Nitric Oxide Synthase (nNOS) content of these nerves in the cerebral arteries of streptozotocin-induced diabetic rats. In the first phase, perivascular nitrergic nerves remain intact while they lose their neuronal NO synthase content. This phase is reversible with insulin treatment. In the second phase, nitrergic cell bodies in the ganglia are lost via apoptosis in an irreversible manner. Throughout the two phases, irreversible thickening of the smooth muscle layer of cerebral arteries is observed. This is the first demonstration of nitric oxide (NO) and to increase the cortical blood flow (6,13–15). It is not known whether the nitrergic nerves in the cerebral vasculature are affected during diabetes. We therefore investigated the effect of diabetes on the morphology and nNOS content of these nerves in the cerebral arteries of streptozotocin (STZ)-induced diabetic rats.

RESEARCH DESIGN AND METHODS

Induction of diabetes. Male Wistar rats (225–250 g) were treated with STZ (75 mg/kg i.p.) or vehicle (saline) as described previously (16,17). Hyperglycemia was defined as a nonfasting blood glucose concentration >20 mmol/l. Insulin was administered 8 and 12 weeks after STZ injection using sustained-release insulin rods (7 mm, -2 units/day; LinBiT, LinShin Canada, Toronto, ON, Canada). Rats were killed 4, 8, 12, 16, and 20 weeks after STZ injection with an overdose of pentobarbitone, and their blood, cerebral arteries, and sphenopalatine ganglia were collected. Some of the animals were perfused through the heart with 4% paraformaldehyde before collecting the cerebral arteries and ganglia. Blood was centrifuged to obtain serum for measuring glucose using a Reffolux-S Glucometer (Boehringer Mannheim, Mannheim, Germany). The blood vessels and ganglia collected from unperfused animals were frozen in liquid nitrogen for protein quantification using Western blotting. The tissues from perfused animals were further fixed in 4% paraformaldehyde for 24 h. The sections were treated with an anti-actin (Sigma; 1:1,000) overnight followed by the following fluorescent conjugated secondary antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-sheep IgG (1:2,000), vesicular acetylcholine transporter (VACHT) (Chemicon; 1:1,000), PGP9.5 (Chemicon; 1:2,000), or smooth muscle specific α-actin (Sigma; 1:1,000) overnight followed by the detection with the following fluorescent conjugated secondary antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-sheep IgG raised in donkey (Chemicon; 1:2,500) or rhodamine-conjugated anti-goat IgG raised in donkey (Chemicon; 1:2,500). The sections were then covered with Mounting Medium (Lilly, Indianapolis, IN) containing 0.1% Triton X-100 and 1% of the serum of the species from which the secondary antibody was obtained. The slides were mounted in a cryostat at −18°C before room temperature and then incubated with PGS containing 0.1% Triton X-100 and 5% of the serum of the species from which the secondary antibody was obtained. The slides were incubated with antibodies against nNOS (raised in sheep, K205 [18]; 1:2,000), vesicular acetylcholine transporter (VACHT) (Chemicon; 1:1,000), PGP9.5 (Chemicon; 1:2,000), or smooth muscle specific α-actin (Sigma; 1:1,000) overnight followed by the detection with the following fluorescent conjugated secondary antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-sheep IgG raised in donkey (Chemicon; 1:2,500) or rhodamine-conjugated anti-goat IgG raised in donkey (Chemicon; 1:2,500). The sections were then covered with Mounting Medium (Lilly, Indianapolis, IN) containing 0.1% Triton X-100 and 1% of the serum of the species from which the secondary antibody was obtained. The slides were incubated with antibodies against nNOS (raised in sheep, K205 [18]; 1:2,000), vesicular acetylcholine transporter (VACHT) (Chemicon; 1:1,000), PGP9.5 (Chemicon; 1:2,000), or smooth muscle specific α-actin (Sigma; 1:1,000) overnight followed by the detection with the following fluorescent conjugated secondary antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-sheep IgG raised in donkey (Chemicon; 1:2,500) or rhodamine-conjugated anti-goat IgG raised in donkey (Chemicon; 1:2,500). The sections were then covered with Mounting Medium (Lilly, Indianapolis, IN) containing 0.1% Triton X-100 and 1% of the serum of the species from which the secondary antibody was obtained. The slides were incubated with antibodies against nNOS (raised in sheep, K205 [18]; 1:2,000), vesicular acetylcholine transporter (VACHT) (Chemicon; 1:1,000), PGP9.5 (Chemicon; 1:2,000), or smooth muscle specific α-actin (Sigma; 1:1,000) overnight followed by the detection with the following fluorescent conjugated secondary antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-sheep IgG raised in donkey (Chemicon; 1:2,500) or rhodamine-conjugated anti-goat IgG raised in donkey (Chemicon; 1:2,500). The sections were then covered with Mounting Medium (Lilly, Indianapolis, IN) containing 0.1% Triton X-100 and 1% of the serum of the species from which the secondary antibody was obtained. The slides were incubated with antibodies against nNOS (raised in sheep, K205 [18]; 1:2,000), vesicular acetylcholine transporter (VACHT) (Chemicon; 1:1,000), PGP9.5 (Chemicon; 1:2,000), or smooth muscle specific α-actin (Sigma; 1:1,000) overnight followed by the detection with the following fluorescent conjugated secondary antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-sheep IgG raised in donkey (Chemicon; 1:2,500) or rhodamine-conjugated anti-goat IgG raised in donkey (Chemicon; 1:2,500).
covered with coverslips after addition of VectaShield mounting medium containing 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector) to visualize the nuclei. No immunostaining was observed when the primary antibodies were omitted. Tdt-mediated dUTP nick-end labeling (TUNEL) staining was performed according to the manufacturer’s instructions (Roche).

