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Arteriolar changes in nitric oxide activity and sensitivity during the course of streptozotocin-induced diabetes

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Abstract

Nitric oxide (NO) may play an important role in the pathogenesis of diabetic microangiopathy. However, arteriolar changes in NO activity and sensitivity to NO may be dependent on both the type of arteriole and the duration of diabetes. Therefore, we assessed, in the in situ spinotrapezius muscle preparation of streptozotocin-diabetic rats and of controls, inside diameters of A2–A4 arterioles and the reactivity to topically applied acetylcholine and nitroprusside, before and after N^G-nitro-L-arginine (L-NNA) at 2, 4, 6 and 12 weeks of diabetes. In A2 arterioles, basal diameters and the contribution of NO to basal diameter were not affected during the course of streptozotocin-induced diabetes. However, the maximal response to acetylcholine in these arterioles was attenuated after 2 until 4 weeks, and from 4 weeks on a sustained decrease in reactivity to sodium nitroprusside was observed. In A3 arterioles, both the basal diameter and the contribution of NO to basal diameter were decreased after 2 weeks and increased after 6 weeks, while the response to sodium nitroprusside was unaffected. In A4 arterioles, a significant increase in basal diameter was observed after 6 weeks only. Thus, this study shows that streptozotocin-induced diabetes causes microvascular changes in NO activity and sensitivity that depend on the type of arteriole. For each order of arteriole, these changes show a specific pattern during the course of diabetes.

Keywords: Hyperglycemia; Microcirculation; Streptozotocin; Acetylcholine; Sodium nitroprusside; Nitric oxide; Diabetes mellitus

1. Introduction

Microangiopathy has a major impact on morbidity and mortality of patients with diabetes mellitus (Anonymous, 1994). An important hypothesis regarding the pathophysiology of diabetic microangiopathy is the haemodynamic concept (Tooke, 1986), which states that an early, reversible increase in microvascular flow eventually leads to an endothelial injury response and microvascular sclerosis (Parving et al., 1983; Zatz and Brenner, 1986; Tooke, 1986).

An increased flow early in diabetes, possibly as a result of enhanced nitric oxide (NO) production, has been described in several microvascular beds (Pieper, 1998; Tooke, 1995). However, these studies addressed changes in overall flow. In order to gain insight in the exact role of NO in microangiopathy, it is necessary to assess its activity and efficacy at different sites of the microcirculation, as several orders of arterioles can react differently to chemical and mechanical vasoactive stimuli (Muller et al., 1996; Jones et al., 1995; Davis, 1993). Indeed, in a previous study, we observed increased basal diameters of A3 and A4, but not A2 arterioles of the rat spinotrapezius muscle after 6 weeks of streptozotocin-induced diabetes. This increase was partly attributable to an increased NO activity in A3 arterioles. At the same time, a decreased reactivity to exogenous NO was observed in A2 arterioles, but not in A3 or A4 arterioles (van Dam et al., 2000).

Several studies provide evidence for a time-dependency of changes in responses to acetylcholine and sodium nitro-

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prusside in diabetic microvessels (Heygate et al., 1996; Furman and Sneddon, 1993; Ralevic et al., 1995; Crijns et al., 1998; Timar-Peregrin and Guy, 2001). However, these studies either addressed changes in endothelial function at one particular site of the microcirculation or after a specific duration of streptozotocin-induced diabetes. Morff (1990) has shown that diabetes causes changes in the responses of the microcirculation to norepinephrine that are both anatomically specific and temporally dependent. Therefore, in order to gain more insight in the exact role of NO in the development of changes in microvascular function in diabetes, it is necessary to assess its activity not only at different sites of the microcirculation, but also after different durations of streptozotocin-induced diabetes.

The aim of the present study was to assess, in vivo, changes in NO activity and reactivity to exogenous NO in different orders of arterioles after different durations of streptozotocin-induced diabetes.

2. Materials and methods

2.1. Experimental animals

All experiments were performed according to the NIH guidelines for the use of experimental animals (Helsinki declaration), after permission of the institutional animal care and use committee. Diabetes was induced in 34 conscious male Wistar rats (~ 250 g; HSD, Zeist, The Netherlands) by a single tail vein injection of 65 mg/kg streptozotocin dissolved in sodium citrate buffer (pH = 4.5). Glucose levels were assessed weekly in each animal with a haemo-glukotest (Accutrend Alpha, Boehringer Mannheim) in blood samples taken from the tail tip. The animals were housed in individual cages and were allowed free access to water and preweighed commercial rat pellet chow. Experiments were performed at 2 weeks ($n = 8$), 4 weeks ($n = 8$), 6 weeks ($n = 6$) and 12 weeks ($n = 12$) after administration of streptozotocin. Untreated male Wistar rats ($n = 8$) were used as controls. These rats were time-matched for 6 weeks of streptozotocin-induced diabetes.

