Graefe's Arch Clin Exp Ophthalmol
(2003) 241:747–756

LABORATORY INVESTIGATION

DOI 10.1007/s00417-003-0740-7

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NADPH diaphorase activity in the rat retina during the early stages of experimental diabetes

Received: 2 April 2003
Revised: 28 May 2003
Accepted: 18 June 2003
Published online: 2 September 2003

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Abstract *Background:* Nitric oxide (NO) plays an important physiological role in inter-cellular communication, but when produced in excess it can become toxic. Our goal was to evaluate possible involvement of NO in the development of retinopathy in diabetic rats. *Methods:* Diabetes was induced in male albino rats by intravenous injection of streptozotocin. Some of the normal and diabetic rats were raised with added L-arginine to increase in vivo NO synthesis, or with added L-NAME to inhibit the rate of in vivo NO synthesis. NADPH diaphorase histochemistry was conducted on retinal whole mounts and transverse sections at different time intervals after induction of diabetes. The electroretinogram (ERG) was recorded to assess retinal function. *Results:* After 6 weeks of diabetes, NADPH diaphorase amacrine cells in the diabetic retinas appeared abnormal in their morphology and the degree of staining was decreased in their processes. In contrast, NADPH diaphorase activity was augmented in Müller cells. Supplementing the rats' diet with L-arginine for 10 weeks slightly reduced NADPH diaphorase activity in amacrine cell in normal rats but had no effect on the diabetic rats. Adding L-NAME for 10 weeks did not alter NADPH diaphorase histochemistry in either normal or diabetic rats. The ERG responses were reduced by L-arginine supplementation in normal and diabetic rats, and were unaffected by adding L-NAME to the drinking water. *Conclusions:* Our findings are consistent with the hypothesis that high glucose levels are deleterious to the rat retina and that excessive synthesis of NO may contribute to the development of diabetic retinopathy.

Keywords Diabetes · Nitric oxide · Rat · Retina · Electroretinogram · Amacrine cells · Müller cells

Introduction

Nitric oxide (NO) has been shown to play a major physiological role in inter-cellular communication [5, 35, 45]. It is produced from L-arginine by the enzyme nitric oxide synthase (NOS) [4] and acts upon the cytosolic form of guanylate cyclase in the target cells [26, 28]. Three isoforms of NOS have been identified [33, 35]. The endothelial (eNOS) and neuronal forms (nNOS) are constitutive enzymes that produce physiological levels of NO for normal functioning of vascular beds and neuronal tissues. The inducible form of NOS (iNOS) produces NO at high rates that can become toxic to living tissue.

One of the primary functions of NO is to control the rate of blood flow via its effects on the vascular smooth muscle [33, 37]; therefore, the NO system has been the focus of numerous studies concerning vascular-related complications of diabetes mellitus. Conflicting findings on NO involvement in animal models of diabetes have been reported over the years. Several studies reported that endothelium-dependent relaxation of aortic rings or isolated mesenteric arteries was reduced [13, 27, 36, 38].

This was attributed to a reduction in NO synthesis from L-arginine [38] or to a reduced response of the vascular smooth muscle to NO [13]. In another study, the endothelium-dependent vascular relaxation was impaired, whereas the response to exogenous application of NO was normal indicating normal responsiveness of the vascular smooth muscle to NO but impaired NO synthesis [50]. In contrast to the above-mentioned reports, the endothelial cells of the mesenteric arteries in rats with prolonged diabetes were found to release normal levels of vasodilator substances, and the arteries were found to respond normally to exogenous application of NO [17]. Human studies also yielded conflicting results; some showed impaired endothelium-derived NO activity with normal response to exogenous NO [49], whereas others reported that both tests for NO-vascular interaction were reduced [6, 51] or normal [2, 44].

The involvement of NO in the development of diabetic retinopathy has been investigated in several studies. Patients with proliferative diabetic retinopathy were characterized by elevated levels of NO in the vitreous [53]. Ocular hemodynamic reactivity to inhibition of NO synthesis was impaired in patients with long-standing IDDM compared with healthy subjects [41]. From these and other studies, it is apparent that the exact role of NO in the development of diabetic complications is not yet clear.

We have recently shown that the functional integrity of the rat retina in streptozotocin (STZ)-induced diabetes is reduced as early as 2 weeks after induction of diabetes [31]. The present study was designed to test the possible involvement of the NO system in the early stages of retinopathy in diabetic rats. Histochemical and immunocytochemical studies of the normal rat retina demonstrated the presence of NOS in amacrine cells and in few displaced amacrine cells [12, 14, 40, 52, 54]. The endothelial form of NOS (eNOS) was also demonstrated in rat Müller cells and endothelial cells [23]. Herein we applied NADPH diaphorase histochemistry to retinal whole mounts and transverse retinal sections of rats at different time intervals after induction of diabetes. The effects of adding L-arginine or L-NAME to the drinking water on retinal function (ERG responses) and NADPH diaphorase activity was studied in normal and diabetic rats.

Materials and methods

Animals

Male Sprague-Dawley rats, weighing approximately 200 g, were used in this study. Sixty rats were studied: 24 in the normal group and 36 in the diabetic group. Diabetes was induced as described previously [31] by intravenous injection (caudal vein) of streptozotocin (STZ; Sigma, St. Louis, Mo.). Diabetes was confirmed by fasting hyperglycemia in excess of 350 mg/dl. Normal rats, matched in age and gender to the diabetic rats, were studied in parallel to the diabetic rats.

