The distinction between metabolic and structural changes occurring in autonomic neurons during diabetes has not been fully clarified. Here we demonstrate that nitric oxide synthase–containing (nitrergic) neurons innervating the penis and gastric pylorus of streptozotocin-induced diabetic rats undergo a selective degenerative process in two phases. In the first phase, nitrergic nerve fibers lose some of their neuronal nitric oxide synthase content and function. In the second phase, nitrergic degeneration takes place in the cell bodies in the ganglia, leading to complete loss of nitrergic function. The changes in the first phase are reversible with insulin replacement; however, the neurodegeneration in the second phase is irreversible. Neurodegeneration is due to apoptotic cell death in the ganglia, which is selective for the nitrergic neurons.

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Diabetes is a common disorder that leads to complications affecting the retina, kidney, vascular, gastrointestinal, peripheral, and autonomic nervous systems. The mechanism through which diabetic complications develop is unclear. The generally accepted view for most diabetic complications is that the disease is accompanied by metabolic changes in the affected organ that, in the long term, result in structural alterations. Thus, if insulin replacement is started before the structural lesions occur, then it should reverse the metabolic changes and prevent the development of the complication. Once the structural lesions occur, however, the process should become less reversible with insulin replacement. Although from early studies in vivo "a point of no return" had been suggested during the course of diabetic peripheral neuropathy, cardiomyopathy, and nephropathy (1–3), most of the subsequent work has concentrated either on cellular dysfunction or on cell death without addressing the distinction between metabolic and structural alterations.

Nitric oxide (NO) is a well-characterized neurotransmitter in the central and peripheral nervous systems. In many tissues of the urogenital and gastrointestinal tract, NO mediates nonadrenergic noncholinergic (NANC) relaxant responses (4). Nerves that release NO are now known as nitrergic (5). NO is generated in these nerves by activation of the neuronal NO synthase (nNOS) and diffuses into the smooth muscle to activate the soluble guanylyl cyclase (sGC), producing an increase in the intracellular cyclic guanosine-3′,5′-monophosphate concentration, leading to relaxation (6).

Impaired nitrergic transmission has been shown to be responsible for erectile dysfunction and gastropathy in diabetes (7–12). To investigate the mechanisms by which the metabolic changes lead to structural disturbances in autonomic neuropathy, we studied the structure and function of nitrergic nerve fibers and neuronal cell bodies in the gastric pylorus, penis, and major pelvic ganglia in streptozotocin (STZ)-induced diabetic rats. In addition, we investigated the effect of different schedules of insulin treatment on this process.

RESEARCH DESIGN AND METHODS

Induction of diabetes. Male Wistar rats (225–250 g) were treated with STZ (75 mg/kg, intraperitoneally) or vehicle (saline) as described previously (10). This dosage was selected from our preliminary experiments in which different doses of STZ (45–95 mg/kg) were tested to find the optimum dose with the least toxicity and the most efficacy within the permitted timeframe. Hyperglycemia was defined as nonfasting blood glucose concentration >20 mmol/l. Insulin was administrated 4, 8, or 12 weeks after STZ injection using sustained-release insulin rods (~2 units/day; LinBit, Scarborough, ON, Canada). Rats were killed at 4, 8, 12, 16, and 20 weeks after STZ injection, and their blood, penis, bilateral anococcygeus muscles, stomach with gastric pylorus, and bilateral major pelvic ganglia (MPG) were collected. Anococcygeous muscles and pyloric sphincter were used for functional in vitro pharmacology studies. The rest of the organs were either fixed in paraformaldehyde for immunohistochemistry or frozen for protein quantification with Western blotting. All animal experiments were conducted according to the rules outlined by the Home Office, Animals (Scientific Procedures) Act 1986 (project no. 70/05161).