2.2. General setup

Anaesthesia was induced in overnight fasted rats by an injection of pentobarbital sodium (Nembutal; 60 mg/kg, ip) and ketamine (70 mg/kg, im). For further administration of Nembutal, an intraperitoneal cannula was inserted. As an indication of the need for additional small doses of the anaesthetic (25% of the original dose), we regularly monitored blood pressure, interdigital and eye reflexes, as well as respiration rate, with the intent of keeping the depth of the anaesthesia stable. In our hands, this anaesthetic protocol causes stable haemodynamics and blood gas values throughout the entire experimental period (approximately 5 h), indicating a constancy of the anaesthetic conditions. Moreover, in previous studies (Heemskerk et al., 1997a,b), we have also found a stable O_2-consumption of the animal and low blood lactate levels throughout the whole experiment. During the experiment, body temperature was kept at 37–38 °C by a heating pad placed under the rat. The trachea was cannulated to maintain a patent airway.

To be informed about systemic haemodynamic variables that could influence the microcirculation, we measured cardiac output in addition to blood pressure. Mean arterial pressure was determined with a catheter (PE_{50}) placed in the right carotid artery. The heart rate was obtained from the blood pressure recording. Cardiac output was measured by thermodilution: saline (0.2 ml) was injected (at room temperature) from a catheter that was inserted in the right jugular vein, with the tip near the right atrium. A thermistor, which was obtained from a Swan-Ganz catheter (Gould, Cleveland, OH), was inserted into the abdominal aorta via a femoral artery. The thermistor was connected to an Edwards 9520A (Santa Ana, CA) cardiac output computer.

Arterial blood samples were taken from a carotid artery at the beginning and the end of an experiment to measure glucose and insulin (by radioimmunoassay; Immunoradiometric Assay, Medgenic Diagnostics, Fleurus, Belgium).

2.3. Spinotrapezius preparation

The left spinotrapezius muscle was approached through a skin incision along the spine, after which subcutaneous fat and fascia were carefully removed. The lateral border of the muscle was freed and lifted with four to five atraumatic sutures to separate it from the underlying muscle layers. It was then draped, with the ventral surface upward, over a perspex pedestal placed on the microscope stage (Fig. 1) as described by Gray (1971) and Marshall (1982). A specific area of interest was selected at the caudo-medial side in which different orders of arterioles were present.

Classification of the vessels (Engelson et al., 1985; Skalak and Schmid-Schonbein, 1986) is also illustrated in Fig. 1. Vessels derived directly from feeding or A1 arterioles and forming main arcades are considered A2 arterioles. Vessels derived from these larger arcade vessels, forming smaller arcades, are considered A3 arterioles. Finally, as A4 arterioles we considered vessels that supply blood from the smallest arcades to terminal arterioles.

The muscle was superfused with tyrode solution (NaCl 128.3, KCl 4.7, $CaCl_2 \cdot H_2O$ 1.36, $MgCl_2 \cdot 6H_2O$ 1.05, $NaHCO_3$ 20.2, $NaH_2PO_4 \cdot 2H_2O$ 0.42 M). During the experiment, the superfusate was equilibrated with 95% N_2 and 5% CO_2 to prevent direct oxygenation of the muscle cells by high levels of oxygen in the surrounding fluid. Only during the dissection was this solution equilibrated with 95% O_2 and 5% CO_2. The temperature of the solution was maintained at ~ 36 °C at the muscle surface; the pH was 7.4.

We used a Zeiss microscope (Zeiss, Germany) with a 25× SW objective (numerical aperture 0.63; Leitz, Germany). The optical magnification at the front plane of the

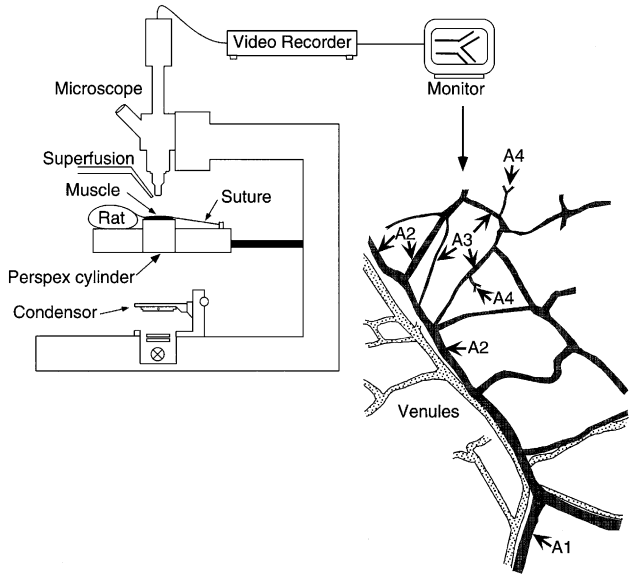

Fig. 1. Experimental setup of the spinotrapezius preparation. The spinotrapezius muscle is draped over a perspex cylinder positioned in the microscope stage over the condensor, such that the muscle is transilluminated. The dorso-medial side of the muscle, with intact feeding arteries, remains connected to the rat, which lies on its side. The preparation is superfused with tyrode solution. The microscope is equipped with a video system to continuously record microvessels on tape. A1: feeding arteriole; A2: main arcade arterioles (>40 μm); A3: smaller arcade arterioles (20–40 μm); A4: arterioles supplying the terminal arterioles (10–20 μm).

video camera was 58.8 ×. The experiments were recorded on videotape (Panasonic AG-6200, Japan) using a black and white Philips LDH 0702/20 camera (Philips, The Netherlands). Inside diameters of A2–A4 arterioles were measured on the monitor from stopped images with an interactive computer program (made in our own workshop) (van den Bos et al., 1991).