The diabetic and normal rats were housed in the same room and were maintained on a standard diet and water ad libitum, under 14/10 h light/dark cycle. In order to increase in vivo synthesis of nitric oxide, L-arginine (1 g/l) was added to the drinking water as a substrate for NO synthesis [36]. N^{ω}-nitro-L-arginine methyl ester (L-NAME; 0.1 g/l) was used as a competitive inhibitor of NO synthesis [39] to reduce in vivo synthesis of NO.

All experimental procedures conformed to the ARVO recommendations for the use of laboratory animals and to institutional guidelines.

Electroretinogram

Rats were anesthetized by intra-muscular injection of a solution containing ketamine hydrochloride solution (10 mg/ml), acepromazine maleate solution (10%), and xylazine solution (2%) in the following volume proportions: 1.0:0.2:0.3, respectively. The dose used (0.5 ml/kg body weight) was sufficient to prevent pain as judged from tail reflex, yet it was not too deep to affect the ERG responses. The pupils were fully dilated with cyclopentolate hydrochloride 1%. Topical anesthesia (benoxinate HCl 0.4%) was administered before ERG recordings.

Prior to the ERG recording session, rats were maintained in complete darkness overnight and prepared for the recordings under dim red illumination. The ERG responses were recorded as described previously [31], from both eyes with corneal electrodes (Medical Workshop, Groningen, Holland). The reference and ground electrodes were made of surgical needles and were inserted into the pinnae. The signals were amplified (×10,000) and filtered (0.3–300 Hz) by differential preamplifiers (Grass, West Warwick, Rhode Island), then digitized by a personal computer equipped with a data acquisition board (Scientific Solutions, Solon, Ohio). To improve signal/noise ratio, 6 ERG responses, which were elicited at 10-s intervals by identical light stimuli, were averaged on-line. Light stimuli were obtained from a Ganzfeld light source (LKC Technologies, Gaithersburg, Md.) with maximum, unattenuated stimulus intensity of 5.76 cd-s/m^2. The ERG responses were elicited by white light stimuli covering intensity range of more than 4 log units using neutral density filters.

The ERG analysis consisted of amplitude and implicit time measurements. The implicit time of the b-wave was measured from stimulus onset to the peak of the b-wave. The amplitude of the a-wave was measured from the baseline to the trough of the a-wave, and the amplitude of the b-wave was determined from the trough of the a-wave to the peak of the b-wave. To assess retinal function, the relationships between the amplitudes of the dark-adapted a- and b-waves and stimulus intensity were fitted to a hyperbolic-type relationship [24]:

$$V = Vmax * \left(I/(I+\sigma)\right) \quad (1)$$

In this equation, V and Vmax are respectively the amplitudes of the ERG wave elicited by a stimulus of intensity I and by a stimulus of super-saturating intensity. The parameter σ is the semi-saturation constant and denotes the stimulus intensity eliciting a response of half-maximal amplitude.

NADPH diaphorase histochemistry

NADPH diaphorase histochemistry is a common method for identifying all three isoforms of NOS [3, 4, 15, 25, 42]. Since NADPH diaphorase activity reflects the ability of NOS to convert the dye nitro blue tetrazolium into an insoluble formazan salt, this method determines the amount of active NOS that is present immediately prior to fixation and not the total amount of NOS [5, 34]. This explains some discrepancies between immunocytochemistry and NADPH diaphorase histochemistry because a large amount of

non-active NOS will lead to intense immunoreactivity and faint NADPH diaphorase activity, and vice versa, a small amount of active NOS will cause faint immunoreactivity and intense NADPH diaphorase activity [34]. Thus, the disadvantage of NADPH diaphorase histochemistry is its lack of specificity of NOS isoforms, but its advantage is its ability to determine the degree to which NOS molecules are activated at the time of tissue fixation.

NADPH diaphorase histochemistry was applied, as described previously [54], to retinal whole mounts and to transverse retinal sections. Rats were anesthetized with sodium pentobarbital (40 mg/kg body weight) and the eyes were enucleated. Then, the rats were killed with an overdose (80 mg/kg body weight) of sodium pentobarbital. The eye was fixed for 10 min in 0.1 M phosphate buffer (pH=7.4) containing 4% paraformaldehyde. The anterior portion of the eye was dissected away and the eyecup was left for additional 10 min of fixation. The eyecup was incubated at 37°C for 135 min in the reaction solution (0.2% Triton X-100, 0.5 mM nitro blue tetrazolium, 1 mM NADPH, 9 mM L-NAME and 50 µm $CaCl_2$ in 0.1 M Tris-Cl, pH=7.4), and then, the eyecup was rinsed twice in 0.1 M Tris-Cl buffer (pH=7.4).

For the whole-mount preparation, the retina was isolated from the eyecup using a fine spatula and mounted on a slide with Immumount medium (Thermo Shandon, Pittsburgh, Pa.). For transverse sections, the eyecup was treated as described previously and then transferred to Karnovsky's fixation medium (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer) for 24 h. The eyecup was embedded in JB-4 resin (Polysciences, Warrington, Pa.), and cut with a microtome (Reichert-Jung, Nussloch, Germany) into sections of 10-µm thickness, and placed on slides.

Results

Normal diet

Diabetic rats were characterized by hyperglycemia that increased within the first few days after injection of streptozotocin (STZ) and remained constant throughout the follow-up period (30 weeks). In the normal rats, blood glucose was around 70 mg/dl and in the diabetic rats above 400 mg/dl [31]. The diabetic rats were also characterized by a slow gradual loss of weight compared with the normal rats that gained weight. Before induction of diabetes, all the rats were approximately 200 g in body weight, whereas towards the termination of the follow-up period (30 weeks), the normal rats weighed approximately 600 g and the diabetic rats approximately 130 g [31].

