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## Nitric Oxide Synthase in the Skeletal Muscle and Arterioles of Rats with Streptozotosin-Induced Diabetes Mellitus

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**Abstract:** Nitric oxide synthase (NOS) that uses NADPH as a cofactor is an enzyme which produces nitric oxide (NO) from L-arginine. Endothelial-derived (eNOS), inducible (iNOS) and neuronal (nNOS) nitric oxide synthase are 3 known isoforms. Endothelial NO regulates the vascular tonus and causes the dilatation of vessels, while NOS localized in the sarcolemma of striated muscle plays a role in the regulation of muscle hemodynamics. Many authors have found differences in NO production in some pathological conditions such as diabetes mellitus (DM), hypertension and atherosclerosis. In this study, NADPH-diaphorase (NADPH-d) was histochemically employed to explore any changes in NO production associated with the degree of DM, by detecting the enzyme in the muscle tissue. Diabetes was induced by injecting Swiss albino rats with 65mg/kg of streptozotosin (STZ). Two, 4, 6 and 12 weeks after the STZ injection, the spinotrapezius muscles of the animals were fixed in paraformaldehyde and NADPH-d histochemistry was applied to cryostat sections. Six and 12 weeks after DM, NOS increased both in the muscles and the endothelial cells of arterioles, being more prominent in the 12-week group in which the NADPH-d reaction was also localized in sarcoplasm together with the sarcolemma of muscle fibers than in controls. NOS was more abundant in the 2nd- and 3rd-order arterioles in diabetic animals than in controls. After the superfusion of the N<sup>W</sup>-nitro-L-Arginine Methyl Ester (L-NMA) that inhibits NOS, the NADPH-d reaction was still present in 12-week DM, while not in controls. ACh superfusion caused endothelial dilation and increased NO production in both the control and diabetic groups. This increase was observed to be more pronounced in diabetics than in controls. We also superfused sodium nitroprusside as a NO donor, and found resulting dilation in arterioles, with differences between the control and DM groups, the latter being more conspicuous.

**Key Words:** Nitric oxide synthase (NOS), diabetes mellitus, skeletal muscle, arteriole

### Sıçanlarda Streptozotosin ile Oluşturulan Diabetes Mellitusda İskelet Kası ve Arteriollerde Nitrik Oksid Sentaz

**Özet:** Nitrik oksid sentaz (NOS), NADPH kofaktörünü kullanarak, L-argininden nitrik oksid (NO) üretilmesini sağlayan enzimdir. Endotel kaynaklı (eNOS), uyarılabilir (iNOS) ve nöronal (nNOS) olmak üzere üç ayrı izoformu mevcuttur. Endotel kaynaklı NO, damar tonusunu düzenler ve damarların gevşemesine neden olur. Çizgili kasın sarkolemmasında izlenen NOS da kas hemodinamiğinin düzenlenmesinde rol oynar. Diabetes mellitus (DM) gibi bazı patolojik durumlarda NO üretiminde farklılıklar ortaya çıkmaktadır. Bu çalışmada, DM un derecesine bağlı olarak NO üretiminin değişip değişmediğini anlamak için NOS kofaktörü olan NADPH-d histokimyasal olarak uygulandı ve enzimin doku düzeyindeki dağılımı gösterildi. Bunun için Swiss albino sıçanlara 65 mg/kg streptozotosin (STZ)