2.4. Experimental protocol

After catheterisation of the rat and preparation of the spinotrapezius muscle had been finished, we moved the animal to the microscope. After a suitable arcade had been found, we waited at least 30 min for stabilisation of the preparation and then started the video microscopic measurements according to the following protocol.

First, the basal inside diameter was assessed. Thereafter, several drugs were added to the superfusate, reaching their final concentrations at the surface of muscle. They were applied until their effect was maximal (usually for 5 min). After the diameter had returned to the control value (usually after 5–15 min), the next concentration of a drug was started. The arteriolar inside diameters were recorded before, during, and after the entire period of superfusion of the drugs.

The integrity of the endothelial vasodilator response was assessed by testing the effect of acetylcholine. The experimental setup did not allow us to perform a complete dose–response relationship of acetylcholine. Therefore, we focused on the capacity of acetylcholine to maximally dilate the vessels. We used three different doses (10^{-5}, 10^{-4}, and 10^{-3} M), giving submaximal or maximal responses, to accurately assess the level of the maximal response and the dose at which this maximal response was reached.

After we had tested the effect of acetylcholine, we administered the NO-generating endothelium-independent vasodilator sodium nitroprusside (10^{-4} M, i.e. a dose high enough to cause maximal dilatation in control animals, as established in pilot experiments). Finally, adenosine, a mainly endothelium-independent vasodilator, was added (Rongen et al., 1996a,b, 1997). The dose of adenosine to obtain maximal dilatation was previously assessed to be 10^{-4} M; the diameter after application of this dose was considered as the passive diameter and this diameter was set at 100%. This approach enabled us to relate basal diameters and the effects of the different drugs to a diameter that was independent of the initial constricted state (Lash and Bohlen, 1991a).

After this series, we added N^G-nitro-L-arginine (L-NNA) 10^{-4} M to the superfusate to block NO production. Twenty minutes after the start of L-NNA, inside diameter was assessed. The contribution of NO activity to basal diameter was defined as the percentage reduction in basal diameter that could be achieved by L-NNA (10^{-4} M). We then repeated the protocol with the vasodilatory drugs while L-NNA superfusion was continued.

2.5. Statistics

For statistical evaluation, we used the average values of all A2, A3, or A4 arterioles (ranging from 2 to 10) per rat. One-way analysis of variance (ANOVA) was used to compare differences among groups after different durations of streptozotocin-induced diabetes. We then used independent-samples *t* tests to compare observations in diabetic rats after a specific duration of streptozotocin-induced diabetes with those in controls in the same order of arterioles. Paired-samples *t* tests were used to assess the effect of L-NNA on basal diameter and on induced vasodilatation within the same rats. As comparisons made between control and diabetic and with and without L-NNA were sufficiently different, no adjustments were performed for multiple comparisons. The level of significance was set at 5%.

3. Results

3.1. General characteristics

Blood glucose level measured 1 week after the streptozotocin injection was 20 ± 5 mM and remained near this value during the entire period of 12 weeks (Table 1). Food consumption in diabetic rats, compared with control rats, almost doubled (~ 150 vs. ~ 300 g/week). Nevertheless,

Table 1
Systemic variables in control and diabetic rats (2, 4, 6, and 12 weeks after injection of streptozotocin)

Systemic variable	Time of measurement	Controls ($n=8$)	Diabetic rats, interval after injection with streptozotocin			
			2 weeks ($n=8$)	4 weeks ($n=8$)	6 weeks ($n=6$)	12 weeks ($n=12$)
Body weight (g)	$t=0$ h	249 ± 75	219 ± 34	216 ± 48	242 ± 32	234 ± 45
Glucose (mM)	$t=-24$ h	5.4 ± 1.1	19.5 ± 2.4[a]	20.6 ± 4.5[a]	19.9 ± 1.9[a]	24.2 ± 2.9[a]
Haematocrit	$t=0$ h	49.7 ± 5.8	53.4 ± 4.3	52.1 ± 4.2	52.8 ± 2.8	49.6 ± 6.4
Mean arterial pressure (mmHg)	$t=0$ h	116 ± 17	ND	124 ± 13	112 ± 15	116 ± 27
	$t=3$ h	123 ± 20		111 ± 28	124 ± 9	109 ± 15
Heart rate (beats/min)	$t=0$ h	395 ± 44	ND	350 ± 28[a]	340 ± 38[a]	354 ± 63
	$t=3$ h	400 ± 24		343 ± 27[a]	347 ± 27[a]	361 ± 51
Cardiac output (ml/min)	$t=0$ h	98 ± 15	ND	81 ± 17	79 ± 14[a]	101 ± 18
	$t=3$ h	85 ± 12		78 ± 13	64 ± 10[a]	90 ± 19

