMA was dissolved in ethanol. The vehicle did not have vasoactive effect.

2.3. Data analysis

Dilatations were expressed as a percentage of the maximal dilation of the vessel defined as the passive diameter at 80 mmHg perfusion pressure in Ca$^{2+}$-free media containing 10$^{-3}$ mol/l EGTA and 10$^{-4}$ mol/l SNP. Constrictions were expressed as a percentage of baseline. From the cumulative dose–response curves of vasoactive agents, the half-maximal effective concentrations (EC$_{50}$) were calculated (sigmoidal non linear least-squares fit). Data are expressed as means±SEM, except for the regression parameters where SD values are given. Statistical analyses were performed by two-way analysis of variance (ANOVA) for repeated measures followed by Tukey’s post hoc test or Student’s t-test. $P<0.05$ was considered statistically significant.
3. Results

3.1. Effects of STZ-induced diabetes on standard parameters of rats

Glycosuria developed within 24 h of STZ administration and was maintained throughout the 12-week period of diabetes. Within 1 wk of injection, fasting blood glucose levels exceeded 20 mmol/l in over 95% of the rats receiving STZ. After 12 weeks all STZ treated rats had glycosuria and elevated plasma glucose levels as compared to controls (C: 6.4±0.5 mmol/l; DM: 25.7±0.7 mmol/l). Body weights of diabetic rats were depressed relative to controls (C: 350±10 and DM: 226±9 grams). There was no significant difference between the systolic blood pressure of control and STZ-diabetic rats (104±3 and 108±3 mmHg, respectively).

3.2. Pressure–diameter relationships of arterioles

Isolated first-order arterioles of gracilis muscle from control rats developed active tone in response to step increases in PP (10–140 mmHg) without the use of any vasoactive agent (Fig. 1, panel A). Initially, the diameter of these vessels increased from ~100 µm to ~150 µm in response to an increase in intravascular pressure from 10 to 40 mmHg. Beyond this point, further increases in pressure resulted in constriction of arterioles. Similarly, pressure–diameter relationships were obtained in arterioles from diabetic animals. Initially, the diameter of diabetic arterioles increased similarly as controls. Further increases in pressure from 40 to 140 mmHg however, elicited a significantly greater constriction of arterioles from diabetic rats (Fig. 1, panel A). In the absence of Ca²⁺ (and in the presence of SNP 10⁻⁴ mol/l) the pressure–passive diameter relationship in each arteriole was also obtained (Fig 1, panel A). Step increases in pressure elicited continuous increases in the diameter of arterioles, reaching a plateau at 100 mmHg. The pressure-passive diameter relationship of arterioles from each group did not differ significantly.

In order to indicate the strength of myogenic tone, the diameter of arterioles in active and passive conditions were compared (see Methods). Fig. 1, panel B depicts the changes in the diameter of arterioles as a percentage of their passive diameter at corresponding perfusion pressures. We found that arterioles from diabetic rats exhibited a significantly decreased normalized diameter at pressure values from 40 to 140 mmHg as compared to controls. To further characterize the alteration of pressure-induced tone in diabetes, the steepness of the descending segment of the pressure–diameter curves were also compared. We found that the slope of this curve was significantly greater in the arterioles from diabetic rats (Fig. 1, panel B, inset) as compared to controls (slopes: C: −0.62±0.08 and DM: −0.93±0.09; p<0.01; data are means±SD).

We also investigated the pressure-induced changes in diameter as a function of time. When the perfusion pressure was increased in one step from 40 to 100 mmHg, both control and diabetic arteriolar diameters increased to the same extent, and then the diameters decreased gradually for 3–5 min as depicted in Fig. 2 Panel A and B. The original records indicate markedly greater pressure-induced constriction of the arteriole from the diabetic rat as compared to control. The summary data of the change in diameter in response to a pressure step in arterioles from control and diabetic rats is shown in Fig. 2 Panel C. A mono-exponential curve was fitted to the time-diameter curves which showed a good fit for all responses. Comparison of the responses revealed that the amplitude ‘a’ of the pressure-induced constriction was significantly greater in arterioles from diabetic rats than those from control rats (C: 26.0±2.8% and DM: 34.1±1.6%, data are means±SD, p<0.05). The plateau diameter ‘d’ was significantly lower