Functional studies using in vitro pharmacology. Bilateral anococcygeous muscles and pyloric sphincter were mounted in horizontal superfusion chambers and superfused (1 ml/min) with modified Krebs’ solution consisting of (in mmol/l) 136.9 NaCl, 2.7 KCl, 0.6 MgSO4, 11.9 NaHCO3, 0.5 KH2PO4, 1.8 CaCl2, 12.5 glucose, 0.01 dexmethylasone, and 0.01 indomethacin at 37°C and bubbled with 95% O2 and 5% CO2. The parameters of the electrical field stimulation (EFS) were 50 V, 0.3 ms pulse duration, 1–50 Hz, for 5 s (anococcygeous muscle) and 30 s (pyloric sphincter). NANC relaxation responses to EFS were recorded after treatment of the tissues with scopolamine (5 μmol/l) and guanethidine (5 μmol/l) followed by elevation of the tone with phenylephrine (3 μmol/l) in the anococcygeous muscle and with serotonin (10 μmol/l) in the pyloric sphincter as described previously (10,13,14).
Immunohistochemistry. After fixation in 4% paraformaldehyde overnight at room temperature, the tissues were transferred into 30% sucrose in phosphate buffer and kept at 4°C overnight. The samples were then frozen in OCT compound (BDH, Poole, U.K.), and serial cryosections at 10- to 20-μm intervals were obtained using a cryostat (−18°C; 2800 Frigoct-E; Leica, Bensheim, Germany). The sections were dried on gelatin-coated slides for 2 h at room temperature and then incubated with PBS containing 0.1% Triton X-100 and 5% of the serum of the species from which the secondary antibody was obtained. Then the slides were incubated with antibodies against nNOS (raised in sheep, K205 [15]; 1:2,000), vesicular acetylcholine transporter (VACHT; Chemicon, Harrow, U.K.; 1:100), tyrosine hydroxylase (TH; Chemicon; 1:200), vasoactive intestinal peptide (VIP; Oncogene, Nottingham, U.K.; 1:100), substance P (SP; Chemicon; 1:100), and PGP9.5 (Chemicon; 1:1,000) overnight followed by detection with appropriate fluorescent conjugated secondary antibodies. TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) staining was performed according to the manufacturer’s instructions (Roche, Lewes, U.K.).

Image analysis. The images were obtained using a laser-scanning confocal microscope (Leica TCS-DMRE). Image analysis was performed using Scion Image software (version 4.02; Scion, Frederick, MD) and Leica Confocal Software (Version 2.00, build0871, Heidelberg, Germany). PGP9.5, nNOS, or TH-positive cell bodies in MPG were counted manually, and the area occupied by the counted cells was measured to give the number of cells per 100,000 μm². In the penis, the numbers of PGP9.5-positive fibers in a cross-section of nerve bundle were counted, and the area occupied by the counted fibers was measured to give the number of nerve fibers per 100,000 μm².

The immunostaining density was measured as the mean amplitude of fluorescence per 1 μm² in selected areas occupied by longitudinal fibers. For avoiding 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.

Western blotting. The frozen tissues were pulverized using a stainless steel pestle and mortar on dry ice, homogenized, and centrifuged as described previously (10). The protein concentration in the supernatant was measured, and equal amounts of protein were run on 7.5% polyacrylamide SDS gels and transferred to nitrocellulose membranes. The blots were incubated overnight with monoclonal nNOS antibody (Transduction Laboratories, Oxford, U.K.; 1:2,000) and then with horseradish peroxidase–conjugated antimosue IgG (Vector Labs, Burlingame, CA; 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.

Statistical analysis. Results are expressed as mean values ± SE from a number of independent experiments or animals. Statistical analyses were performed using Prism software (version 3.0; GraphPad Software, San Diego, CA). Data were compared by Student’s unpaired, two-tailed t test. P < 0.05 was considered significant.

RESULTS

Presentation of the results. Throughout the article, STZ-induced diabetic animals without any insulin replacement are referred to as Xw, where X is the number of weeks that the animal was kept alive after STZ treatment. STZ-induced diabetic animals with insulin replacement are referred to as XY, where X is the number of weeks that the animal was kept alive and Y is the number of weeks that it received insulin. Insulin treatment was always given for the last Y weeks of the study.