enjekte edilerek DM oluşturuldu. Enjeksiyondan 2, 4, 6 ve 12 hafta sonra hayvanların spinotrapezius kasları paraformaldehid ile tesbit edilerek, alınan kriyostat kesitlerine NADPH-d yöntemi uygulandı. DM un altıncı ve 12nci haftasından sonra hem kaslarda hem de arteriollerin endotel hücrelerinde NOS in arttığı gözlemlendi. DM lu kas fibrillerinde sarkolemma yanında sarkoplazmada da NADPH-d reaksiyonu mevcuttu. Arteriollerin ikinci ve üçüncü dallanmalarında NOS miktarı kontrolden fazla idi. NOS inhibitörü olan L-NMA süperfüzyonundan sonra kontrolden NOS görülmezken, diabetin onikinci haftasında NADPH-d reaksiyonu gözlenmekteydi. ACh süperfüzyonu endotel kaynaklı gevşemeye neden oldu ve NO üretimi hem kontrol grubunda hem de diabetik grupta arttı. Fakat bu durum diabetik grupta çok daha belirgindi. Bir NO vericisi olan sodyum nitroprusid uygulandığında arteriollerde gevşeme gözlemlendi ve NOS reaksiyonu bakımından kontroller ile diabetik grup arasında fark vardı. Diabetik grupta bu reaksiyon da oldukça belirgindi.

**Anahtar Sözcükler:** Nitrik oksid sentaz (NOS), diabetes mellitus, iskelet kası, arteriol

## Introduction

Nitric oxide synthases are enzymes which produce nitric oxide (NO) from L-Arginine. Three major isoforms of this enzyme have been isolated and characterized: endothelial (eNOS), inducible (iNOS) and neuronal (nNOS). nNOS occurs in several cell types, including neurons and skeletal muscle cells (1). In all vertebrate species, nNOS is present in the sarcolemma of muscle fibers (2, 3, 4). Recent reports indicate that skeletal muscle tonically produces NO and that NO functions as an endogenous modulator of muscle physiology, at both the tissue and the cellular levels. NO derivatives appear to mediate cell-cell interactions, e.g., by modulating vascular control. NO also acts directly on skeletal muscle fibers to influence both muscle metabolism and contractile function (5). Endothelial NOS is widely expressed in endothelial cells throughout the vascular bed. It is generally accepted that endothelium-derived NO is an important factor in the control of basal vascular tone (6). In response to agents such as bradykinin and acetylcholine or to mechanical forces such as shear stress, endothelial cells produce NO, which diffuses into vascular smooth muscle cells and causes vasorelaxation. Arginine analogs such as N<sup>G</sup>-monomethyl-L-arginine (L-MMA) and L-nitroarginine are competitive inhibitors of NOS enzymes (7).

Abnormalities in endothelial production of NO occur in atherosclerosis, diabetes and hypertension (8). In patients with insulin-dependent diabetes mellitus (IDDM), increased forearm and skin blood flow are found, indicating vasodilation (9,10,11). It is not known whether microvascular changes in IDDM are linked to changes in endothelium-dependent vasoactive mechanisms. When the production of NO rises, peripheral resistance falls and organ blood flow increases. An increase in NO production has been suggested to occur in IDDM and to explain the vasodilation that is typically observed (12,13). Other investigators, however, found the production or efficiency of NO to be diminished in experimental and human IDDM (14,15,16). The role of NO with respect to changes in vasoreactivity in IDDM is controversial.

The aim of this study was to determine the NOS differences in skeletal muscle and the arterioles of muscle during DM.

## Materials and Methods

Experimental animals: Diabetes was induced in 36 male Swiss albino rats (250 g) by a single intraperitoneal injection of 65 mg/kg Streptozotocin (STZ) dissolved in serum physiologic solution. The animals were housed in individual cages and received commercial rat pellet chow and water ad libitum. The rats were separated into 5 groups: I: 2-week DM (n=6), II: 4-week DM (n=6), III: 6-week DM (n=6), IV: 12-week (n=6) DM and V: 12-week DM, which were also separated into 3 groups superfused with  $10^{-4}$  M Acetylcholine (ACh, n=4),  $10^{-4}$  M sodium nitroprusside (NP, n=4) or  $10^{-4}$  M N<sup>W</sup>-Nitro-L-Arginine Methyl ester (L-NMA, n=4) Time-matched untreated rats were used as controls (n=10) for the first 4 groups. Controls of group V were also separated into 3 subgroups which were superfused with ACh, NP or L-NMA. Glucose levels were determined weekly in all animals by glucometer (Bayer) in blood samples taken from tail tip.

