Alterations in Nitric Oxide Activity and Sensitivity in Early Streptozotocin-induced Diabetes Depend on Arteriolar Size

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Changes in NO activity may play an important role in the early increase in microvascular flow that has been implicated in the pathogenesis of diabetic microangiopathy. We assessed, in the in situ spinotrapezius muscle preparation of 6 weeks' streptozotocin-diabetic rats (n = 6) and of age-matched controls (n = 8), basal inside diameters of A2–A4 arterioles and the reactivity to topically applied acetylcholine and nitroprusside, before and after N\(^\text{G}\) -nitro-L-arginine. In diabetic rats, cholinergic vasodilatation in A2–A4 arterioles was intact. Basal diameter in A3 and A4 arterioles was significantly higher in streptozotocin-diabetic rats. The increased basal diameter in A3 arterioles was partially due to an increased contribution of NO to basal diameter. The response to nitroprusside was impaired in streptozotocin-diabetic rats in A2, but not in A3 and A4 arterioles. Thus, this study shows that NO activity and sensitivity are altered after 6 weeks of streptozotocin-induced diabetes. These streptozotocin-induced changes are anatomically specific and, for arterioles, depend on their position within the vascular tree.

Keywords: Hyperglycemia; Microcirculation; Streptozotocin; Acetylcholine; Nitroprusside; Nitric oxide; Diabetes mellitus

INTRODUCTION

Microangiopathy has a major impact on morbidity and mortality of patients with diabetes mellitus.\(^{[1]}\) An important hypothesis regarding the pathophysiology of diabetic microangiopathy is the hemodynamic hypothesis,\(^{[2]}\) which states that an early, reversible increase in microvascular flow eventually leads to an endothelial injury response and microvascular sclerosis.\(^{[2-4]}\)

Using a mathematical model and experimental data on nitric oxide (NO) production and degradation rates, Vaughan et al., showed that the range of NO action may exhibit significant spatial heterogeneity in vivo and that the microcirculation is the optimal site for NO to exert its regulatory function.\(^{[5,6]}\) An increased flow early in diabetes, possibly as a result of enhanced NO production, has been described in several microvascular beds.\(^{[7,8]}\) However, those studies

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addressed only overall changes in flow. In order to gain insight in the exact role of NO in microangiopathy, it is necessary to assess its activity and effectivity at different sites of the microcirculation, as several orders of arterioles can react differently to chemical and mechanical vasoactive stimuli.\textsuperscript{[9–11]} Recently, it was shown that the small arterioles that control capillary perfusion are capable of responding differently in a spatially organised way and that metabolic changes are able to change this pattern of diameter change.\textsuperscript{[12]}

The earliest changes in the microvasculature caused by the metabolic disturbance in type 1 diabetes can most likely be observed in striated muscle, which is metabolically the most active tissue. Indeed, changes in reactions in different orders of arterioles of striated muscle to several vasoactive substances have been observed in rats 6 weeks after the induction of diabetes by streptozotocin (STZ).\textsuperscript{[13]}

To gain more insight regarding the role of NO in the pathophysiology of diabetes-induced microangiopathy, we investigated its contribution to vascular tone in arcade (A2) arterioles and the smaller transverse (A3) and precapillary (A4) arterioles in the \textit{in situ} spinotrapezius muscle preparation\textsuperscript{[14, 15]} of control rats and rats with STZ-induced diabetes. We chose the spinotrapezius muscle because it is easily accessible without much trauma, because different orders of arterioles can be studied with intravital microscopy, and because drugs can be applied topically, thus preventing systemic changes.

The aim of the present study, therefore, was to assess, \textit{in vivo}, the contribution of NO to basal tone and the reactivity to nitroprusside in different orders of arterioles after 6 weeks of STZ-induced diabetes, a widely studied model of type 1 diabetes.\textsuperscript{[18]}

\textbf{MATERIAL AND METHODS}

\textbf{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 6 conscious male Wistar rats (~250 g; HSD, Zeist, The Netherlands) by a single tail vein injection of 65 mg/kg STZ 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 pre-weighed commercial rat pellet chow. Experiments were performed 6 weeks after administration of STZ. Untreated male Wistar rats (\(n = 8\)) were used as controls.