The enzyme NOS was found mainly in rat amacrine cells [12, 14, 40, 52, 54] that are best visualized in the retinal whole-mount preparation. Figure 1 shows NADPH diaphorase histochemistry of retinal whole mounts from 2 normal (Fig. 1A, C) and 2 diabetic (Fig. 1B, D) rats that were killed 6 weeks (Fig. 1A, B) or 25 weeks (Fig. 1C, D) after induction of diabetes. The NADPH diaphorase active amacrine cells are easily identified in all four micrographs (arrows); however, their shape and the pattern of staining clearly differ between the normal and diabetic retinas. The NADPH diaphorase amacrine cells in the normal retina are characterized by densely stained cell bodies and long stained processes (Fig. 1A, C). All retinas from normal rats, that were treated for NADPH diaphorase histochemistry (n=12), and studied at different time intervals of follow-up (up to 25 weeks), exhibited similar pattern of NADPH diaphorase activity in amacrine cells indicating no age-dependent changes. In contrast, NADPH diaphorase amacrine cells in the diabetic retinas (Fig. 1B, D) appeared to be smaller in size, were spherical in shape,

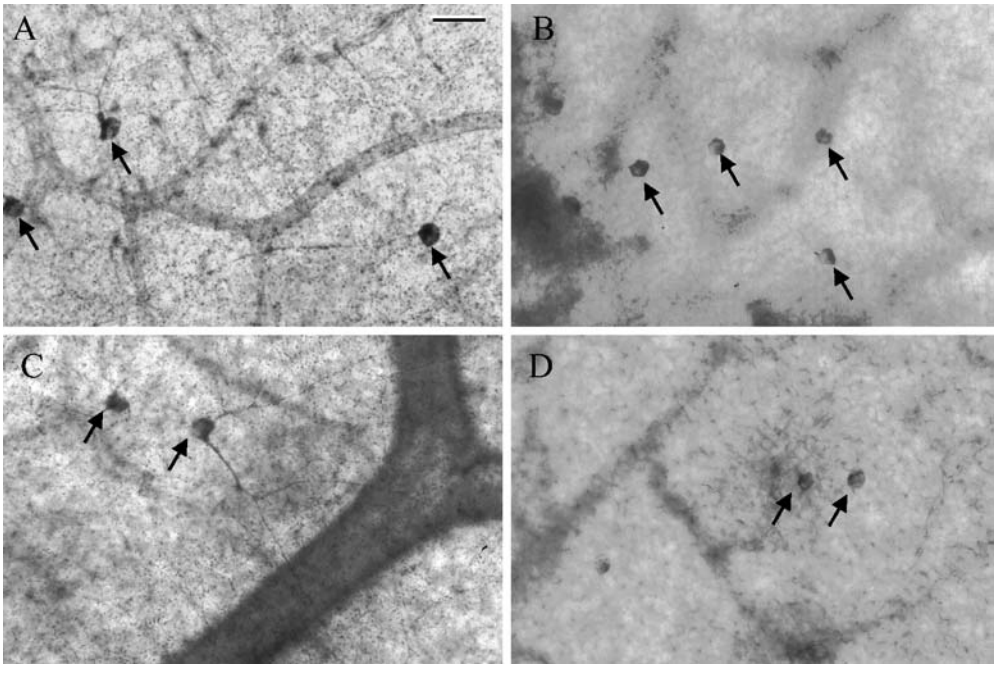

Fig. 1A–D NADPH diaphorase histochemistry of retinal whole mounts from two normal (**A, C**) and two diabetic (**B, D**) rats. The rats were studied 6 weeks (**A, B**) and 25 weeks (**C, D**) after induction of diabetes. NADPH diaphorase amacrine cells are seen in all four micrographs (*arrows*). They are strongly stained and contain long stained processes in the normal retinas (**A, C**). In the diabetic retina, the amacrine cells are characterized by small cell bodies of spherical shape and they lack stained processes (**B, D**). Calibration bar for all micrographs: 25 µm