Delayed insulin treatment corrects serum glucose and body weight loss. Diabetes was induced in male rats by treating them with STZ. Eight weeks and 12 weeks after STZ injection, some of the animals received insulin rod implants (delayed insulin treatment; groups 20/8 [20-week diabetic animal that received insulin in the last 8 weeks], 20/12 [20-week diabetic animal that received insulin in the last 12 weeks], 16/4 [16-week diabetic animal that received insulin in the last 4 weeks], and 16/8 [16-week diabetic animal that received insulin in the last 8 weeks]). Some animals received insulin implants immediately after STZ injection (continuous insulin treatment; groups 20/20, 16/16, 12/12, 8/8, and 4/4). Body weight and serum glucose concentration (Fig. 1) showed that the untreated diabetic rats lost weight or did not gain as much weight as control animals, and their serum glucose concentration remained high. The animals with continuous insulin treatment had similar body weight and serum glucose patterns to control animals (Fig. 1). The animals with delayed insulin treatment gained weight, and their serum glucose concentrations decreased to nondiabetic levels shortly after implantation of insulin rods (Fig. 1).

nNOS protein expression decreases with progression of diabetes. The nNOS protein content of the penis and gastric pylorus, measured by Western blotting, showed a gradual decrease during the course of diabetes (Fig. 2). In the animals that were treated with insulin continuously for the whole duration of diabetes, there was no decrease in nNOS (Fig. 2). The nNOS content recovered in diabetic animals whose insulin treatment started at the 8th week (groups 20/12 and 16/8); however, no recovery was observed in animals that received insulin rod implants at the 12th week (groups 20/8 and 16/4; Fig. 2).
Effect of insulin replacement on loss of nitrergic function. NANC relaxation responses were measured in the anococcygeus muscle in the presence of scopolamine and guanethidine to inhibit cholinergic and noradrenergic responses, respectively, and after elevation of the tone with phenylephrine. These NANC responses were purely nitrergic in nature and neurogenic in origin because they were completely inhibited in the presence of an inhibitor of NOS (L-N-nitro-L-arginine methyl ester, L-NAME; Fig. 3) or tetrodotoxin (TTX). The magnitude and the duration of these nitrergic relaxation responses decreased gradually during the course of 20 weeks of diabetes. Insulin treatment prevented the loss of nitrergic responses in group 20/12 but not in group 20/8 (Fig. 3).

In the pyloric sphincter, NANC relaxation responses were obtained in the presence of scopolamine and guanethidine and after elevation of the tone with serotonin. Nitrergic relaxation responses were recorded during EFS, which were inhibited by L-NAME (500 μmol/l) or TTX (1 μmol/l, not shown). "8w," "12w," and "20w" denote the animals that were diabetic for 8, 12, and 20 weeks, respectively. "20/8" and "20/12" denote the animals that were diabetic for 20 weeks and received insulin for the last 8 and 12 weeks, respectively. B: Relaxation responses in the anococcygeus muscle expressed as percentage of the maximum tone elicited with 2.5 Hz (black), 5 Hz (gray), or 10 Hz (white) EFS. *P < 0.05 significantly different from control.

In the pyloric sphincter, NANC relaxation responses were obtained in the presence of scopolamine and guanethidine and after elevation of the tone with serotonin. Nitrergic relaxation responses were recorded during EFS, which were inhibited by L-NAME (500 μmol/l) or TTX. However, a rebound contraction was observed after termination of EFS (Fig. 4). These contractions were resistant to L-NAME or TTX. The magnitude and duration of nitrergic relaxations decreased and those of rebound contractions increased gradually during the course of 20 weeks of diabetes. Insulin treatment prevented the loss of nitrergic responses and the increase in rebound contractions in group 20/12 but not in group 20/8 (Figs. 4 and 5).