Fig. 1. Panel A: Diameters of isolated skeletal muscle arterioles from control (C, n=10, open symbols) and STZ-diabetic rats (DM, n=10, filled symbols) as a function of perfusion pressure in the presence (circles) and absence (triangles) of Ca in the superfusion solution. Panel B: Changes in normalized diameter of arterioles as a function of perfusion pressure (C: open symbols, DM: filled symbols). Changes in diameter are normalized to the passive diameter at the corresponding pressure. Inset: The slopes of selected segments of the pressure–diameter curves of arterioles are significantly different. Data are mean±SEM. * indicates significant (p<0.05) differences from control.
induced constriction is greater to a pressure step, but also the speed of its development is faster in arterioles of diabetic rats as compared to controls.

To assess the possible modulatory role of endothelium in the enhanced constrictions to pressure increases, we removed the endothelium of arterioles. We found that infusion of air resulted in loss of the endothelium, as indicated by the absence of dilation to ACh, while dilation to SNP remained intact (arteriolar diameters at 80 mmHg after removal of the endothelium: C: 149±8 μm, DM: 113±6 μm). We found that removal of the endothelium did not significantly affect the pressure-diameter curve of arterioles from either group of rats and did not change the difference between the pressure-induced constriction of arterioles from control and diabetic rats (Fig. 3, slopes in inset: C: -0.56±0.07; DM: -0.81±0.08; p<0.05; M±SD).

3.3. Responses to ACh and SNP

In a dose-dependent manner ACh (10⁻⁹ to 10⁻⁶ mol/l) elicited significantly greater dilations of arterioles isolated from control than those from diabetic rats (Fig. 4, panel A; EC₅₀ = 4.0±0.9 x10⁻⁹ mol/l and 4.8±2.0 x10⁻⁸ mol/l (p<0.01), respectively). After preincubation (for 30 min) and in the presence of the nitric oxide synthase inhibitor L-NNA (10⁻⁴ mol/l) ACh-induced dilations of arterioles from control (EC₅₀ = 1.5±0.5 x10⁻⁸ mol/l; Fig. 4, panel B), but not from diabetic rats (EC₅₀ = 8.5±1.7 x10⁻⁹ mol/l; Fig. 4 panel C) decreased significantly. In a dose-dependent manner SNP dilated arterioles of control and diabetic rats (Fig. 4; EC₅₀ = 8.9±1.1 x10⁻⁹ mol/l and
1.4±0.4×10⁻⁸ mol/l, respectively). L-NNA did not affect dilations to SNP in either group (EC₅₀ for C: 8.1±1.3×10⁻⁸ mol/l, DM: 8.3±1.2×10⁻⁸ mol/l, Fig. 4).

3.4. Responses to norepinephrine

In a dose-dependent manner norepinephrine (10⁻⁹ to 10⁻⁵ mol/l) elicited significantly greater constrictions in arterioles from diabetic than those from control rats (EC₅₀ = 8.0±2.0×10⁻⁷ mol/l and 6.2±0.6×10⁻⁷ mol/l (p<0.01), respectively. (Fig. 5, panel A). Endothelium removal enhanced constrictions of arterioles of control rats (EC₅₀ = 2.1±0.5×10⁻⁷ mol/l, Fig. 5, panel B) whereas it had no significant effect in arterioles from diabetic rats (EC₅₀ = 4.9±1.3×10⁻⁸ mol/l, Fig. 5, panel C). Nevertheless, after removal of the endothelium, arterioles from diabetic rats were still significantly more responsive to norepinephrine than arterioles from control rats (Fig. 5, panel D).