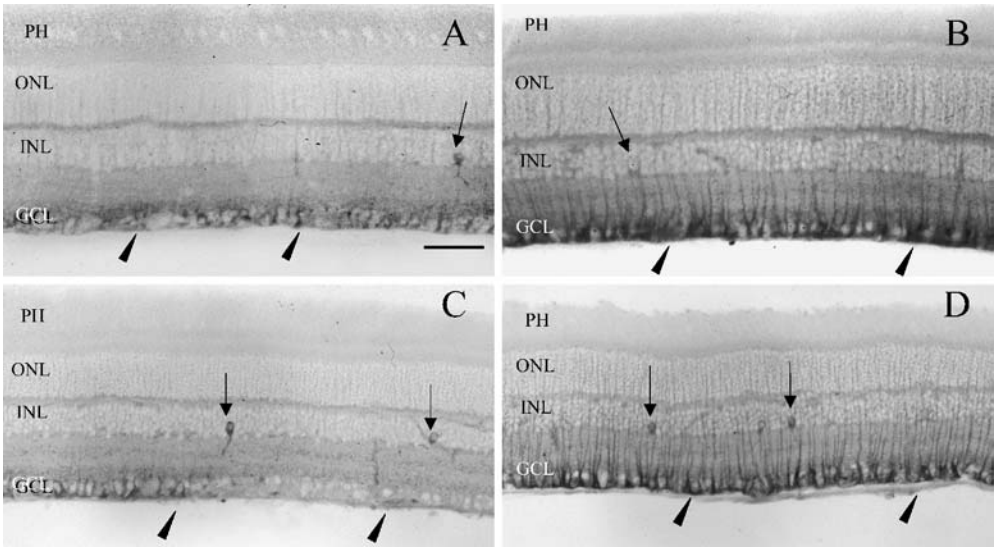

Fig. 2A–D NADPH diaphorase activity in transverse retinal sections of two normal (**A, C**) and two diabetic (**B, D**) rats that were studied 7 weeks (**A, B**) or 14 weeks (**C, D**) after onset of diabetes. Stained amacrine cells can be seen in the proximal border of the INL in all four micrographs (*arrows*). In the 7-week diabetic retina, strong NADPH diaphorase staining is observed in the endfeet of Müller cells (*arrowheads* in **B**). With time of diabetes, NADPH diaphorase activity expanded to the entire length of the Müller cells **D**. In contrast, the Müller cells in the normal retina of any age are labeled to a lesser extent and NADPH diaphorase staining is confined to their endfeet (*arrowheads* in **A** and **C**). Marked retinal layers: outer segments of photoreceptors (*PH*); outer nuclear layer (*ONL*); inner nuclear layer (*INL*); and ganglion cell layer (*GCL*). Calibration bar for all micrographs: 50 μm

and lacked stained processes. All retinas from diabetic rats, that underwent NADPH diaphorase histochemistry (n=24), exhibited the same changes in amacrine cells as shown in Fig. 1B, D), indicating early onset of pathology (6 weeks), and no further deterioration within 25 weeks of follow-up. Another difference between diabetic and normal rats was noted in the background appearance of the retinal whole mounts. We suspect that this difference arises from differences in tissue consistency (water content), due to the high (>350 mg/dl) glucose levels.

Transverse retinal sections were used to observe diabetic-induced changes in NADPH diaphorase activity of deeper retina structures. Transverse sections of 2 normal (A and C) and 2 diabetic (B and D) rats that were prepared for NADPH diaphorase histochemistry 7 weeks (A and B) and 14 weeks (C and D) after onset of diabetes are shown in Fig. 2. The cells that are most easily identified as NADPH diaphorase positive are the amacrine cells that are seen in the proximal border of the inner nuclear layer of all four micrographs (arrows in Fig. 2). Other structures in the INL that are less prominently stained may be small-caliber blood vessels [54]. The major difference between the normal and diabetic retina is seen in the Müller cells. In the 7-week diabetic retina, the endfeet of the Müller cells (arrowheads in Fig. 2B) are stained more than those in the retina of the normal rat (arrowheads in Fig. 2A). This difference between the diabetic and normal retinas seems to increase with period of diabetes. The transverse retinal section from the 14-week diabetic rat (Fig. 2D) shows intense NADPH diaphorase activity in the endfeet of the Müller cells (arrowheads) that extends throughout the entire length of the Müller cells. In the normal retina, only faint NADPH diaphorase activity is seen, confined to the endfeet of the Müller cells (arrowheads in Fig. 2C).

The NADPH diaphorase activity was also observed along the walls of the blood vessels (both arteries and veins), reflecting the presence of eNOS in endothelial cells [23]; therefore, we could use the retinal whole mounts that had been treated for NADPH diaphorase histochemistry, to assess pathological changes in the retinal vasculature. In all the rats that were studied at periods of diabetes shorter than 20 weeks, the blood vessels of the diabetic retina appeared similar to those of the normal retina at the light microscopic level (not shown here). In rats suffering from longer periods of diabetes, differences became evident. In Fig. 3, NADPH diaphorase of retinal whole mount from a rat suffering from diabetes for 25 weeks (Fig. 3B) is compared with a normal rat retina (Fig. 3A). These micrographs were taken from similar retinal regions. The vascular tree appears very distinct in the normal retina and exhibits intense staining (Fig. 3A), whereas the blood vessels in the diabetic retina appear thinner, less robust, and are stained more faintly for NADPH diaphorase (Fig. 3B).

Nitric oxide diet

Several studies have suggested that manipulation of the retinal level of NO in diabetic animals can prevent, or at

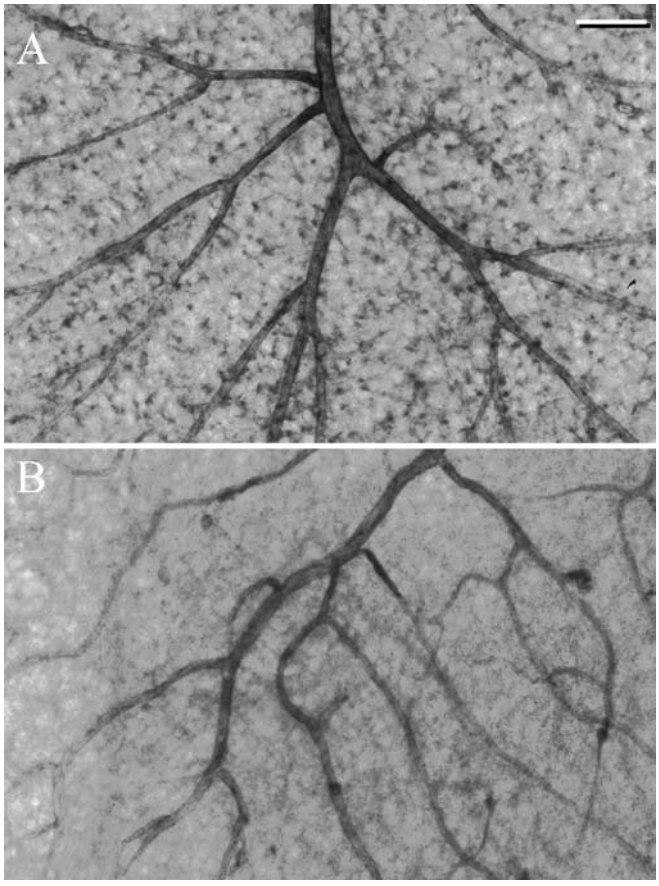

Fig. 3A, B The retinal vascular bed, as seen by NADPH diaphorase histochemistry, of normal **A** and diabetic **B** rats which were studied 25 weeks after induction of diabetes. NADPH diaphorase activity is clearly visible in the walls of the blood vessels in both retinas. The blood vessels appear more densely stained in the normal retina (**A**) compared with the diabetic retina (**B**). Calibration bar: 50 μm