Image analysis. The images were obtained using a laser-scanning confocal microscope (TCS-DMIRE, Leica). Image analysis was performed using Scion Image software (version beta 4.02; Scion) and Leica Confocal Software (Version 2.0.0, build05871; Leica) as described previously (17,19).

The immunodensity of perivascular nerve fibers was measured as the mean amplitude of fluorescence per 1 μm² in areas occupied by nerve fibers. To avoid day-to-day variation in fluorescence intensity, several sections from different experimental groups were immunostained and analyzed in the same batch on the same day. The laser intensity and gain functions were set according to the control tissue; thereafter, these settings were applied to all sections from all experimental groups within the same batch. The results were expressed as percentage of control to avoid the variation between different batches.

nNOS⁺ cell bodies in the ganglia were counted manually in a blinded fashion, and the area occupied by the counted cells was measured to give the number of cells per 100,000 μm².

The thickness of smooth muscle layer was determined in sections immunostained with an antibody raised against smooth muscle–specific α-actin. The distance covered by the smooth muscle cells was measured in 10 random areas in each section using the Leica Confocal Software. The measurements were then pooled for each group.

Western blotting. The frozen tissues were pulverized using a stainless steel pestle and mortar on dry ice, homogenized in 100 μl homogenization buffer (20 mmol/l HEPES, pH 7.2, 1 mmol/l EDTA, 5 mmol/l dithiothreitol, 0.1 mmol/l phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin and soya bean trypsin inhibitor, pepstatin, E-64, bestatin, aprotonin, and 5 μg/ml 3,4-dichloroisocoumarin) and centrifuged at 13,000g for 30 min at 4°C as described previously (16,17). The protein concentration in the supernatant was measured, and equal amounts of protein were run on 8% polyacrylamide SDS gels and then transferred to nitrocellulose membranes. The blots were incubated overnight with monoclonal nNOS antibody (raised against the 10951-289 region of human nNOS; Transduction Laboratories; 1:2,000) and then with horseradish peroxidase–conjugated anti-mouse IgG (Vector Laboratories; 1:2,000) for 2 h. The reactive bands were detected with a luminol-based kit. The optimal X-ray exposure was selected and scanned, and the density of each band corresponding to nNOS was measured using Scion Image software. The density of each band was then expressed as a percentage of the band in that gel from a control (nondiabetic) animal. Nondiabetic rat brain cytosol was used as a positive control.

Presentation of the results. Nondiabetic age-matched animals that were injected with saline and killed at the end of 20 weeks were referred to as the control group. STZ-induced diabetic rats without any insulin treatment are referred to as “20/0” group, STZ-induced diabetic animals at week 20 without insulin were treated as the “20/20” group, and STZ-induced diabetic animals at week 12 without insulin were treated as the “20/12” group. These animals were killed at the end of 20 weeks after STZ injection and they received insulin for the last 8 and 12 weeks, respectively.

A

FIG. 1. Delayed insulin treatment normalizes body weight and serum glucose levels. The weight (A) and serum glucose (B) of rats during 20 weeks of diabetes are shown. STZ was injected at week 0. The control group (●), diabetic group (□), and diabetic groups with insulin treatment begun at the 8th (●) and 12th (□) week are shown. Data are means ± SE.