$t=-24$ h: 24 h before the experiments; $t=0$: at the start of the experiments; $t=3$ h: at the end of the experiments.
Data are expressed as mean ± S.D.
ND: not determined (for logistical reasons).
[a] $P<0.05$ for streptozotocin diabetes vs. control.

the body weight of diabetic rats remained lower than that of the controls (Table 1). There was no relationship between body weight and either passive diameter, baseline diameter, or diameter during L-NNA superfusion in the different arterioles (data not shown). Systemic haemodynamic variables and haematocrit in control rats and after different durations of diabetes are also presented in Table 1. Baseline blood pressure was not significantly different among the groups, but heart rate was significantly lower after 4 and 6 weeks of diabetes. A similar picture was seen for cardiac output, with the values after 6 weeks of diabetes being significantly lower than controls (Table 1). Haematocrit was not significantly different between control and diabetic rats. During the actual microcirculation experiments (lasting ~ 3

Table 2
Inside diameters (μm) of A2, A3, and A4 arterioles in the spinotrapezius of control and streptozotocin-diabetic rats at baseline, after blocking the NO production with L-NNA, and after dilatation with 10^{-4} M sodium nitroprusside (SNP), 10^{-4} M acetylcholine (ACh), or 10^{-4} M adenosine (AD)

Order of arteriole	Condition	L-NNA (10^{-4} M)	Controls ($n=8$)	Diabetic rats, interval after injection with streptozotocin				
				2 weeks ($n=8$)	4 weeks ($n=8$)	6 weeks ($n=6$)	12 weeks ($n=12$)	ANOVA (P)
A2	Basal	−	39.1 ± 11.7	30.8 ± 9.7	31.4 ± 10.2	36.5 ± 10.1	35.0 ± 11.7	0.515
		+	31.6 ± 9.3[a]	24.8 ± 10.0	23.1 ± 4.1[a,b]	24.2 ± 4.5[a]	28.3 ± 11.6[a]	0.344
	SNP (10^{-4} M)	−	61.8 ± 11.7	54.5 ± 12.7	45.6 ± 7.5[c]	48.9 ± 4.7[b]	50.6 ± 8.3[b]	0.020
		+	62.9 ± 12.5	50.2 ± 17.3	44.7 ± 6.9[c]	44.7 ± 8.7[b]	46.9 ± 10.9[a,b]	0.026
	ACh (10^{-4} M)	−	61.5 ± 14.8	47.1 ± 10.3[b]	40.0 ± 11.9[b]	52.2 ± 9.6	50.0 ± 12.5	0.037
		+	48.0 ± 17.1[a]			33.7 ± 11.2[d]		
	AD (10^{-4} M)	−	63.7 ± 11.8	57.3 ± 9.7	51.6 ± 6.0[b]	56.4 ± 6.4	57.6 ± 8.7	0.136
		+	63.0 ± 11.9	54.7 ± 12.2	46.5 ± 9.0[b]	51.4 ± 5.6[b,d]	51.9 ± 11.3[d]	0.058
A3	Basal	−	15.5 ± 5.2	10.7 ± 3.8	19.3 ± 6.3	23.1 ± 5.5[b]	19.5 ± 9.0	0.028
		+	12.3 ± 3.1[a]	11.3 ± 3.1	14.8 ± 5.4	13.6 ± 2.4[a]	15.6 ± 5.5	0.320
	SNP (10^{-4} M)	−	26.2 ± 4.1	28.2 ± 5.4	27.6 ± 3.2	31.6 ± 3.7[b]	27.5 ± 4.6	0.295
		+	26.2 ± 5.0	26.2 ± 3.1	28.6 ± 5.8	26.1 ± 7.4	25.2 ± 6.6	0.850
	ACh (10^{-4} M)	−	25.6 ± 4.9	23.0 ± 5.0	23.9 ± 7.6	32.0 ± 3.4[b]	26.6 ± 8.8	0.195
		+	15.4 ± 4.3[d]			21.3 ± 5.3[a]		
	AD (10^{-4} M)	−	27.7 ± 3.5	29.2 ± 2.8	31.0 ± 2.5	32.8 ± 2.2[b]	29.3 ± 4.8	0.131
		+	24.9 ± 6.7[a]	28.2 ± 3.1	28.7 ± 3.2	29.9 ± 5.1	26.7 ± 4.2	0.384
A4	Basal	−	7.9 ± 2.8	7.9 ± 6.4	7.4 ± 1.6	11.6 ± 2.1[b]	11.2 ± 5.2	0.179
		+	6.3 ± 2.6	6.0 ± 3.2	5.9 ± 2.0	7.3 ± 3.7[d]	8.0 ± 3.2	0.651
	SNP (10^{-4} M)	−	15.0 ± 3.1	15.1 ± 1.5	12.9 ± 2.3	16.7 ± 4.4	16.3 ± 3.0	0.244
		+	13.7 ± 3.2	14.3 ± 3.9	12.9 ± 3.4	14.6 ± 4.8[a]	13.5 ± 3.5	0.945
	ACh (10^{-4} M)	−	14.9 ± 2.8	11.6 ± 3.5	10.3 ± 4.4[b]	16.0 ± 3.0	15.5 ± 4.5	0.051
		+	9.0 ± 5.3[d]			11.0 ± 2.7[a]		
	AD (10^{-4} M)	−	15.4 ± 3.4	15.8 ± 3.4	13.8 ± 2.4	18.0 ± 7.4	16.4 ± 3.6	0.571
		+	14.4 ± 3.4	14.1 ± 2.0	13.1 ± 4.4	16.0 ± 6.0[a]	15.2 ± 3.3	0.780