least slow down, the development of retinopathy; however, there is no agreement as to whether NO synthesis should be reduced or augmented. Some authors have hypothesized that reduced NO production and/or reduced responsiveness to NO occurred in diabetes, and therefore, the level of NO should be augmented, whereas others have assumed that excess of NO synthesis in the diabetic retina was deleterious. Thus, treatment of diabetic rats with a nitrovasodilator (adding NO) was found to prevent the reduction in nerve conduction velocity and in blood flow [7]. In contrast, treating diabetic rats with guanidine compounds, that inhibit NO synthesis by iNOS, prevented the elevation in the permeability of vascular vessels to albumin [47], and the development of diabetic retinopathy [20].

To test these possibilities, we raised normal rats and diabetic rats with added L-arginine to stimulate in vivo NO synthesis [36], or L-NAME, to inhibit in vivo NO synthesis [39], to the drinking water. These manipulations were expected to affect the blood pressure, but it was not monitored here. Instead, we assumed that the effects were similar in diabetic and normal rats and compared the two groups that were treated similarly. The ERG responses were measured at different time intervals to assess retinal function, and after 10 weeks of follow-up, the rats were killed and their retinas were prepared for NADPH diaphorase histochemistry. Figure 4 shows ERG responses from three normal rats that were maintained for 8 weeks on normal, L-arginine, or L-NAME diet. The ERG responses of the rat receiving L-arginine in the drinking water were smaller in amplitude compared with those of the rat on normal drinking water and those of the rat with L-NAME added to the water. The diabetic rats exhibited similar trend of diet effect on the ERG responses; reduction by adding L-arginine to the drinking water (not shown here).

The ERG responses were recorded from 12 normal and 12 diabetic rats that were raised for 8–10 weeks with water containing either 0.1% L-arginine or 0.01% L-NAME (6 rats in each group). The response–intensity curves for the a-wave and b-wave were constructed from these ERG responses and were fitted to Eq. (1) in order to derive the amplitude of the maximum response (Vmax) and the semi-saturation constant (σ) [24]. The average (±SD) values of these ERG parameters and of blood glucose for these groups of rats are summarized in Table 1 and compared with similar data from diabetic and normal rats that were raised with regular tap water. Statistical analysis was conducted by Student's *t* test. In normal rats, supplementing the drinking water with L-arginine induced a slight, but not significant, increase in the semi-saturation constants of the ERG a-wave and b-wave, but significantly ($p<0.01$) reduced their maximum amplitudes (Table 1). This indicates a reduction in the functional integrity of the distal rat retina. Adding L-NAME to the drinking water exerted no effect on the dark-adapted ERG responses of normal rats (Table 1). The NO diet also affected the level of blood glucose in normal rats. It was significantly higher in rats maintained on water with added L-arginine ($p<0.01$) and in those kept on L-NAME enriched water ($p<0.05$). In the diabetic rats, adding L-arginine to the drinking water slightly reduced ($p<0.05$) the maximum amplitude of the a- and b-waves, whereas L-NAME exerted no significant effects (Table 1). L-arginine or L-NAME did not affect the levels of blood glucose of the diabetic rats.

Micrographs of retinas from normal rats that were treated for 10 weeks with L-arginine or L-NAME and were processed for NADPH diaphorase histochemistry are shown in Fig. 5. In the retinal whole mounts, NADPH diaphorase amacrine cells are clearly visible (arrows in Fig. 5) in the retina of both normal rats that were raised on L-arginine (A) or L-NAME (B) enriched diet. The cell bodies of these amacrine cells appear