\textbf{General Set Up}

Anesthesia was induced in overnight fasted rats by an injection of pentobarbital sodium (Nembutal\textsuperscript{R}; 60 mg/kg, intraperitoneally) and ketamine (70 mg/kg, intramuscularly). For further administration of Nembutal\textsuperscript{R}, an intraperitoneal cannula was inserted. As an indication of the need for additional small doses of the anesthetic (25% of the original dose), we frequently monitored blood pressure, interdigital and eye reflexes, as well as respiration rate with the intent of keeping the depth of the anesthesia stable. In our hands, this anesthetic protocol caused stable hemodynamics and blood gas values throughout the entire experimental period (approximately 5 hrs), indicating a constancy of the anesthetic conditions. Moreover, in previous studies\textsuperscript{[17, 18]} we have also found a stable O\textsubscript{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 the systemic hemodynamic variables which could influence the microcirculation we measured cardiac output in addition to blood pressure. Mean arterial pressure (MAP) was determined with a catheter
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(PE50) placed in the right carotid artery. The heart rate (HR) was obtained from the blood pressure recording. Cardiac output (CO) was measured by thermodilution: saline (0.2 ml) at room temperature was injected 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).

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 4 to 5 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[14] and Marshall.[19] A specific area of interest was selected at the caudo-medial side in which different orders of arterioles were present.

Classification of the vessels[20,21] is also illustrated in Figure 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, CaCl₂·H₂O 1.36, MgCl₂·6H₂O 1.05, NaHCO₃ 20.2, NaH₂PO₄·2H₂O 0.42 mol/L). During the experiment the superfusate was equilibrated with 95% N₂ and 5% CO₂, 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% O₂ and 5% CO₂. 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 25xSW objective (numerical aperture 0.63; Leitz, Germany). The optical magnification at the front plane of the video camera was 58.8x. The experiments were recorded on video tape (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).[22]

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. After that, 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 (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 ACh. The experimental set up did not allow us to perform a complete dose-response relationship of ACh. Therefore, we focused on the capacity of ACh to dilate the vessels maximally. We used 3 different doses (10⁻⁵, 10⁻⁴ and 10⁻³ mol/L), giving submaximal or maximal
responses, to accurately assess the level of the maximal response and the dose at which the maximal response was reached.

After we had tested the effect of ACh, we administered the NO-generating endothelium-independent vasodilator nitroprusside (NP: $10^{-4}$ mol/L, i.e., a dose high enough to cause maximal dilatation in control animals, as established in pilot experiments). Finally, adenosine (Ad), a mainly endothelium-independent vasodilator was added.\cite{23-25} The dose of Ad to obtain maximal dilatation was previously assessed at $10^{-4}$ mol/L; 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 from the initial contractile state.\cite{26}

After this series, we added L-NNA ($10^{-4}$ mol/L) 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 ($10^{-4}$ mol/L). We repeated the protocol with the dilatory drugs while L-NNA superfusion continued.

Analysis and Statistics

For statistical evaluation, we used the average values of all A2, A3, or A4 arterioles (ranging from 2 to 10) per rat. In 1 STZ rat muscle preparation no A3 arterioles were evaluated; the number of observations for statistical analysis was thus 6 for the STZ-treated rats in A2 and A4 arterioles, 5 for the STZ-treated rats in the A3
arterioles and 8 for the control group. We used two-tailed non-parametric tests with level of significance set at 5%.