RESULTS

Body weight and serum glucose. The STZ-injected rats, which did not receive any insulin treatment, lost weight or did not gain as much weight as control animals (Fig. 1A), and their serum glucose concentrations were significantly higher (Fig. 1B). The animals that received insulin treatment beginning 8 or 12 weeks after STZ injection gained weight (Fig. 1A), and their serum glucose concentrations decreased to control levels shortly after initiation of insulin treatment (Fig. 1B).

nNOS immunofluorescence in cerebral perivascular nerves. In pilot studies, we compared nNOS immunofluorescence in different parts of the cerebral vasculature, such as the anterior, middle, and posterior cerebral arterioles and the basilar artery, in nondiabetic rats. Because the middle cerebral artery showed the most consistent immunostaining (not shown), it was used for the rest of the study. In control (nondiabetic) animals, nNOS immunostaining was observed in the nerve fibers surrounding the middle cerebral artery (Fig. 2A and F). These fibers run irregularly around the arterial wall. The density of nNOS⁺ fibers decreased gradually during 20 weeks of diabetes (Fig. 2B and C and Fig. 3). By 20 weeks, very few or no nNOS⁺ fibers were observable (Fig. 2C). In the 20/8 (8 weeks of insulin) group, the density nNOS⁺ fibers were similar to those in the 20w group (Fig. 2D and Fig. 3). There was no significant difference between the control and 20/12 group (12 weeks of insulin) (Fig. 2E and Fig. 3).

nNOS and VACHT colocalization. Because nNOS and the cholinergic nerve marker, VACHT, are known to be colocalized in parasympathetic perivascular nerve fibers, we studied VACHT immunostaining throughout 20 weeks of diabetes. In control animals, all of the nNOS⁺ nerves were also positive for VACHT (Fig. 2F). Diabetic animals at 4, 8, and 12 weeks showed a gradual decline in nNOS immunostaining, whereas VACHT staining remained unchanged (Fig. 3). At 12 weeks, VACHT⁺ but nNOS⁻ nerve fibers were visible (Fig. 2J), but thereafter, as demonstrated by the 16w and 20w groups, VACHT staining declined rapidly (Fig. 2J and Fig. 3). The VACHT immuno-
staining showed significant recovery in the 20/12 group but not in the 20/8 group (Fig. 2K and L and Fig. 3).

**PGP9.5 immunostaining.** To ascertain the change in the total amount of nerve fibers, we immunostained the middle cerebral artery sections with a nonspecific neuronal marker, PGP9.5. The immunostaining density with PGP9.5 did not show any significant change until 16 weeks, after which it gradually decreased and reached a significant level at the end of 20 weeks. The 20/8 group had a similar decline. The 20/12 group, however, showed a significant recovery of PGP9.5 immunostaining (Fig. 3).

**Sphenopalatine ganglia.** To determine whether the cell

![Image](image_url)
bodies of nitrergic nerves were affected by diabetes, we immunostained the sphenopalatine ganglia with antibodies raised against nNOS, VAChT, and PGP9.5. Two different cell types were observed: large (secretomotor) cells with a diameter of $30-50 \mu m$, which were positive for PGP9.5 and VAChT, and small (vasodilator) cells with a diameter of $20-30 \mu m$, which were positive for PGP9.5, VAChT, and nNOS (Fig. 4A). After 16 weeks of diabetes, there was a significant reduction in the number of nNOS$^+$ small cells (Fig. 4B and C and Fig. 5A). This reduction was prevented if the insulin treatment was initiated at the 8th week (20/12 group; Fig. 4E and Fig. 5A). The nitrergic cell loss was, however, not prevented in the 20/8 group (Fig. 4D and Fig. 5A). In the 16w and 20w groups, some of the nNOS$^+$ cells were also TUNEL$^+$ (Fig. 4F and Fig. 5B). We did not observe any TUNEL$^+$ cells in the control group or in the diabetic groups before 16 weeks (Fig. 5B).

**nNOS Western blotting.** Western blotting of cerebral artery homogenates with nNOS antibody revealed a pattern of decline in nNOS protein amount throughout 20 weeks of diabetes. Western blotting of sphenopalatine ganglia homogenates showed a trend of increase by 12 weeks followed by a decrease in nNOS protein amount. The decrease in both cerebral artery and ganglia samples was prevented in the 20/12 group but not in the 20/8 group (Fig. 6).