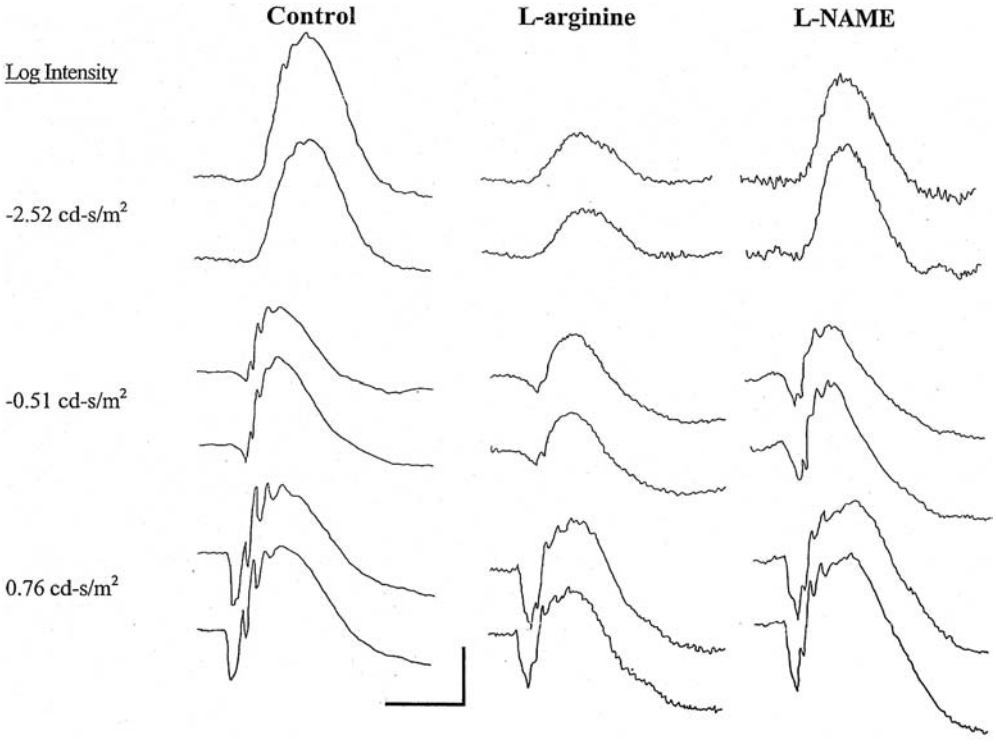

Fig. 4 Electroretinogram (ERG) responses of three normal rats that were raised for 8 weeks with a diet containing normal tap water (*first column*), L-arginine (0.1%) water (*second column*), and L-NAME (0.01%) water (*third column*). The ERG responses were recorded in the dark-adapted state using light stimuli of different intensities as denoted to the left of each row of responses. Each pair of traces represents the ERG responses of the left (*upper trace*) and right (*lower trace*) eyes. Calibration bars: vertical 100 μV, horizontal 100 ms

Table 1 The effects of adding L-arginine or L-NAME to the drinking water (8–10 weeks) on retinal function and blood glucose of normal (a) and diabetic (b) rats. Each group contained six rats. The average±SD of Vmax and σ were calculated by fitting the ERG response–intensity curves to a hyperbolic function (Eq. 1). Statistical tests (Student's *t* test) were performed between the experimental groups (L-arginine and L-NAME) and the corresponding regular-water group

		Regular water	L-arginine (0.1%)	L-NAME (0.01%)
(a) Normal rats				
a-wave	Vmax (μV)	364±63	249±47**	432±143
	σ (cd-s/m²)	0.260±0.105	0.351±0.125	0.297±0.092
b-wave	Vmax (μV)	757±205	475±113**	762±193
	σ (cd-s/m²)	0.0055±0.0029	0.0081±0.0076	0.0041±0.0014
Blood glucose (mg/dl)		69±5	190±37**	146±53*
(b) Diabetic rats				
a-wave	Vmax (μV)	270±6856	202±62*	276±104
	σ (cd-s/m²)	0.312±0.118	0.288±0.098	0.233±0.094
b-wave	Vmax (μV)	439±105	297±94*	344±97113
	σ (cd-s/m²)	0.0047±0.0022	0.0148±0.0115*	0.0095±0.069
Blood glucose (mg/dl)		448±33	420±72	424±41

*$p<0.05$, **$p<0.01$

similar in shape and degree of staining to those found in normal rats raised under normal conditions (Fig. 1A, C); however, the NADPH diaphorase amacrine cells in the retina of the rat raised with L-arginine diet (Fig. 5A) lack stained processes, like those of diabetic rats. The NADPH diaphorase activity in Müller cells was also slightly affected by the type of diet. In the transverse retinal section of the rat that was raised on L-arginine diet (Fig. 5C), the endfeet of the Müller cells were stained more heavily compared with the transverse sections from normal rats that were raised on L-NAME diet (Fig. 5D) or normal tap water (Fig. 2A). Similar NADPH diaphorase histochemistry was seen in three rats of each experimental group (L-arginine diet and L-NAME diet) which were studied after 10 weeks on NO-related diet.