Loss of nNOS in nerve fibers is followed by cell body loss. The morphology of the nerve fibers in the penis and pyloric sphincter was studied using immunohistochemistry with antibodies against nNOS, PGP9.5 (a nonspecific neuronal marker), VACHt (a cholinergic nerve marker), VIP, SP (an excitatory nerve marker in the gastrointestinal tract), and TH (a noradrenergic nerve marker). A decrease in nNOS immunostaining was evident in both cross-sections of nerve bundles (Figs. 6 and 7A) and longitudinal sections (Figs. 7A and 8) in the diabetic animals. This effect was reversible with insulin in groups 20/12 and 16/8 but not in groups 20/8 and 16/4 (Figs. 6, 7A, and 8).

PGP9.5 stains all nerve fibers, and its staining intensity

FIG. 2. Western blot (A) showing protein bands corresponding to nNOS in homogenates from penis (P) and pylorus of the stomach (S) of animals. Eighty micrograms of protein was loaded for each sample. Brain homogenate (B) from a control animal (20 μg) was used as a positive control. "4w," "8w," "12w," "16w," and "20w" denote the animals that were diabetic for 4, 8, 12, 16, and 20 weeks, respectively. "16/4" and "16/8" denote the animals that were diabetic for 16 weeks and received insulin for the last 4 and 8 weeks, respectively. "20/8" and "20/12" denote the animals that were diabetic for 20 weeks and received insulin for the last 8 and 12 weeks, respectively. B and C: Quantitative representation of the densitometric analysis of the Western bands in the penis and stomach, respectively, expressed as a percentage of the control. □, STZ-induced diabetic animals that received no insulin; ▲, STZ-induced diabetic animals that had insulin implants at the 8th week; ▼, STZ-induced diabetic animals that had insulin implants at the 12th week.

FIG. 3. Nitrergic responses in the anococcygeus muscle of diabetic animals with or without insulin replacement. A: Mechanical responses of the anococcygeus muscles from six different groups elicited with EFS (50 V, 0.3 ms pulse duration, 10 Hz, for 5 s) after treatment of the tissues with scopolamine, guanethidine, and phenylephrine. These relaxation responses were nitrergic because they were blocked by L-NAME (500 μmol/l) or TTX (1 μmol/l, not shown), "8w," "12w," and "20w" denote the animals that were diabetic for 8, 12, and 20 weeks, respectively. "20/8" and "20/12" denote the animals that were diabetic for 20 weeks and received insulin for the last 8 and 12 weeks, respectively. B: Relaxation responses in the anococcygeus muscle expressed as percentage of the maximum tone elicited with 2.5 Hz (black), 5 Hz (gray), or 10 Hz (white) EFS. *P < 0.05 significantly different from control.
in sections with mixed nerve fiber populations remained unchanged until the 16th week. From then on, the PGP9.5 staining showed a small decrease (~20% at 20 weeks; Fig. 7A). However, in the cross-sections of richly nitrergic nerve bundles in the penis, up to 50% nerve fiber loss was detected using PGP9.5 staining (Fig. 7B). This indicates that nNOS in the axons is merely downregulated during early stages of diabetes, whereas in the later stages, a degenerative process seems to have begun.

To investigate this further, we studied VAChT and VIP, which colocalize with nNOS in the penis and pyloric sphincter, respectively (16,17). When nNOS and VAChT

FIG. 4. The mechanical responses of the pyloric sphincter from five different groups elicited with EFS (50 V, 0.3 ms pulse duration, for 30 s) at 2.5 Hz (■), 5 Hz (▲), and 10 Hz (●) after treatment with scopolamine, guanethidine, and serotonin. The relaxation responses during EFS were blocked by l-NAME (○) or TTX (not shown). The contractile responses after the nitrergic response were resistant to TTX.

FIG. 5. EFS-induced relaxation responses in the pyloric sphincter were expressed as magnitude of relaxation as a percentage of the maximum tone (2.5 Hz [black], 5 Hz [gray], or 10 Hz [white]). *P < 0.05 significantly different from control.