At the end of the diabetic periods, anesthesia was induced by injection of sodium pentothal (70 mg/kg) intraperitoneally. Body temperature was kept at 37-38°C by a heating table under the rats during the experiment.

The 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 4-5 atraumatic sutures to separate it from the underlying muscle layers, as described by Gray (17) and Marshall (18). Tissues were superfused with saline solution during muscle preparation and drug application. The temperature of this solution was maintained at 36°C in groups I, II, III and IV and their controls, and the muscle was removed next to the spine and fixed with 4% paraformaldehyde. In the other groups (subgroups of V) and their controls, the muscle was superfused with NO-dependent ACh for 3 minutes (n=4); NO donor NP for 6 minutes (n=4); and NOS inhibitor L-NMA for 15 minutes (n=4). All drugs were prepared with saline solution and after superfusions, muscles were removed and fixed.

NADPH-d histochemistry: Skeletal muscle was fixed in 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4 for 2 hours, and tissue was cryoprotected in 20% sucrose in 0.1M phosphate buffer (pH 7.4) at 4°C overnight. Ten-micrometer sections were cut with cryostat and mounted on slides. These sections were incubated with 1mMβ-NADPH (Sigma), 0.5 mM Nitroblue tetrazolium (Sigma) and 0.2% triton X-100 in 50 mM Tris-HCL pH 8, at 37°C for 45 minutes, and then rinsed with 0.1M phosphate buffer (pH7.4) and distilled water. Finally they were mounted in glycerol (19).

## Results

Glucose levels of control rats and before STZ injection were  $98 \pm 2$  mg/dl (n:16). One week after, glucose levels were significantly increased ( $P < 0.0001$ ). It was  $352 \pm 23$  mg/dl (n:17) in the first week and it remained at similar levels for 12 weeks (Table 1).

	Glucose (mg/dl)
Control	98±2 (n:16)
1 week	352±23 (n:17)
2 weeks	322±19 (n:7)
4 weeks	275±11 (n:7)
6 weeks	431±25 (n:7)
12 weeks	377±16 (n:7)

Table 1. Glucose levels of control and STZ injected animals during experimental periods.

± :SE.

A control was prepared for NADPH-d reaction, treated with only nitroblue tetrazolium and triton X-100 without NADPH-d. There was no histochemical reaction for NOS (Fig. 1). NADPH-d reactions were localized exclusively at the sarcolemma and endothelial line of arterioles in control animals (Fig. 2a). In diabetic rats, a positive staining was observed at the sarcolemma of muscle fibers, which did not occur in controls. In addition, several muscle fibers showed granular and diffuse sarcoplasmic NADPH-d reactions. These events are more clear in 6- and 12-week diabetic animals (Fig. 2b). The arteriolar reaction was also similar in both 2- and 4-weeks DM. There was no significant increase as compared to control slides (Fig. 3a, b). However, when we looked at the eNOS histochemistry 6 weeks after STZ injection, endothelial NADPH-d reaction was increased, and 2nd- and 3rd-order arterioles were more reactive, the branchial points in particular having strong eNOS (Fig. 4). In 12-week DM, the histochemical reactions of endothelial cells were greater than those of controls and other groups (Fig. 5a,b).

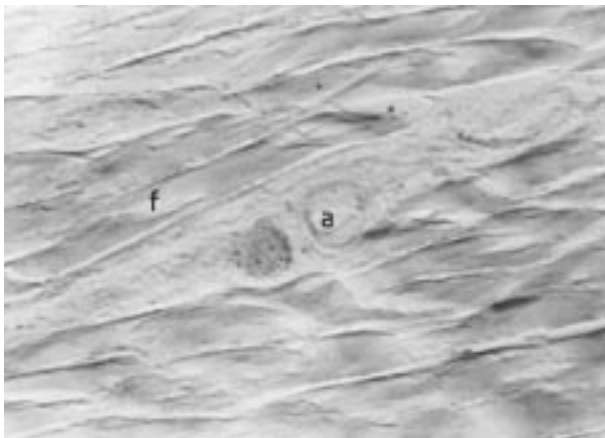


Figure 1. When cofactor was not applied to the tissue, there was no NADPH-d reaction. This shows that NADPH detects NOS. Muscle fiber (f) and arteriole (a) X 400.