Data are expressed as mean ± S.D.
[a] $P<0.05$ for with vs. without L-NNA.
[b] $P<0.05$ for streptozotocin diabetes vs. control.
[c] $P<0.01$ for streptozotocin diabetes vs. control.
[d] $P<0.01$ for with vs. without L-NNA.

h), there was a decrease in cardiac output of about 15% in both control and diabetic rats. Mean arterial pressure and heart rate did not change significantly during the experiments (Table 1).

3.2. Inside arteriolar diameter in different orders of arterioles

In A2 arterioles, absolute or relative basal diameters were not significantly different between rats with different durations of diabetes and controls. In A3 arterioles, there was an overall change in both absolute ($P=0.028$) and relative ($P=0.047$) basal diameters, as shown in Table 2 and Fig. 2, respectively. After 2 weeks, diabetic rats had nonsignificantly decreased absolute basal diameters, as compared to control rats (10.7 ± 3.8 vs. 15.5 ± 5.2 μm, $P=0.084$) and significantly decreased relative basal diameters ($37 \pm 12\%$ vs. $56 \pm 16\%$, $P=0.033$). After 6 weeks, this early decrease in basal diameters was followed by significantly increased absolute inside diameters (23.1 ± 5.5 vs. 15.5 ± 5.2 μm, $P=0.028$) and nonsignificantly increased relative inside diameters ($70 \pm 14\%$ vs. $56 \pm 16\%$, $P=0.138$). In A4 arterioles, absolute inside arteriolar diameter was increased after 6 weeks of diabetes (11.2 ± 2.1 vs. 7.9 ± 2.8 μm, $P=0.019$). Nonsignificant increases in relative inside arteriolar diameters were observed after 6 and 12 weeks of diabetes ($72 \pm 22\%$ vs. $54 \pm 23\%$, $P=0.164$ and $68 \pm 24\%$ vs. $54 \pm 23\%$, $P=0.260$).

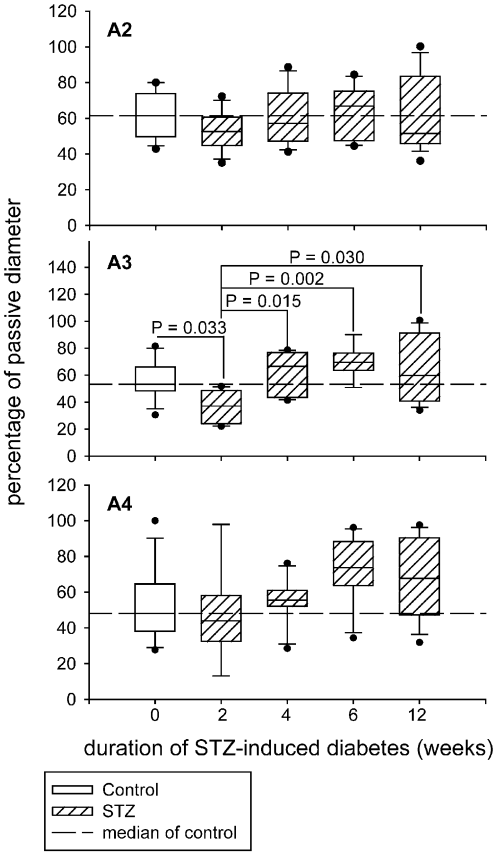

Fig. 2. Inside arteriolar diameter in the basal state. Inside diameters, expressed as percentage of the passive diameter (i.e., after 10^{-4} M adenosine; set at 100%), of A2–A4 arterioles in the spinotrapezius muscle in control (open plots) and diabetic rats (filled plots), after 2, 4, 6, and 12 weeks of streptozotocin-induced diabetes. The data are expressed as box-and-whisker plots: the central line is the median, the lower and upper quartiles are indicated by the box, the 2.5 and 97.5 centiles are indicated by whiskers and black dots indicate the range. P values for differences between diabetic and control rats over time (ANOVA): A2 arterioles: $P=0.760$; A3 arterioles: $P=0.047$; A4 arterioles: $P=0.353$. P values for significant differences between control rats and after specific durations of streptozotocin diabetes are shown in the figure.