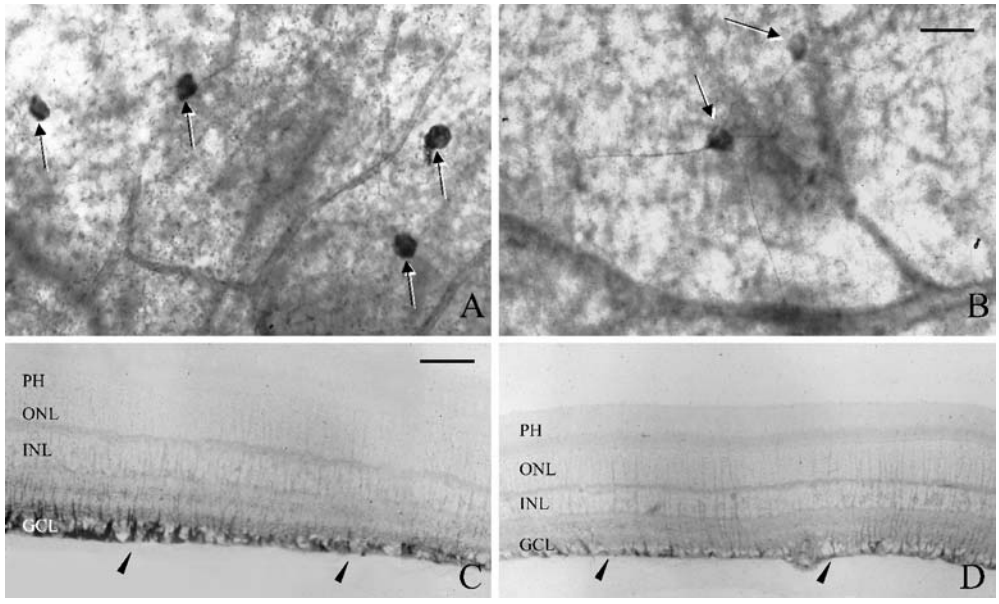

Fig. 5A–D NADPH diaphorase histochemistry of retinal whole mounts (**A, B**) and transverse retinal sections (**C, D**) of four normal rats that were raised for 8 weeks on a diet containing L-arginine (**A**, **C**) or L-NAME (**B, D**) in the drinking water. The cell bodies of NADPH diaphorase amacrine cells are similarly stained in both retinal whole mounts (*arrows* in **A** and **B**), but those in the L-arginine-treated rat lack stained processes (**A**). In the transverse sections, the endfeet of the Müller cells exhibit more profound NADPH diaphorase activity in the retina from the L-arginine treated rat (*arrowheads* in **C**), compared with the L-NAME-treated rat (*arrowheads* in **D**). Calibration bar: 25 μm for **A** and **B**, 50 μm for **C** and **D**

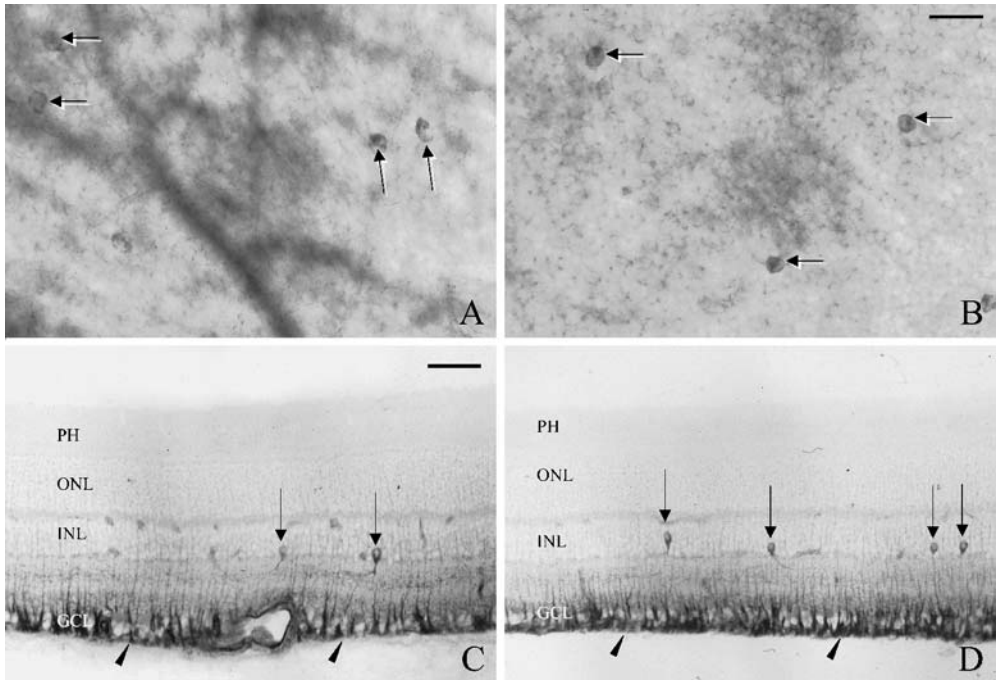

Fig. 6A–D NADPH diaphorase histochemistry of retinal whole mounts (**A, B**) and transverse retinal sections (**C, D**) of four diabetic rats that were raised for 8 weeks on a diet containing L-arginine (**A**, **C**) or L-NAME (**B**, **D**) in the drinking water. The NADPH diaphorase amacrine cells are similarly stained in both retinal whole mounts (*arrows* in **A** and **B**). In the transverse sections, the endfeet of the Müller cells are intensely stained for NADPH diaphorase (*arrowheads* in **C** and **D**). Calibration bar: 25 μm for **A** and **B**, 50 μm for **C** and **D**

Adding L-arginine or L-NAME to the drinking water of diabetic rats did not affect the pattern of NADPH diaphorase histochemistry as shown in Fig. 6. In the retinal whole mounts from both rats, NADPH diaphorase amacrine cells can be identified (arrows in Fig. 6A, B). The bodies of the amacrine cells in both retinas appear smaller in size compared with those in the normal rats (Fig. 5A, B), and are characterized by abnormal shape (spherical) and lack stained processes. Also, the transverse sections from the retinas of both diabetic rats appear similar (Fig. 6C, D). Stained amacrine cells can be seen in the proximal region of the INL (arrows), whereas staining in the distal region of the INL probably belong to small-caliber blood vessels [54]. In both retinas (arrows in Fig. 6C, D), NADPH diaphorase staining in Müller cells is stronger than in retinas from normal rats (Fig. 5). Similar results were seen in retinas from three diabetic rats treated with L-arginine and three diabetic

rats treated with L-NAME, that were prepared for NADPH diaphorase histochemistry after 10 weeks on NO diet.

Discussion

The data presented herein clearly show that the pattern of NADPH diaphorase activity in the rat retina is affected by diabetes. Assuming that NADPH diaphorase is a marker for nitric oxide synthase [3, 15, 21, 25], and that the degree of staining reflects only active NOS molecules at the time of tissue fixation [5, 34], these observations indicate significant changes in the NO system of the diabetic rat retina. In the normal retina, the neuronal cells that consistently exhibit NADPH diaphorase activity are amacrine cells (Fig. 1A, C) in agreement with previous reports [12, 14, 40, 52, 54]. We also found NADPH diaphorase activity in the endfeet of the Müller cells (Fig. 2A, C), whereas a previous study demonstrated eNOS immunoreactivity throughout most of the length of the Müller cells [23]. This difference probably reflects the different types of measurements. While immunocytochemistry stains all NOS molecules, NADPH diaphorase stains only active NOS molecules [34]; thus, Müller cells in the normal rat retina probably contain eNOS but not in an active form. We also found NADPH diaphorase activity in the walls of the retinal blood vessels (arterial and venous parts) (Fig. 3), as reported previously [23], and in small-caliber vessels deep in the INL [54].