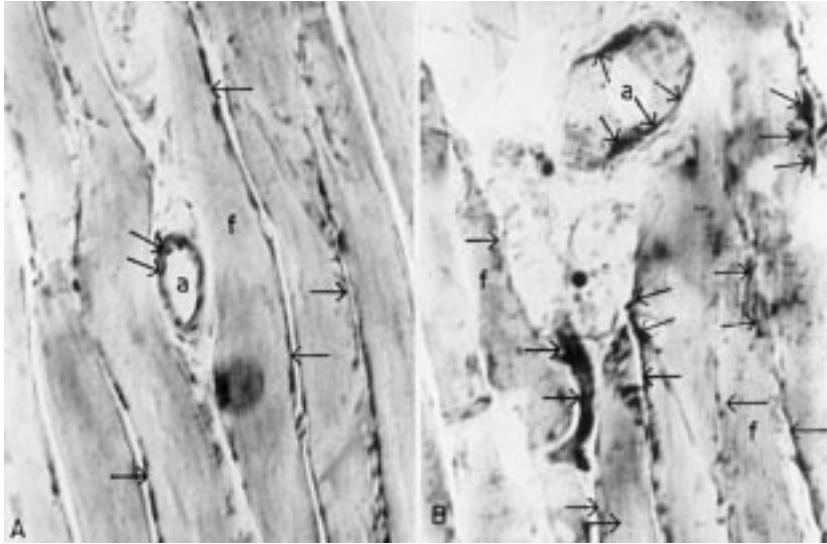


Figure 2. We saw less NADPH-d reaction (→) in muscle fibers (f) and arterioles (a) in control groups (A). (B) shows increased NADPH-d reaction (→) in the sarcolemma, sarcoplasm and arteriole (a) of muscle in 12-week DM X 800.

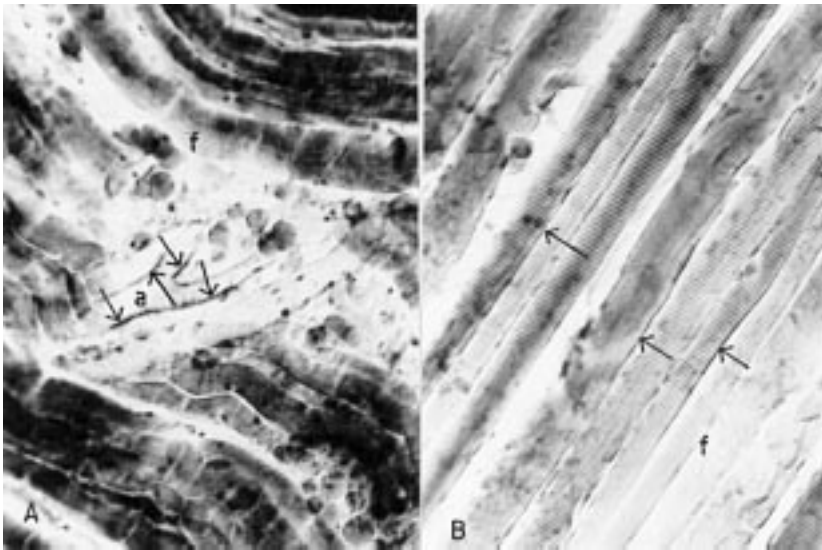


Figure 3. There are no significant differences between control, 2-week and 4-week DM groups. In 2-weeks DM, the NOS reaction (→) was almost the same as in the control group (A), and a similar reaction to those of 2-weeks DM and controls was found in 4-week DM (B) X 400.