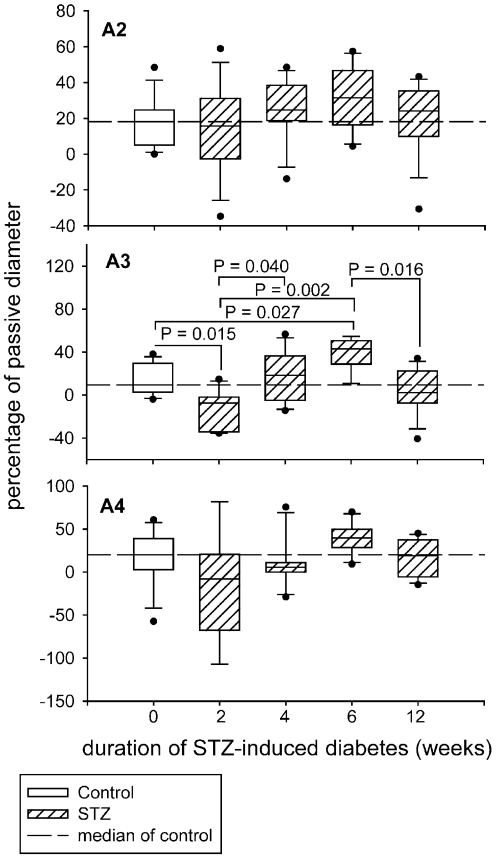

Fig. 3. Contribution of endogenous NO to basal inside diameter. Percentage reduction of the basal diameter of A2–A4 arterioles in the spinotrapezius muscle caused by superfusion of L-NNA (10^{-4} M) in control (open plots) and in diabetic rats (filled plots); box-and-whisker plots. P values for differences between diabetic and control rats over time (ANOVA): A2 arterioles: $P=0.634$; A3 arterioles: $P=0.006$; A4 arterioles: $P=0.230$. P values for significant differences between control rats and after specific durations of streptozotocin diabetes are shown in the figure.

3.3. Contribution of endogenous NO to basal inside diameter

Overall, superfusion of L-NNA (10^{-4} M) caused a decrease in basal arteriolar diameter in both control and diabetic rats (Table 2), confirming the role of NO in maintaining basal diameters in this model. After blockade of the endogenous NO production, inside arteriolar diame-

ters were significantly different in diabetic rats as compared with control rats after 4 weeks of streptozotocin-induced diabetes only (Table 2). In A2 and A4 arterioles, diabetes did not significantly affect the contribution of NO to basal tone (Fig. 3). In A3 arterioles however, diabetes induced time-specific changes in the contribution of NO to basal inside diameter ($P=0.006$). Basal NO activity was significantly attenuated in A3 arterioles of diabetic rats after 2 weeks ($-12 \pm 20\%$ vs. $15 \pm 16\%$, $P=0.015$), followed by an enhancement ($38 \pm 17\%$ vs. $15 \pm 16\%$, $P=0.027$) after 6 weeks (Fig. 3).

3.4. Response to sodium nitroprusside

After addition of sodium nitroprusside in the basal state, a significantly higher inside diameter was found in all types of arterioles in both control and diabetic rats (Table 2). However, this increase was smaller in A2 arterioles after 4, 6, and 12 weeks of streptozotocin-induced diabetes as compared to controls. In contrast, inside diameters in A3 arterioles were higher with sodium nitroprusside after 6 weeks of streptozotocin-induced diabetes as compared to controls, but not at other time points (Table 2). In order to correct for differences in basal NO production, responses to sodium nitroprusside relative to passive diameters were assessed after addition of L-NNA (Fig. 4). The relative response to 10^{-4} M sodium nitroprusside superfusion after blocking endogenous NO production was significantly lower in A2 arterioles after 4, 6, and 12 weeks of streptozotocin-induced diabetes, but not in A3 and A4 arterioles.