3.5. Responses to extracellular Ca²⁺

Arterioles maximally dilated in a Ca²⁺-free PS solution. Administration of Ca²⁺ (10⁻⁴ to 7.5×10⁻³ mol/l) constricted arterioles from control and diabetic rats in a dose-dependent manner. The dose–response curve to Ca²⁺ was characterized by a steep part indicating substantial constriction (from 10⁻⁴ to 10⁻³ mol/l) and a plateau phase (from 10⁻³ to 7.5×10⁻³ mol/l), where the arteriolar tone
was maintained. In arterioles of diabetic rats, Ca\textsuperscript{2+} elicited significantly greater constrictions than those from controls (Fig. 6, panel A). After removal of the endothelium, constrictions to Ca\textsuperscript{2+} remained significantly increased in arterioles from diabetic rats compared to those from controls (Fig. 6, panel B).

3.6. Responses to voltage-dependent Ca\textsuperscript{2+}-channel opener and protein kinase C activator

In a dose-dependent manner the voltage-dependent Ca\textsuperscript{2+} channel activator Bay K 8644 (10\textsuperscript{-11} to 10\textsuperscript{-7} mol/l) elicited significantly greater constrictions in endothelium-denuded arterioles from diabetic than those from control rats (EC\textsubscript{50}=4.2±1.5\times10\textsuperscript{-9} mol/l and 1.7±0.8\times10\textsuperscript{-9} mol/l (p<0.05), respectively; Fig. 7, panel A). The PKC activator phorbol 12-myristate 13-acetate (10\textsuperscript{-10}–3\times10\textsuperscript{-7} mol/l) induced slow, concentration-dependent constrictions. In endothelium-denuded arterioles of diabetic rats PMA elicited significantly greater constrictions than those from controls (EC\textsubscript{50}=6.1±2.0\times10\textsuperscript{-9} mol/l and 2.0±1.0\times10\textsuperscript{-8} mol/l (p<0.05), respectively; Fig. 7, panel A).

4. Discussion

The novel findings of our study are that (1) in skeletal muscle arterioles of diabetic rats the pressure-sensitive myogenic response is enhanced, (2) this enhancement is present in the absence of endothelium, (3) and most likely due to the increased activation of voltage-dependent Ca\textsuperscript{2+} channels and/or of protein kinase C in the arteriolar smooth muscle. Both the impairment of nitric oxide-mediation, as indicated by reduced dilations to acetylcholine, and alteration of smooth muscle function are likely to contribute to the enhanced constrictions of skeletal muscle arterioles to norepinephrine in diabetes mellitus.
Several mechanisms have been proposed to explain the microvascular disease observed in diabetes. Previous studies described how diabetes alters vessel wall morphology (e.g. nonenzymatic glycosylation reactions), increases platelet activity, and promotes low density lipoprotein modification [27]. However, many of the data describing the morphological [27,28] and functional [14,29–31] alterations in diabetes were obtained in studies of large vessels. Less data are available to assess the possible alterations in regulation of vasomotor tone of microvessels in diabetes independently from neural and hormonal factors. One of the primary regulators of arteriolar tone is the pressure-sensitive myogenic response [7,8,20]. Because the skeletal muscle microcirculation represents a major part of peripheral resistance and determine tissue blood flow, the dysfunction of myogenic mechanisms may contribute to the development of peripheral vascular disease in skeletal muscle observed frequently in diabetes mellitus [1–3]. Thus, in the present study we have utilized isolated gracilis muscle arterioles from STZ-diabetic and normal rats and examined the changes in the myogenic response and assessed the possible role of endothelium and smooth muscle.

4.1. Effect of diabetes on pressure-induced constrictions

In the present study we found that arterioles from diabetic rats exhibited enhanced constrictions to increases in intraluminal pressure compared to that of controls as indicated by a steeper active pressure–diameter relation.
ship (more negative slope) and a higher maintained tone (Fig. 1). Zimmerman et al. [19] also reported an enhanced vascular tone during increases in perfusion pressure in isolated rat cerebral arteries of STZ-diabetic female rats. Contrary to our findings in mesenteric arteries [12] and cremaster arterioles [10] an impaired myogenic response was reported.