RESULTS

General Characteristics

Blood glucose level measured 1 week after the STZ injection was 20 ± 5 mmol/L and remained so during the entire period of 6 weeks. Food consumption in DM rats, compared with control rats, almost doubled (≈150 vs. ≈300 g/wk). Nevertheless, the body weight of DM rats remained less than that of the control counterparts (242 ± 32 g vs. 350 ± 36 g for 6 weeks' diabetic rats and control rats, respectively; P < 0.05). There was no relationship between body weight and either passive diameter, baseline diameter or diameter during L-NNA in the different arterioles (data not shown). Systemic hemodynamic and biochemical variables at the start and at the end of the experiments are presented in Table I. At the day of the experiment, after an overnight fast, the glucose levels of both the DM and control rats had been decreased by ≈40% as compared to the non-fasted state. Baseline blood pressure was not significantly different between the groups, but heart rate and cardiac output were significantly higher in control rats. Hct was not significantly different between control rats and diabetic rats. During the actual microcirculation experiments (lasting ≈3 hr), there was a decrease in CO of about 15% in both control and diabetic rats. MAP and HR did not change significantly during the experiments (Tab. I).

Inside Arteriolar Diameter in Different Orders of Arterioles

As compared to control rats, DM rats had significantly increased inside diameters of A3 and A4, but not of A2 arterioles in the basal state (Fig. 2, Tab. II). Basal diameters relative to passive diameter did not differ significantly between DM and control rats (Fig. 2).

Contribution of Endogenous NO to Basal Inside Diameter

Overall, superfusion of L-NNA (10^-4 mol/L) caused a decrease in basal diameter in both control and diabetic rats (Tab. II), confirming the role of NO in maintaining basal diameters in this model. After blockade of the endogenous NO production, inside arteriolar diameters were not significantly different in DM rats as compared with control rats (Tab. II). However, the contribution of NO activity to basal inside diameter was significantly higher in diabetic rats than in control rats in A3 arterioles (Fig. 3).

Response to Nitroprusside

After addition of nitroprusside in the basal state, a significantly higher inside diameter was found in all types of arterioles in both control and diabetic rats (Tab. II). However, this increase was smaller in A2 arterioles of diabetic rats as
compared to controls. By contrast, in A3 arterioles inside diameter remained higher under nitroprusside in diabetic animals as compared to controls, while in A4 arterioles similar inside diameters were found (Tab. II). In order to correct for differences in basal NO production, responses to nitroprusside were also assessed after addition of L-NNA (Fig. 4). In contrast to control rats, in DM rats the response to $10^{-4}$ mol/L nitroprusside superfusion was significantly lower in A2, but not in the A3 and A4 arterioles, when endogenous NO production was blocked by L-NNA. We have tested in 3 additional experiments whether higher doses
TABLE II  Inside diameters (in μm) of A2, A3 and A4 arterioles in the spinotrapezius of control and diabetic (DM) rats at baseline, after blocking the NO production with L-NNA, and after dilatation with 10^{-4} M nitroprusside (NP), 10^{-3} M acetylcholine (ACh) or 10^{-4} M adenosine (Ad). Data are expressed as mean ± SD.

| Order of arteriole | Condition   | Control (n=8) | DM (n=6) |
|--------------------|-------------|---------------|----------|
| A2                 | basal       | 39.1 ± 11.7   | 36.5 ± 10.1 |
|                    | L-NNA 10^{-4} M | 31.6 ± 9.3    | 24.2 ± 4.5  |
|                    | NP 10^{-4} M  | 61.8 ± 11.7   | 48.9 ± 4.7* |
|                    | ACh 10^{-3} M | 62.4 ± 14.1   | 54.5 ± 8.9  |
|                    | Ad 10^{-4} M  | 63.7 ± 11.8   | 56.4 ± 6.4  |
| A3                 | basal       | 15.5 ± 5.2    | 23.1 ± 5.5* |
|                    | L-NNA 10^{-4} M | 12.3 ± 3.1    | 13.6 ± 2.4  |
|                    | NP 10^{-4} M  | 26.2 ± 4.1    | 31.6 ± 3.7* |
|                    | ACh 10^{-3} M | 25.4 ± 5.8    | 32.3 ± 3.0* |
|                    | Ad 10^{-4} M  | 27.7 ± 3.5    | 32.8 ± 2.2* |
| A4                 | basal       | 7.9 ± 2.8     | 11.6 ± 2.1* |
|                    | L-NNA 10^{-4} M | 6.2 ± 2.7     | 7.3 ± 3.7   |
|                    | NP 10^{-4} M  | 15.0 ± 3.1    | 16.7 ± 4.4  |
|                    | ACh 10^{-3} M | 15.2 ± 3.4    | 15.9 ± 2.8  |
|                    | Ad 10^{-4} M  | 15.4 ± 3.4    | 18.0 ± 7.4  |