**Smooth muscle–specific α-actin immunostaining.** To measure the smooth muscle thickness of cerebral arteries, middle cerebral arteries from each group were immunostained using an antibody raised against smooth muscle–specific α-actin. The thickness of smooth muscle layer was then measured using confocal microscopy, as shown in Fig. 7A. The thickness of the middle cerebral artery in the control group was $35.65 \pm 1.25 \mu m$, which gradually increased after 8 weeks and reached a significant level at 16 weeks ($43.04 \pm 1.04 \mu m$). Insulin treatment in either the 20/8 or 20/12 groups did not affect this increase (Fig. 7B).

**DISCUSSION**

Our results demonstrated that nitrergic nerve fibers lost their nNOS content gradually throughout 20 weeks of diabetes. This decrease was reversible with insulin treatment initiated at the 8th week. However, insulin treatment began at the 12th week was not able to reverse the nNOS depletion. This suggests that there are two phases of nNOS depletion: a first phase when the nNOS depletion is reversible and a second phase when nNOS depletion is irreversible and there is loss of axons and cell bodies.

Because the loss of PGP9.5$^+$ and VAChT$^+$ nerve fibers around the middle cerebral artery and the decrease in the number of nitrergic neuronal cell bodies in the sphenopal-
atine ganglia were observed in the second phase, the irreversible component seems to be due to degeneration of the nitrergic neurons. This is further supported by the observation of the TUNEL^+ nitrergic cells in the ganglia during the second, irreversible phase, which suggests that cholinergic-nitrergic neurodegeneration occurs by apoptotic cell death. To our knowledge, this is the first demonstration of degeneration of perivascular cholinergic-nitrergic autonomic neurons innervating diabetic cerebral blood vessels.

In the reversible first phase, axons were intact but showed a significantly decreased nNOS content. The mechanism by which nNOS is depleted in the axons, but not in the cell bodies, is unclear. Previously, a deficiency in androgen (20), insulin, and/or related growth factors (21) has been suggested to cause depletion of nNOS in the distal axons of nitrergic nerves of the penis during diabetes. However, if this were the case, in the present study, a decrease in nNOS in the ganglia would also be expected. Alternatively, and perhaps most likely, axonal transport of nNOS from the cell body to the axons (22) might be compromised in diabetes. This possibility is currently under investigation in our laboratory.

The second phase was characterized by irreversible degeneration and cell body loss due to apoptosis. The neuronal cell death in this phase could be due to hyperglycemia as suggested by others for sensory nerves (22). However, the blood glucose levels of diabetic animals, which received insulin from the 12th week and had nerve loss, were comparable to nondiabetic animals. This suggests that hyperglycemia per se is unlikely to be the direct reason for autonomic neurodegeneration. More probably, advanced glycation end products (AGEs), which are known to accumulate irreversibly in the blood and tissues (i.e., delayed administration of insulin fails to lower AGE levels), are involved in this process (23,24). Indeed, we have recently shown that AGEs, but not high glucose, cause apoptosis in nNOS^+ cholinergic neurons in vitro (24). We are currently investigating the accumulation of AGEs in the cerebral blood vessels and sphenopalatine ganglia of diabetic rats.

We have recently made a similar observation of biphasic degeneration of nitrergic nerve fibers in diabetic penis and pyloric sphincter (17). Together with the present findings, these suggest a novel mechanism for degeneration of nitrergic autonomic nerves during diabetes. The model we are proposing suggests that nitrergic nerves degenerate in two phases during diabetes. In the first phase, possibly because of a defective axonal transport, nNOS is depleted in the axons (nerve fibers) while its concentration remains unchanged or slightly elevated in the neuronal cell bodies (ganglia). This phase is reversible with insulin treatment. In the second phase, the accumulation of nNOS protein in the proximal axon and the cell body coincides with the accumulation of AGEs, which results in increased oxidative stress and activation of caspase signaling pathways in the cell body leading to apoptosis (24). It is not known whether other nitrergic nerves in the body such as those...
innervating the heart and coronary arteries are similarly affected in both types of diabetes. Nevertheless, our biphasic model highlights the importance of blood glucose control in the prevention of diabetic complications, since uncontrolled blood glucose levels leading to AGE accumulation and neurodegeneration would result in irreversible damage.