It is thought that one of the roles of myogenic mechanism is to prevent sudden increases in perfusion pressure in the distal part of microcirculation by increasing arteriolar resistance when pressure increases. Thus, we investigated the time-dependent behavior of the myogenic response as well. A step increase in perfusion pressure from 40 to 100 mmHg resulted in an increase in diameter followed by a constriction (Fig. 2). The pressure step was accomplished in less than two seconds, a time much faster than the time constant of the myogenic response preventing the myogenic mechanism to counteract the passive increase in diameter [26]. We found that in arterioles from diabetic rats the time constant was significantly shorter and the amplitude of the response was significantly greater than in arterioles from control rats (Fig. 2). The faster and greater constrictions to increases in intravascular pressure may exaggerate myogenic response in vivo during changes in blood pressure which may result in temporal reductions of blood supply to the skeletal muscle in diabetes [4].

Since myogenic mechanism is determined by passive and active properties of the arteriolar wall, it can be hypothesized that changes in myogenic response observed in the present study are due to alterations in the structure of vessels. In our study the pressure–diameter curve of arterioles from control and diabetic rats in Ca²⁺ free PS solution were not significantly different (Fig. 1), suggesting no change in the passive compliance of arterioles which may modify active responses. Interestingly, Hill and Ege [10] showed changes in the passive mechanical properties of cremasteric arterioles and hypothesized that this could be due to advanced glycosylation or other changes in the structure of the vessel wall and contribute to the impaired myogenic response. However, it is likely that alterations of cremasteric arterioles are different in diabetes than those of skeletal muscle or other tissues [19].

Endothelial factors, such as NO, are known to importantly modulate vascular tone. Impaired endothelium-dependent, flow-mediated vasodilation and impaired vasorelaxation to endothelium-dependent agents (such as acetylcholine) have been described both in diabetic patients [14,15] and in animal models of diabetes [10,13,16,18,19]. A reduced release of NO from endothelium in diabetes has been proposed at least in part to be responsible for the increased responses of large vessels and mesenteric microvessels to constrictor agents [27,32,33]. Furthermore, the increased myogenic tone of cerebral arteries from STZ-diabetic rats has been recently attributed also to such impairment of endothelial function [19]. In the present study, we found that ACh-mediated vasodilation is reduced in arterioles isolated from skeletal muscle of diabetic rats, an impairment which is likely to be due to the reduced biological availability of NO as suggested by previous studies [12,13]. Dilatations in response to the NO donor SNP were similar in vessels from control and diabetic rats.

On the basis of the above mentioned studies and findings in small cerebral arteries of diabetic rats showing that the pressure-induced tone is enhanced due to the lack of NO [19] one can assume that the impaired function of endothelium may contribute to the enhanced myogenic constriction of gracilis arterioles in diabetes. However, in the present study we found that removal of the endothelium did not significantly affect the pressure-induced responses of gracilis arterioles either from control or diabetic rats (Fig. 3). Earlier reports demonstrated little modulatory role of endothelium on the myogenic tone of gracilis muscle arterioles of male rats under no-flow conditions [8]. This may explain why the lack of NO release in endothelium-denuded vessels has no significant effect on the pressure–diameter curve of skeletal muscle arterioles from diabetic rats. The reason for the greater modulatory role of NO on the basal tone of cerebral vessels than on arterioles of skeletal muscle may be due to the fact that the cerebral vessels were isolated from female rats [19] whereas in our study we used vessels from male rats. Indeed, previous studies showed a gender difference, i.e. greater release and effect of NO on myogenic tone of rat in female arterioles compared to those of males [21,22]. Thus, diabetes may affect the female and male cardiovascular system differently as it was also shown in hypertension [22]. As the increase in pressure-induced tone in diabetic arterioles could not be related to the altered function of endothelium, we hypothesized that the enhancement is due to factors intrinsic to arteriolar smooth muscle.