FIG. 6. Effect of diabetes on nitrergic nerve fibers in the penis. The cross-sections of nerve bundles in the penis were immunostained for nNOS (green) and PGP9.5 (red). A and B: Control. C and D: 12 weeks. E and F: 20 weeks. G and H: 20/12 group. I and J: 20/8 group. Magnification ×200 in A, B, E, F, G, and H; ×160 in C and D; and ×100 in I and J.
were studied in the same sections of the penis, we observed a decrease in nNOS immunostaining throughout the 20 weeks (Figs. 7A and 8). VAChT, however, started to decrease only after the 16th week (Figs. 7A and 8). The decrease in both stainings was reversible by delayed insulin treatment in group 20/12 but not in group 20/8 (Figs. 7A and 8). In the pyloric sphincter, nNOS immunostaining decreased gradually throughout the 20 weeks of diabetes (Figs. 7A and 8). Just like VAChT, VIP immunostaining remained unchanged for up to 16 weeks and then decreased gradually (Figs. 7A and 8). In some sections before 16 weeks, VIP-positive and nNOS-negative nerve fibers were evident (Fig. 8V). The decreases in nNOS and VIP immunostaining were reversible with delayed insulin treatment in group 20/12 but not in group 20/8 (Figs. 7A and 8). nNOS-negative nerve fibers, such as TH-positive excitatory noradrenergic fibers in the penis and SP-positive excitatory fibers in the pyloric sphincter, were not affected by diabetes (Fig. 7A). These results suggested that nNOS protein is initially downregulated in the axons (first 12 weeks) and that this is followed by a selective nitrergic neurodegeneration process.

We then studied the cell bodies of the penile nitrergic nerves, which are located in the MPG, to obtain further evidence for the degenerative phase. In the MPG, nNOS was colocalized with VAChT and PGP9.5 (not shown) but not with TH, in accordance with previous studies (18). TH-positive cells were larger than nNOS-positive cells (Fig. 9). nNOS-immunostaining intensity was not changed; however, the number of nNOS-positive cell bodies started to decrease in number, coinciding with nerve fiber loss. This phase was not reversible by insulin treatment. 

**Nitrergic cell body loss is due to apoptosis.** We then...
investigated, using TUNEL staining, whether the loss of cell bodies in the MPG during the second phase was due to apoptosis. TUNEL staining was evident in some of the nNOS-positive cells in 16- and 20-week diabetic animals and in group 20/8 (Figs. 11 and 12). TUNEL staining was not observable in control animals or in diabetic animals up to 12 weeks and was reduced in group 20/12 (Figs. 11 and 12). The number of nNOS-negative, TH-positive cells that were TUNEL-positive did not significantly change throughout the 20 weeks of diabetes (Fig. 12). These results suggested that nitrergic cell body loss in the second phase was due to apoptosis.

DISCUSSION

Our results demonstrate the depletion of nNOS content from nitrergic nerve fibers in the early stages of STZ-induced diabetes. This was associated with a significant reduction in nitrergic function in both the anococcygeus muscle and gastric pylorus, in accordance with previous studies (7–12,19–25). The loss of nNOS content was reversible with early insulin replacement, suggesting that the decrease in protein content in early diabetes was not due to structural damage to the nitrergic nerves. The reversible decrease in nNOS content could be due to a decrease in androgen levels (19) or to withdrawal of insulin and/or related growth factors necessary for nNOS expression (26,27). In such a case, one would have expected reduced nNOS expression in the cell bodies as well as in the axons. We did not, however, detect any change in

FIG. 10. Diabetes reduces nitrergic cell body count and the nNOS/TH cell ratio in the MPG. A: nNOS-positive cell bodies in MPG were counted manually, and the area occupied by the counted cells was measured to give the number of cells per 100,000 μm². B: The ratio of the number of nNOS-positive nerves to the number of TH-positive nerves in the MPG. * P < 0.05 significantly different from control.