Another plausible mechanism for the apoptotic death of the nitrergic neurons in the second phase could involve N-methyl-D-aspartate (NMDA) receptors whose mRNA is expressed in several autonomic ganglia, including sphenopalatine ganglia (25). Exogenous application of NMDA relaxes cerebral blood vessels via activation of nNOS, suggesting that NMDA receptors might be involved in activation of autonomic nitrergic nerves (26,27). NMDA receptor activation has been suggested to induce neuronal apoptosis in an NO-dependent manner (28). Moreover, NMDA receptor subunits in the central neurons are modified transcriptionally and post-translationally by diabetes (29). Therefore, abnormal signaling through modified NMDA receptors might alter NO production in the ganglia, leading to apoptosis in diabetes.

The implications of this biphasic model are twofold. First, the model suggests “a point of no return” after which the nitrergic neurons are lost irreversibly. This emphasizes the importance of early intervention and indicates that new treatment options for autonomic neuropathy are needed for patients who pass this point. Such treatments such as AGE-crosslink breakers should be able to arrest the pathology and preserve the existing function. Second, new diagnostic approaches that will detect nitrergic nerve function and integrity need to be developed to assess whether a diabetic patient has reached this point.

Perivascular nitrergic nerves around the cerebral arteries have been shown to contain other neurotransmitters such as vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating peptide (PACAP) (12). Interestingly, earlier studies suggested a reduction of VIP-ergic nerve fibers in the cerebral blood vessels of STZ-induced diabetic rats (30). Our study, showing that the nitrergic nerve fibers undergo a biphasic degenerative process during diabetes, highlights the relevance of those early studies.

In the current study, we have shown two different cell types in the sphenopalatine ganglia: large nNOS cells and small nNOS cells. This is in accordance with a previous study where the size and the targets of nerve cells in the cat sphenopalatine ganglion were investigated (31). The study suggested that the large cells are secretomotor neurons innervating the palatine glands, whereas small cells are vasodilator cells innervating the cerebral vasculature (31). In our study, we have shown that the small vasodilator neurons are nNOS. We have also demonstrated that large nNOS neurons underwent apoptosis in diabetic animals, although not to the same extent as the nitrergic neurons. It is known that besides the palatine salivary glands, the sphenopalatine ganglion also innervates lacrimal glands and vasculature in the orbit (32,33). Our results therefore might suggest a pathophysiological mechanism by which autonomic neuropathy could be involved in xerostomia (34), glaucoma (35), and decreased lacrimation (36) observed in diabetic patients.

NO is released not only by nitrergic nerves but also by
the endothelial cells in the cerebral arteries. Both types of diabetes have been shown to impair endothelial NO-dependent relaxation of cerebral blood vessels (37–41) while vasoconstrictor responses are preserved (42). Together with our findings, these studies suggest an overall reduction in neurogenic and endothelial NO-dependent vasodilation in diabetic cerebral arteries, which could contribute to the cerebrovascular pathologies associated with diabetes.

Interestingly, the thickness of the smooth muscle layer of the middle cerebral artery increased gradually after 12 weeks of diabetes. Although we did not observe any increase in the number of smooth muscle cells to cause the thickening, this could be due to the technique we used for this measurement. Therefore, we are not certain whether the increase in smooth muscle layer thickness is due to hyperplasia or hypertrophy of smooth muscle cells. Moreover, we did not use planimetry to measure the area occupied by the smooth muscle cells, which is more accurate than measuring the thickness. Therefore, these results should be interpreted with caution. The thickening of smooth muscle layer could be another factor in the pathogenesis of diabetic stroke, as suggested previously (43). Deficiency of endogenous vasodilator substances such as NO or sympathetic denervation could account for enhanced growth of smooth muscle cells (44,45).

In conclusion, our biphasic nitrergic nerve degeneration model suggests a significant decrease in the nNOS protein during diabetes. Together with endothelial dysfunction, this could result in severe deficiency of NO and vasodilation response to both nerve stimulation and shear stress in cerebral arteries. This might further lead to thickening of smooth muscle layer and an increase in vasoconstrictor response. The imbalance between dilatory and constrictive aspects might eventually increase the susceptibility to strokes.

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FIG. 7. Diabetes causes irreversible thickening of smooth muscle layer of middle cerebral arteries. Middle cerebral arteries were immunostained using smooth muscle–specific α-actin antibody. The thickness of the smooth muscle layer was measured as shown (A) in 10 random areas from each section and pooled for each group, giving mean smooth muscle thickness in micrometers (B). Data are means ± SE. *P < 0.05, significantly different from control; ANOVA followed by Dunnett’s test.


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