Figure 4. Increased NOS histochemistry (→) in 6-week diabetes was observed when compared to the control and 2- and 4-week DM. The reaction was more dense in the 2nd- and 3rd-order arterioles of experimental animals X 800.

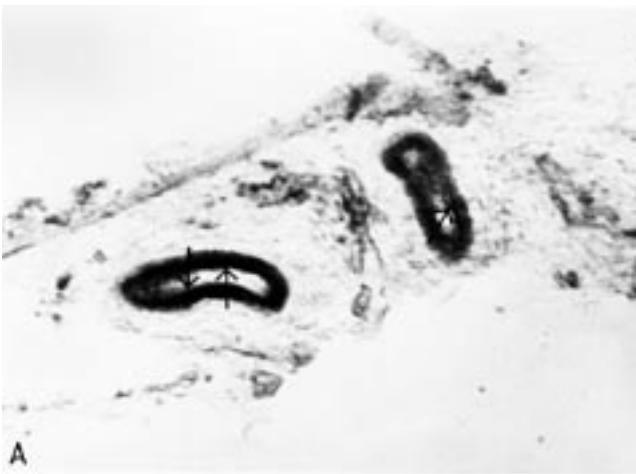


Figure 5. NOS was firmly increased with STZ injection for 12 weeks. (A) and (B) show increased NADPH-d reaction (→) of different-sized arterioles in 12-week DM X 400.

After blocking the NO production with L-NMA, there was no NADPH-d reaction in either the arterioles or the sarcolemma of the muscle in the control animals. Some arterioles were also contracted in these groups (Fig. 6a), whereas we observed eNOS reactions in some parts of the arterioles and muscle fibers in 12-week diabetic rats that were superfused with L-NMA (Fig. 6b). After ACh and NP superfusion, the dilation of vessels was clear, but NP superfusion caused more relaxation in both arterioles and muscles. NOS reactions were also significantly increased after treatment with these drugs in the experimental groups (Figs. 7-8).

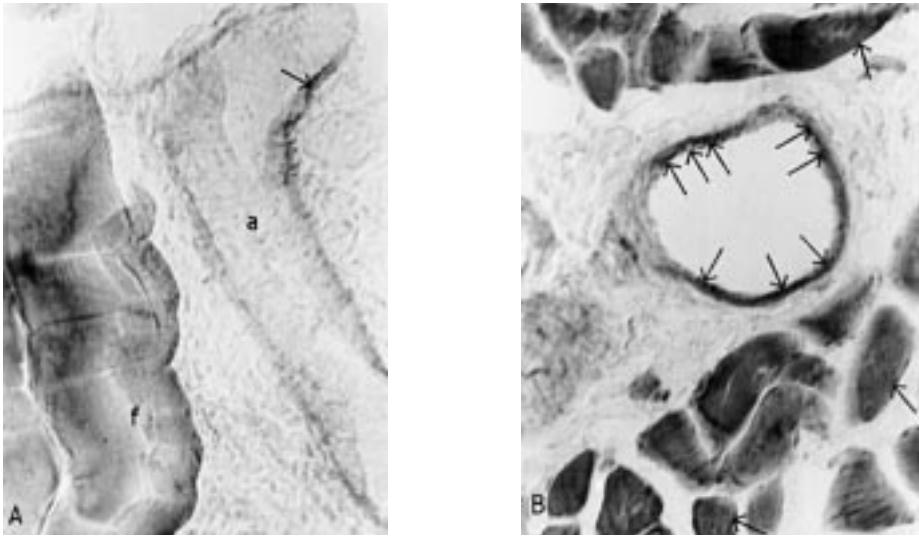


Figure 6. After L-NMA superfusion, since L-NMA inhibited NOS in the control group, there was no NADPH-d reaction in both muscle fibers (f) and arterioles (a) (A). But L-NMA did not completely inhibit NOS in severe diabetes, resulting in NADPH-d reaction (→) in some sarcolemma of muscle fibers (f) and endothelial cells of arterioles (a) in 12-week (B) DM X 800.