FIG. 11. Nitrergic neurons in the MPG undergo apoptosis. TUNEL-positive nuclei (red arrows) were evident in the nitrergic neurons in the MPG of 20-week diabetic animals but were fewer in the 20/12 group. Some nNOS-negative nonneuronal cells, most probably proliferating fibroblasts, were also TUNEL-positive, regardless of the diabetic status (white arrows). Scale bar = 45 μm.
nNOS expression in the MPG, where the cell bodies of nitrergic nerves innervating the penis are located. An alternative and more likely possibility is that the axonal transport of nNOS protein from the cell body to the axons (28) is altered during the early stages of diabetes.

As the diabetes progressed, structural damage to nitrergic nerve fibers became evident; cotransmitters in the nitrergic nerves such as VACHt and VIP started to decrease, as did the nonspecific neuronal marker PGP9.5. The change in transmitter content in the nerve fibers and the decrease in nerve fiber number coincided with a decrease in the number of nitrergic neurons in the ganglia. This resulted in total loss of nNOS content and was not reversible with insulin treatment, suggesting a degenerative process.

The decrease in neurotransmitter content was evaluated using semiquantitative immunohistochemical techniques with intrinsic methodologic problems, such as day-to-day variation in fluorescence intensity. Although we took control tissues as reference point and measured all other groups relative to controls, the quantitative value of these results should be regarded with caution.

Peripheral motor and sensory neuropathy in diabetes has been suggested to be caused by metabolic and vascular disturbances leading to impaired neural function and to loss of neurotrophic support and in the long-term to apoptosis of neurons and Schwann cells (29–32). Increased oxidative and nitrosative stress caused either directly by metabolic disturbances within the neuron or indirectly by vascular dysfunction has been implicated in this pathology (32). Although the available information specifically for the autonomic nerves is relatively limited compared with that for motor and sensory nerves, a similar consensus seems to be emerging. Oxidative stress has been suggested to be involved in autonomic dysfunction in diabetic rat heart, penis, and gut (33–35), and blood flow to the autonomic ganglia has been shown to be reduced by diabetes (36). Our results, suggesting a degenerative process that most probably originates in the ganglia, further support and add to this proposed mechanism.

Nitrergic dysfunction in diabetes has been shown to lead to an enhanced excitatory neurotransmission (37–39). Noradrenergic hyperactivity in the penis (10) and an increased excitatory SP innervation in the gastrointestinal tract (12) have been demonstrated previously in diabetic animals and humans. Our results showing selective nitrergic degeneration and sparing of excitatory TH-positive neurons in the MPG and penis and SP-positive nerve fibers in the gastric pylorus further support this and provide a basis for the pathogenesis of the nitrergic-excitatory imbalance in diabetes.

We have observed contraction in the pyloric sphincter, which started immediately after the nitrergic relaxation response. This contraction is more likely to be a rebound contraction than a neurotransmitter-mediated response because it was resistant to scopolamine, guanethidine, and TTX. We do not think that these responses were a direct myogenic effect of EFS because they were observed only after the tone of the tissue was elevated with serotonin. Moreover, they were resistant to treatment with L-NAME, suggesting that they are not NO-mediated rebound contractions observed by others (40). Therefore, we assume that these rebound contractions are due to EFS-induced changes in the sensitivity of smooth muscle to exogenous serotonin. It is possible that these contractions were offsetting the nitrergic relaxations, hence the increased rebound contraction and decreased nitrergic relaxation in the diabetic animals. Therefore, the decrease in relaxation responses in the pyloric sphincter of diabetic animals should be interpreted with caution.

We have demonstrated by TUNEL staining that the nitrergic neuronal loss was due to apoptotic cell death. This is in accordance with previous studies in which an increase in apoptosis in retinal neuronal cells and sensory neurons in diabetic human and STZ-diabetic rats was reported (29–31). However, in our study, the apoptosis was specific to nNOS-positive cells, indicating a role for endogenous NO in the apoptotic cell death.