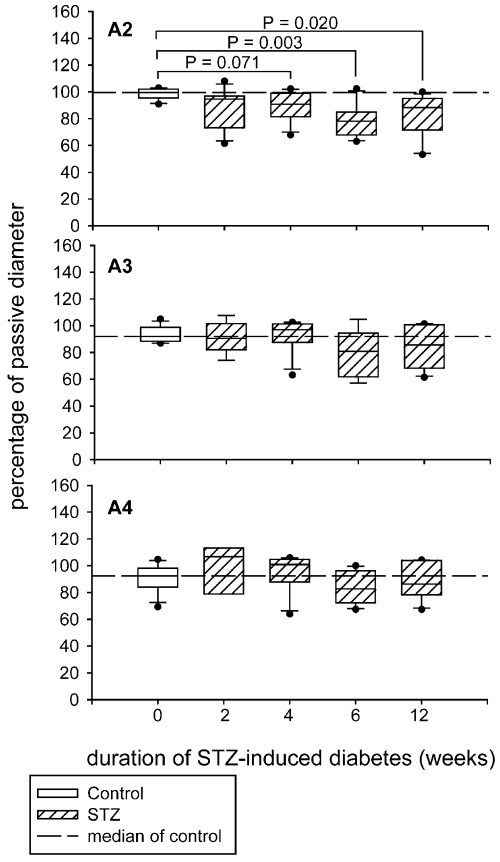

Fig. 4. Arteriolar response to exogenous NO. Inside diameters, expressed as percentage of the passive diameter (i.e., after 10^{-4} M adenosine; set at 100%), of A2–A4 arterioles in the spinotrapezius muscle after superfusion of 10^{-4} M sodium nitroprusside during blockade of NO production with L-NNA (10^{-4} M) in control rats (open plots) and after different durations of streptozotocin-induced diabetes (filled plots); box-and-whisker plots. P values for differences between diabetic and control rats over time (ANOVA): A2 arterioles: $P=0.091$; A3 arterioles: $P=0.376$; A4 arterioles: $P=0.725$. P values for significant differences between control rats and after specific durations of streptozotocin diabetes are shown in the figure.

3.5. Response to acetylcholine and adenosine

In the early phase of streptozotocin-induced diabetes, responses to acetylcholine were attenuated in A2 arterioles. Absolute, but not relative, inside diameters after acetylcholine were significantly lower in diabetic rats after 2 and 4 weeks of streptozotocin-induced diabetes (Table 2). L-NNA was able to attenuate acetylcholine-induced vasodilatation in control as well as diabetic rats in all orders of arterioles (Table 2). Acetylcholine, as compared with adenosine, was able to cause maximal dilatation in all orders of arterioles in both control rats and after 6 and 12 weeks of streptozotocin-induced diabetes. No differences were found between acetylcholine doses of 10^{-4} and 10^{-3} M.

There were no significant differences in the absolute responses to adenosine during the course of streptozotocin-induced diabetes. However, after 6 and 12 weeks, L-NNA could significantly attenuate adenosine-induced vasodilatation (Table 2).

4. Discussion

This study aimed to follow alterations in NO activity and sensitivity at different sites of the microcirculation during the course of streptozotocin-induced experimental diabetes. Using intravital microscopy, we found changes in both NO activity and sensitivity that were not only anatomically specific, but also changed during the course of streptozotocin-induced diabetes. In A2 arterioles, basal diameters and the contribution of NO to basal diameter were not affected during the course of streptozotocin-induced diabetes. However, the maximal response to acetylcholine in these arterioles was attenuated after 2 until 4 weeks, and from 4 weeks on a sustained decrease in reactivity to exogenous NO was observed. In A3 arterioles, both the basal diameter and the contribution of NO to basal diameter were decreased after 2 weeks and increased after 6 weeks, while the response to exogenous NO was unaffected. In A4 arterioles, a significant increase in basal diameter was observed after 6 weeks only.

The present study is not in line with the hypothesis that an increased NO production early in diabetes can cause a

decreased response to NO or compounds that cause an increased production of NO later (Corbett et al., 1992; Pieper, 1999), despite the fact that our findings confirm previous observations that changes in microvascular reactivity due to streptozotocin-induced diabetes are both temporally and anatomically specific (Morff, 1990).

An early microvascular dysfunction resulting in a decreased response to acetylcholine in A2 arterioles and decreased basal diameters of A3 and A4 arterioles may be caused by several mechanisms. Firstly, NO bioactivity may be decreased via increased quenching either by advanced glycosylation end products (Bucala et al., 1991), or by superoxide. This radical interacts with NO forming peroxynitrite. Diabetes can both increase the production of superoxide (Tsao et al., 1998) and decrease the endothelial capacity to eliminate this radical (Kamata and Kobayashi, 1996). However, this quenching mechanism cannot explain the temporary nature of decreased responses to acetylcholine in A2 arterioles and of decreased basal diameters in A3 arterioles. Secondly, arteriolar NO production may decrease in early diabetes. Hyperglycemia can decrease Na^+/K^+-ATPase activity, resulting in a decreased NO production (Gupta et al., 1992). This mechanism is also expected to result in sustained, rather than temporary, decreases in arteriolar relaxation. A mechanism that may account for the temporary nature of the observed attenuated arteriolar reactivity is that metabolic changes due to diabetes may decrease the availability of L-arginine, the substrate for endothelial nitric oxide synthase (eNOS) (Reyes et al., 1993; Suanarunsawat et al., 1999), or increase the amount of endogenous inhibitors of eNOS, such as asymmetric dimethylarginine and N^G-monomethyl-L-arginine (Masuda et al., 1999). Muscle wasting occurring in a later phase of the disease may compensate for a decreased availability of L-arginine by an increased release of L-arginine from protein catabolism. This possibility requires further investigation. Thirdly, although acetylcholine is often used as a means to measure stimulated NO-mediated vasodilatation, it is well known that acetylcholine not only releases NO, but also causes an increased production of the vasodilator endothelium-derived hyperpolarizing factor and prostacyclin as well as several vasoconstricting mediators. Streptozotocin-induced diabetes can cause an increase in acetylcholine-induced prostacyclin production (Kamata and Hosokawa, 1997). As we focused on possible alterations in NO production and sensitivity, we cannot exclude that inside diameter differences between control and diabetic rats in this study are partly caused by changes in production of other vasorelaxing or constricting agents.