*P < 0.05 for DM vs. control.

Responses to Acetylcholine and Adenosine

Acetylcholine, as compared with adenosine, was able to cause maximal dilatation in both control and diabetic rats in all orders of arterioles. No differences were found between acetylcholine doses of 10^{-4} and 10^{-3} mol/L. In A3 arterioles, inside diameters were significantly higher in diabetic rats after both after acetylcholine and adenosine (Tab. II). However, when endogenous NO production was blocked by L-NNA, inside diameters after adenosine or acetylcholine were of NP could further increase the arteriolar diameters in DM rats, but this was not the case (data not shown). Superfusion of higher doses of NP, however, had a complicating disadvantage, because at these doses the NP caused systemic effects (i.e., a decrease in blood pressure).
no longer significantly different between diabetic and control rats in these arterioles (data not shown), suggesting that an increased basal production of NO could still cause a further increase in diameter in diabetic rats after either adenosine or acetylcholine.

**DISCUSSION**

Induction of diabetes mellitus by streptozotocin treatment in rats is an accepted model of human type 1 diabetes. This model allowed us to investigate differential microcirculatory changes...
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in DM, which cannot be studied in man. We intended and achieved incomplete destruction of pancreatic beta-cells and therefore the diabetic rats had some residual insulin production and thus a comparatively moderate hyperglycemia (∼20 mmol/l).\[15,27\]

Using intravital microscopy, we found an increase in basal diameter in A3 and A4, but not in A2 arterioles after 6 weeks of STZ-induced diabetes mellitus (Fig. 2). There was a greater change in diameter after L-NNA in the diabetic rats as compared to the control rats, especially in A3 arterioles (Fig. 3). This suggests that the increase in basal diameter observed in diabetic rats is at least partially due to an increased contribution of endogenous NO to basal diameter in the A3 arterioles. Although NO production was increased significantly in A3 arterioles only, it cannot be excluded that also in A2 and A4 arterioles there was some increase in NO production in diabetic rats. These findings support the hypothesis of Williamson et al., that an increased NO production occurs in the early phase of DM.\[29\] An increased NO production was also observed in the kidney of STZ rats.\[29\] However, several studies also report weaker relaxation responses to endothelium-dependent agents after induction of diabetes with STZ.\[30–33\] These differences might be explained by variations in experimental set-up, the type of endothelium-dependent relaxation response that was investigated, the type of vessel, the duration of diabetes and differences in the elevation in glucose levels that were obtained in STZ rats.

Although NO has been indicated as an antiatherosclerotic molecule,\[34,35\] several studies provide evidence that the increase in NO activity we observed in the early phase of STZ-induced diabetes may be a mediator of vascular damage. An increased NO synthase activity can induce chemical injury to the vessel wall via the production of peroxynitrite, a toxic radical.\[36–39\] An enhanced NO activity in the early phase of diabetes which preceded impaired function in the later phase was described by Pieper et al.\[38\] Moreover, they showed that NOX-101, a scavenger of NO, prevented endothelial dysfunction in STZ-induced diabetic rats.\[39\] However, it has also been shown that chronic treatment with an NO donor can ameliorate diminished responses to ACh in aorta and kidney vasculature caused by streptozocin.\[40\] Whether the observed increase in basal NO activity shown in our model also causes microvascular damage in a later phase of the disease needs further elucidation.