Other forms of NOS that may be involved in the neurodegenerative process by producing high amounts of NO are inducible NOS (iNOS) and endothelial NOS (eNOS). In our studies, however, we did not detect induction of iNOS in the penis or pyloric sphincter of diabetic rats. Peroxynitrite, the product of the reaction between NO produced by eNOS and superoxide in arterioles that provide circulation to the sciatic nerve, has been suggested to be involved in diabetic neuropathy (41,42). Although we did not detect any increase in nitrotyrosine immunostaining in diabetic rat penis (10), it is possible that NO generated by eNOS from nearby blood vessels to produce NO may contribute to nitrergic degeneration. Therefore, we suggest that endogenous NO derived from nNOS and/or eNOS is involved in the apoptotic cell death.

We did not observe any apoptosis in TH-positive sympathetic neurons in the MPG. This is in accordance with a previous study (43) in which severe untreated STZ-induced diabetes in rats for 40 weeks failed to produce sympathetic neuron loss or apoptosis in their superior mesenteric or superior cervical sympathetic ganglia. Sympathetic dysfunction in diabetes has been suggested to be due to neuroaxonal dystrophy rather than to apoptosis (44). This suggests that the underlying neuropathology for nitrergic dysfunction might differ from that for sympathetic dysfunction in diabetes.

In 20-week diabetic animals whose insulin treatment was begun at the 12th week (group 20/8), irreversible nitrergic dysfunction and nitrergic degeneration were observed, although their blood glucose levels were normalized. This suggests that sustained high glucose level is not a prerequisite for the development of nitrergic degeneration. "Hyperglycemic memory," which refers to persistence or progression of hyperglycemia-induced alterations during subsequent periods of normal glucose homeostasis (45), may account for this. One such example has been reported in which diabetic dogs developed severe retinopathy after 2.5 years of euglycemia, which was preceded by 2.5 years of hyperglycemia (46). An alternative explanation could be the accumulation of factors that cannot be reversed by insulin treatment and that will lead to irreversible nerve damage. One such factor might be AGEs. We have recently shown that AGEs steadily rise in the serum and accumulate in the tissues during the course of diabetes and that this increase is not reversible by insulin treatment. Moreover, AGEs and endogenous NO synergize
to lead to neuronal apoptosis in a cell culture model (S. Cellek and S. Moncada, unpublished observations). Therefore, we propose that synergistic action of endogenous NO and AGEs leads to nitrergic degeneration.

Although we observed a decrease in nNOS in the nerve fibers at the early stages of the disease, the cell bodies still contained nNOS at this time. Structural damage was evident only after the 12th week and occurred at the same time as cell body damage was observed. This suggests the possibility that nitrergic neuropathy results from the co-location and interaction between AGES and NO, primarily in the cell body, and that the axonal damage is a result of cell body damage.

If NO is an important component of the factors in diabetic damage, then it is likely that cells other than nitrergic neurons that express NOS may be susceptible to damage in diabetes. Indeed, in the cardiovascular system, NO scavengers have been shown to protect against endothelial dysfunction during diabetes (47). In the kidney, a NOS inhibitor or treatment with insulin has been shown to prevent development of glomerular hyperfiltration in diabetic animals (48). It is interesting that in the central nervous system, diabetes-related dysfunction is localized to areas where nNOS is most concentrated, i.e., those associated with learning and memory (49), and diabetes has been shown to decrease nNOS content in these areas of the brain (50).

In conclusion, we have shown that there are two distinct phases of diabetic autonomic neuropathy. The first phase, with loss of nNOS in the nerve fibers and nitrergic dysfunction, was reversible with insulin replacement. The second phase was characterized by neuronal apoptosis, possibly as a result of the synergistic action of AGEs and endogenous NO in the cell bodies of nitrergic neurons, and was irreversible by insulin replacement.

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