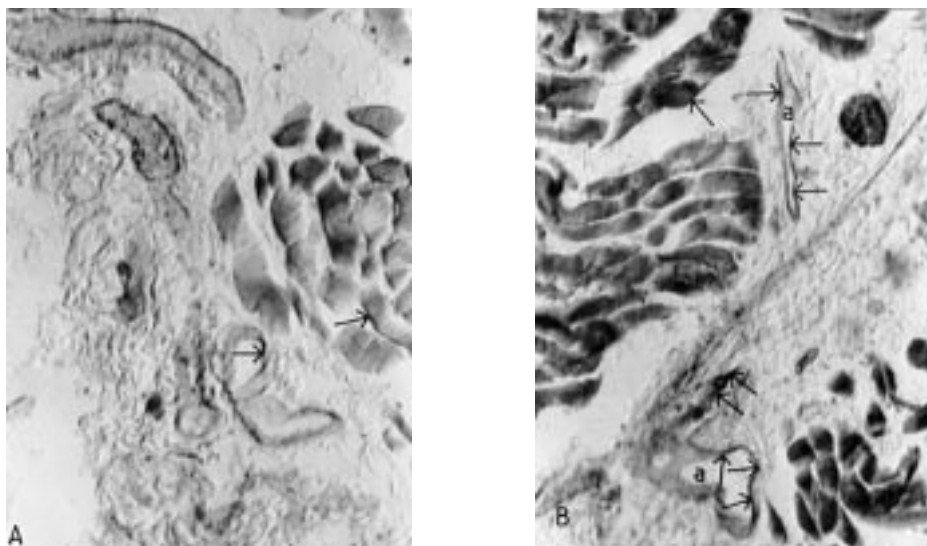


Figure 7. After ACh superfusion, NOS reaction was lower in controls than in diabetics (A) and in 12-week DM (B). NOS activity (→) was stronger X 400.



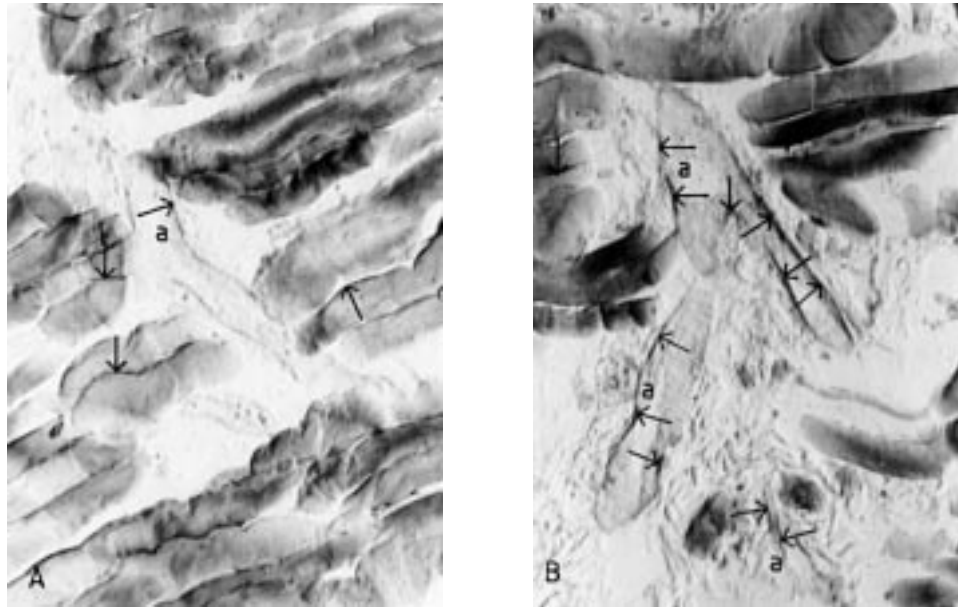


Figure 8. Dilated arterioles with NP in control group (A). Less reaction is seen. In (B) NOS activity is persistent in 12-week DM because of the increased NO production X 400.