4.2. Effect of diabetes on arteriolar responses to norepinephrine

Arteriolar smooth muscle contractility can be characterized by responses of arterioles to norepinephrine, an agent known to elicit receptor-mediated influx of extracellular Ca²⁺ [25]. The findings that diabetes enhances arteriolar constrictions to NE (Fig. 5, panel A) is in accordance with earlier data reporting increased responses to NE in aorta [27] and mesenteric arteries [32,34,35] of STZ-induced diabetic rats. Present (Fig. 5, panel B) and previous studies in skeletal muscle arterioles demonstrated that there is concomitant release of endothelial factors upon administration of NE [36]. NE-induced responses of arterioles from diabetic rats, however, remained unchanged after removal of the endothelium (Fig. 5, panel C), suggesting an impaired endothelial function. Our finding that in diabetes constrictions to NE are significantly greater even in the absence of the endothelium (Fig. 5, panel D) extend previous reports on mesenteric arteries from STZ-diabetic rats.
rats [23,37] to skeletal muscle arterioles, and suggest that the enhanced responsiveness of microvessels to α-adrenergic agonists cannot be attributed only to dysfunction of endothelium but also due to the altered Ca\(^{2+}\)-signaling mechanisms in the smooth muscle [25,35].

4.3. Altered calcium sensitivity and myogenic response in diabetes

Earlier findings demonstrated that the maximum contractile response to Ca\(^{2+}\) increases in renal [24] and mesenteric [38,35] arteries of diabetic rats. Similarly, increased responses to Bay K 8644 in renal [24] and mesenteric [35,39] arteries of diabetic rats have been reported. It was also shown that both resting and NE-induced \(^{45}\)Ca\(^{2+}\) uptake increases in renal arteries of diabetic rats [24]. Furthermore, there are studies suggesting that in diabetes mellitus the enhanced contraction of smooth muscle to various agonists may be attributed to the enhanced activation of protein kinase C [40–42].

Previous studies established that pressure-induced myogenic constriction of skeletal muscle arterioles, at least in part, depends on the influx of extracellular Ca\(^{2+}\) through voltage dependent Ca\(^{2+}\) channels [43,44] and activation of PKC [20]. Thus, we compared the constrictions of gracilis arterioles to increasing concentrations of extracellular Ca\(^{2+}\) and to pharmacological activation of voltage-gated Ca\(^{2+}\) channels and PKC in the presence of myogenic tone developed in response to an intravascular pressure of 80 mmHg. We found that constrictions to increasing concentrations of extracellular Ca\(^{2+}\) were significantly greater in arterioles from diabetic rats than in those of control rats, a difference which was independent of the endothelium (Fig. 6). Similarly, constrictions to pharmacological stimulation of voltage-gated Ca\(^{2+}\) channels by Bay K 8644 (in the absence of the endothelium) were also enhanced in arterioles from diabetic rats compared to those of controls (Fig. 7 panel A), suggesting that the enhanced activity of these channels contribute to the enhanced myogenic response in diabetes.

The mechanism by which PKC activation increases vascular tone is still unclear; it may involve sensitization of contractile proteins to Ca\(^{2+}\), increase in [Ca\(^{2+}\)], and increase of myosin light chain phosphorylation [40] mechanisms that are important in the development of myogenic response [43,45–47]. Because in the present study arteriolar constrictions to PMA, a known activator of PKC, were enhanced in arterioles from diabetic rats (Fig. 7 panel B), the increased activity of PKC may also be responsible for the development of enhanced myogenic response in diabetes mellitus.

In summary, the novel findings of our study are that (1) experimental diabetes mellitus results in enhanced pressure-induced myogenic constrictions in rat skeletal muscle arterioles, (2) this enhancement seems to be independent of the impaired endothelial function present simultaneous-ly, (3) and it is likely due to the increased activity of voltage-dependent Ca\(^{2+}\) channels and/or protein kinase C in arteriolar smooth muscle. These alterations could lead to an increase in arteriolar tone in vivo eliciting reduced microvascular perfusion of skeletal muscle, which may be of importance in the development of skeletal muscle microangiopathy associated with diabetes mellitus.

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