In the control rats, the maximal responses to ACh were comparable to those found by other investigators using the same spinotrapezius muscle preparation.\[15\] In the diabetic rats of the present study, ACh still had the capacity to dilate the arterioles maximally after 6 weeks of diabetes. The maximal vasodilation caused by ACh could only partly be blocked by L-NNA, indicating that in this study the maximal response to ACh was mainly independent from nitric oxide production. Although ACh is often used as a means to measure stimulated NO-mediated vasodilatation it is well known that ACh not only releases NO, but also causes an increased production of the vasodilator endothelial derived hyperpolarizing factor (EDHF) and prostacyclin (PGI2) as well as several vasoconstricting mediators. Streptozotocin diabetes can cause an increase in ACh-induced PGI2 production.\[40\] As we focussed 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: a decrease in the effect of 10−4 mol/L nitroprusside in diabetic rats in the A2 arterioles. 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 NP effect is impaired, a defect in the mechanism by which
NO stimulates the smooth muscle cells through activation of guanylate cyclase and production of cGMP is assumed.\[^{[41]}\] The dose of nitroprusside we used (10^{-4} \text{ 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 arterioles of diabetic rats. The cause of this phenomenon is presently unclear, but it is possible that the mechanism via which NO activates guanylate cyclase in smooth muscle cells is impaired after 6 weeks of STZ induced diabetes. An argument in favor of this hypothesis is the fact that A2 arterioles contain a relatively high amount of smooth muscle cells. An early increase in NO production may cause a decreased expression of guanylate cyclase or a desensitisation of guanylate cyclase.\[^{[42]}\]

In this study we investigated changes in arteriolar reactivity after 6 weeks of STZ induced diabetes. We found an increased basal diameter in A3 and A4 arterioles and an unchanged diameter in A2 arterioles. This would be expected to result in a decreased pressure gradient from A2 arterioles to precapillary arterioles and thus in an elevated capillary pressure, which indeed has been observed in patients with type 1 diabetes mellitus.\[^{[43]}\]

There are several possible causes for the changes in arteriolar reactivity in streptozotocin diabetes. Firstly, moderate hyperglycemia can cause changes in oxidative stress and increased production of reactive glycation products. Both for acute and chronic hyperglycemia it has been shown that hyperglycemia increases NO production and decreases the bioavailability of NO.\[^{[44, 45]}\] Secondly, changes in arteriolar reactivity might be primarily due to the decreased delivery of nutrients to the muscle cells, causing an autoregulatory dysfunction leading to vaso-dilatation.\[^{[2-4]}\] Thirdly, streptozotocin-induced diabetes causes marked atrophy of hindlimb skeletal muscles, which is accompanied by a relative increase in flow capacity per tissue mass.\[^{[46]}\] It cannot be excluded that the changes in arteriolar reactivity that were observed in this study might have been partly caused by an attenuation of muscle development that is caused by STZ diabetes, but that is also part of the clinical picture of type 1 diabetes. However, the exact mechanism via which STZ diabetes can cause the observed changes in arteriolar reactivity is beyond the scope of this study.

In conclusion, after 6 weeks of STZ-induced diabetes in rats, we found an increased basal diameter of small transverse arterioles of the spinotrapezius muscle but not of the larger arcade arterioles. There was a significantly increased contribution of endogenous NO to basal tone in A3 arterioles. The reactivity to exogenous NO was impaired in A2, but not in A3 and A4 arterioles. Thus, this study suggests a role of alterations in NO activity and sensitivity to NO in the pathogenesis of diabetic microvascular complications. Furthermore, our results show that these diabetes-induced changes are anatomically specific and, for arterioles, depend on their position within the vascular tree.

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