## Discussion

We have shown that the enzyme NOS was increased in the skeletal muscle at 6 and 12 weeks of diabetes mellitus. Moreover, we have seen that the NADPH-d reaction in diabetic rats was often localized also in the cytoplasm of muscle fibers in addition to the normal plasmalemmal localization. Cappani et al. (1998) reported that in aged rats the amount of nNOS in the skeletal muscle increased both in the soluble and microsomal fractions and that an additional intracytoplasmic localization appeared (4). Neuronal NOS was localized to the sarcolemma of fast fibers; eNOS was associated with mitochondria. Isolated skeletal muscle produces NO at low rates under resting conditions. NO appears to mediate cell-cell interactions in muscle, including vasodilation and inhibition of leukocyte adhesion. Muscle metabolism also appears to be NO-sensitive at several sites, including glucose uptake, glycolysis, mitochondrial oxygen consumption and creatine kinase activity (5). Young and Leighton (1998) have shown that NO stimulates glucose transport and glucose oxidation in isolated, incubated rat skeletal muscle preparations. Increased NO can stimulate glucose metabolism in skeletal muscle (20). On the other hand, Way et al. (1999) studied anococcygeus muscle from diabetic rats, measuring NOS activity and NADPH-d activity in the tissue, but found no significant differences between DM and normal smooth muscles (21).

We observed eNOS differences between groups and, especially 6 and 12 weeks after STZ administration, NADPH-d reaction was increased. These results were clear in 2nd- and 3rd-order arterioles. We recently showed that vasoreactivity of arterioles were more pronounced in larger arterioles than in smaller ones in DM (22). The vascular endothelium shows many abnormalities in diabetes; among these, there seems to be a substantial defect in the normal vasodilatory capacity of the endothelial cells. According to some investigators, the underlying defect points towards a reduction in NO synthesis (8,14,15,16, 23, 24). However, some others have reported that NO synthesis increases in the endothelial cells during diabetes mellitus (12,13, 25,). Our previous (22, 26) and present results showed that NO synthesis increased in arterioles during the development of diabetes mellitus. Through the elevation of endothelial cell calcium, D-glucose may lead to increased synthesis of NO (27). This mechanism may contribute to the vasodilation and reduced peripheral resistance in the early stages of diabetes and the associated increase in blood flow, and cause shear stress, which stimulates NO synthesis (28, 29). Hyperglycemia also stimulates the polyol pathway, leading to an increase in sorbitol flux and fructose synthesis through enhanced aldose reductase activity (30). Activation of the polyol pathway is associated with increased utilization of NADPH, which may lead to reduced availability of cellular NADPH, an essential cofactor of NO synthase (31). We observed increased NADPH-d reaction in the endothelial cells of arterioles in 6- and 12-week diabetic animals, but we saw no clear differences between controls and 2- 4-week diabetics. These results may be related to utilization of NADPH in DM.

After blocking the NO production with L-NMA, saw no reaction to NADPH-d in control animals, whereas there were some reactions in the endothelial cells of arterioles 12 weeks after the injection of STZ. This means NO production was increased in DM. ACh and NP superfusion also showed similar results in 12-week DM. ACh, which is an endothelial dependent vasodilatory agent, causes the release of NO from endothelial cells, and NO stimulates cGMP, which causes dilatation of the smooth muscle of the arterioles. The arterioles were dilated both in controls and 12-week DM after adding ACh to the superfusate, but the NOS reaction was present in some parts of the arteriole, causing even maximal dilatation. NP is a NO donor, directly causing dilatation in the smooth muscle cells of the arterioles. When we superfused the muscle with NP, the NOS reaction was more pronounced in 12-week DM than in controls.

It can be concluded that NO production increases in DM after 6 weeks.

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