In the diabetic retina, the distribution of NADPH diaphorase-positive amacrine cells across the retina did not differ from that in the normal retina, but the cells differ from those in the normal retina by their smaller size, spherical shape, and the lack of stained processes. This was seen as early as 6 weeks after induction of diabetes (Fig. 1B) and did not change markedly with time up to 25 weeks of follow-up (Fig. 1D). Similar observation has been reported previously [39]. This effect of diabetes can be an indirect consequence of the high glucose level (>350 mg/dl) that may lead to ketoacidosis and dehydration. Alternatively, a direct link between high glucose and cellular function needs to be considered [32]. The effects of L-arginine-enriched diet on the NADPH diaphorase amacrine cells in the normal rat (Fig. 5A) tend to support the latter alternative. Furthermore, a recent study showed abnormal photopic ERG responses in healthy volunteers suffering from high blood glucose levels during glucose-loading experiments [16].

In contrast to the reduced NADPH diaphorase activity in amacrine cells, we found an increase in the activity of NADPH diaphorase in Müller cells of the diabetic rat retina (Fig. 2). Since NADPH diaphorase can detect equally all NOS isoforms, we could not define the type of NOS that was expressed in the Müller cells of the diabetic retina. Based on previous findings, we suggest that NADPH diaphorase activity in the Müller cells reflects the inducible isoform of NOS. A recent study of retinas from diabetic patients showed increased immunoreactivity for iNOS in cells that were identified as Müller cells [1]. Furthermore, NO synthesis in the diabetic rat retina could be significantly reduced by adding iNOS antagonist, whereas antagonists for cNOS were considerably less effective [10].

The induction of iNOS in Müller cells of the diabetic retina can reflect toxicity of streptozotocin, a possibility that was not tested directly here. Alternatively, it can reflect a response to subnormal rate of NO synthesis due to loss of NADPH diaphorase amacrine cells. The experiments in which L-NAME or L-arginine were added to the drinking water of normal and diabetic rats tend to exclude this possibility. Reducing NO synthesis in the normal retina (adding L-NAME) did not induce NADPH diaphorase activity in Müller cells (Fig. 5D), whereas raising NO synthesis in the diabetic retina (adding L-arginine) did not prevent the increase in NADPH diaphorase activity in the Müller cells (Fig. 6C). The most likely possibility attributes the diabetic-induced changes in NADPH diaphorase in retinal Müller cells to the high glucose level that can act directly to induce NOS expression and/or indirectly by increasing the levels of cytokines (e.g., IL-1β). Interleukin-1β was found to induce the expression of NOS in colonic smooth muscle cells [30] and to stimulate NO production and uptake of L-arginine in vascular smooth muscle cells [8, 18]. A stimulatory effect of hyperglycemia on nitric oxide synthase activity was demonstrated in isolated rat and human cells [43, 46] and on the rate of NO synthesis in an isolated rat retina [29].

The deleterious effect of hyperglycemia on the rat retina [31] is emphasized by the subnormal ERG responses that were recorded from normal rats that were raised on water with added L-arginine (Fig. 4; Table 1). The levels of blood glucose in these rats (190±37 mg/dl) were significantly (p=0.0002) higher than in rats that were maintained on regular tap water (69±5 mg/dl), but lower than in our diabetic rats (>350 mg/dl). Thus, added L-arginine caused elevation in blood glucose that was probably sufficient to cause a reduction in retinal function as assessed from the ERG responses, and slight changes in the NADPH diaphorase amacrine cells (Fig. 5A, C). The concentration of plasma glucose also increased in normal rats raised on L-NAME enriched diet (146±53 mg/dl), but to a lesser extent compared with the effects of the L-arginine diet (p=0.015), and was probably insufficient to affect the ERG responses (Fig. 4) and NADPH diaphorase activity (Fig. 5B, D). The exact pathway leading to elevation of blood glucose due to added L-arginine was not studied here; however, increased activity of gluconeogenesis is one possibility.

The data presented herein and in previous reports support a role for NO in the development of diabetic reti-

nopathy. We suggest that in the early stages of diabetes, before vascular changes become evident, the activity of cNOS in amacrine cells decreases but induction of iNOS in Müller cells may lead to an overall increase rate of NO synthesis. Increased synthesis of NO was found in diabetic patients with no signs of diabetic retinopathy [22] and in diabetic rats [10, 29]. The excess production of NO can strengthen the deleterious effects of hyperglycemia and thus, contributes to the development of diabetic retinopathy. NO has been shown to contribute to the breakdown of the blood-retinal barrier [9, 11] and to the VEGF-induced hyperpermeability of blood vessels [48]. Elevated levels of NO have been suggested to interact with elevated levels of glutamate to further exacerbate the oxidative stress that is induced by diabetes [29]. Furthermore, NO is a reactive radical that by itself can cause death of retinal neurons [19]; thus, the induction of iNOS in retinal Müller cells in the early stages of diabetes seems to play a positive feedback role by augmenting the deleterious effects of hyperglycemia.

Acknowledgements Acknowledgements. This study was partially supported by grants from the Chief Scientist, Israel Ministry of Health (no. 3633), and from Teva Pharmaceutical Industries, Israel.

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