After blocking endogenous NO production, we found a further difference between diabetic and control rats; namely, a decrease in the effect of 10^{-4} M sodium nitroprusside in the A2 arterioles after 4, 6, and 12 weeks of streptozotocin-induced diabetes. Although most studies show unaltered microvascular responses to exogenous NO (Kamata and Hosokawa, 1997; Lash and Bohlen, 1991b; De Vriese et al., 2000), decreased responses to sodium nitroprusside have been shown in cremaster arterioles after at least 2 weeks of streptozotocin-induced diabetes (Timar-Peregrin and Guy, 2001). Sodium nitroprusside, as a direct donor of NO to smooth muscle cells, is often used to establish whether the response of smooth muscle cells to NO is still intact. When the sodium nitroprusside effect is impaired, a defect in the mechanism by which NO stimulates the smooth muscle cells through activation of guanyl cyclase and production of guanosine 3′,5′-cyclic monophosphate is assumed (Murad, 1986). The dose of sodium nitroprusside used (10^{-4} mol/l), which was high enough to cause maximal dilatation in the arcade arterioles (A2) of control rats, was not able to cause maximal dilatation in the same order of arterioles of diabetic rats. The cause of this phenomenon is presently unclear, but it is possible that streptozotocin-induced diabetes causes an impairment of the mechanism via which NO activates guanyl cyclase in smooth muscle cells. Although NO can desensitize guanyl cyclase to activation by NO (Brandes et al., 2000), increases in NO production are unlikely to be the cause of the decreased responses to sodium nitroprusside in this study. Hyperglycemia can also directly attenuate guanyl cyclase (Craven and DeRubertis, 1989). Another factor that could explain the impaired responses to sodium nitroprusside in A2 arterioles is an increased superoxide production (Kamata and Kobayashi, 1996; Tsao et al., 1998), which may also explain the early decreased vasodilatory responses in A2 and other arterioles.

In the present study, we did not find a correlation between body weight and arteriolar reactivity. Furthermore, previous studies did not observe any change in arteriolar reactivity after different time intervals in nondiabetic rats (Morff, 1990; Timar-Peregrin and Guy, 2001). Consequently, we think that our conclusions regarding the time specificity of streptozotocin-induced changes are valid, despite the fact that changes in arteriolar reactivity in streptozotocin-induced diabetes were compared with controls that were time-matched for 6 weeks.

Adenosine was used as a mainly endothelium-independent relaxant to estimate maximal arteriolar dilatation. However, as shown in Table 2, adenosine-induced vasodilatation was significantly inhibited by L-NNA after 6 and 12 weeks of streptozotocin-induced diabetes in A2 arterioles and after 6 weeks in A4 arterioles. A possible explanation for this observation is that L-NNA nonspecifically blocked responses to all vasorelaxants, including sodium nitroprusside. This is unlikely, as L-NNA was not able to block responses to adenosine in A2 or A4 arterioles of control rats. Also, responses to sodium nitroprusside were decreased in A2 arterioles of diabetic rats both in the absence and in the presence of L-NNA. Another option is that part of the adenosine-mediated vasodilatation in A2 arterioles is endothelium-dependent after 6 and 12 weeks of streptozotocin-induced diabetes. This is in line with recent observations in aortic rings of control as well as nonhypertensive diabetic rats (Fahim et al., 2001). A rightward shift in the concen-

tration–response curve of adenosine after 6 weeks of streptozotocin-induced diabetes can explain why L-NNA was able to block adenosine-induced vasodilatation in diabetic, but not in control rats. In the absence of L-NNA, adenosine-induced vasodilatation is not altered significantly during the course of streptozotocin-induced diabetes, indicating that adenosine is still capable of inducing maximal vasodilatation. Therefore, differences observed for other agents were not due to their being expressed as a percentage of adenosine-induced vasodilatation.

In conclusion, although streptozotocin-induced diabetes in rats caused a sustained decrease in reactivity to exogenous NO in A2 arterioles after 4 weeks, this change could not be attributed to an earlier increase in NO activity. After 2 weeks, reactions to acetylcholine were impaired in A2 arterioles. In A3 arterioles, an early decrease followed by an increase in basal diameter was observed, which could at least partially be attributed to changes in basal NO activity in these arterioles. Thus, this study shows that streptozotocin-induced diabetes causes changes in NO activity and sensitivity in the microcirculation that depend on the type of arteriole. For each order of arteriole, these changes show a specific pattern during the course of diabetes.

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