Neuronal Nitric Oxide Synthase Mediates Statin-Induced Restoration of Vasa Nervorum and Reversal of Diabetic Neuropathy

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Background—Peripheral neuropathy is a frequent and major complication of diabetes.

Methods and Results—Severe peripheral neuropathy developed in type II diabetic mice, characterized by significant slowing of motor and sensory nerve conduction velocities. Rosuvastatin restored nerve vascularity, including vessel size, and nerve function also recovered to the levels of nondiabetic mice. Neuronal nitric oxide synthase expression in sciatic nerves was reduced in diabetic mice but was preserved by rosuvastatin. Coadministration of a nitric oxide synthase inhibitor with rosuvastatin attenuated the beneficial effects of rosuvastatin on nerve function and limited the recovery of vasa nervorum and nerve function. In vitro, rosuvastatin inhibited downregulation of neuronal nitric oxide synthase expression induced by high-glucose conditions in cultured Schwann cells. Furthermore, Akt phosphorylation in Schwann cells, downregulated by high-glucose conditions, was also restored by rosuvastatin, consistent with the change of neuronal nitric oxide synthase expression. Akt inhibition independently reduced neuronal nitric oxide synthase expression in Schwann cells in low-glucose cultures.

Conclusions—These data indicate that the HMG-CoA reductase inhibitor rosuvastatin has a favorable effect on diabetic neuropathy independent of its cholesterol-lowering effect. Our data provide evidence that this effect may be mediated in part via neuronal nitric oxide synthase/nitric oxide and phosphatidylinositol 3-kinase/Akt-signaling pathways and also suggest that restoration or preservation of the microcirculation of the sciatic nerve may be involved. (Circulation. 2005;112:93-102.)

Key Words: diabetes mellitus ■ nervous system ■ nitric oxide synthase

Peripheral neuropathy is one of the major complications of diabetes that can lead to significant morbidity. Sensory abnormalities predominate,1 leading to a failure to detect minor trauma of the lower extremities, ultimately contributing to skin ulcerations. Indeed, some reports indicate that 20% of all hospital admissions among diabetic patients in the United States are for foot problems.2 Even with intensive therapy, including insulin and more effective oral agents, the incidence of neuropathy has increased to as much as 16%3 of the 17 million diabetic patients in the United States alone. The pathogenic mechanisms of diabetic neuropathy that have been considered include the following: degeneration of proteins critical to neural function by nonenzymatic glycosylation,4 altered neural polyol metabolism,5,6 reduction of neurotrophic factors,1,7 and microvascular disease with impaired blood flow.6,8,9 Ischemia in diabetic nerves10,11 has also been considered in the pathogenesis of diabetic complications. Indeed, we have shown previously that experimentally induced diabetes causes physiological dysfunction in the peripheral nervous system associated with destruction of vasa nervorum. These prior studies have also documented that vascular endothelial growth factor (VEGF) gene transfer successfully modifies the neuropathy by restoring microcirculation in vasa nervorum.12,13 In addition, prior studies from our laboratory and others have revealed that HMG-CoA reductase inhibitors, or “statins,” can exert an angiogenic effect in ischemic tissues.14–16 Moreover, recent data have suggested a potential beneficial effect of statins that extends beyond the traditional indications of hyperlipidemia and that may be especially prominent in people with diabetes.17–22 Accordingly, we investigated the hypothesis that diabetic neuropathy can be reversed by administration of statins. In the present report we document that the disordered peripheral nerve physiology resulting from experimental diabetes is associated with marked destruction of the vasa nervorum of the sciatic nerve and that neuronal function may be successfully restored by administration of rosuvastatin, a new HMG-CoA reductase inhibitor. Nerve recovery induced by rosuv-
astatin is accompanied by replenishment of the vasa nervorum; however, our results also suggest that this statin may have neurogenic effects that augment the demonstrated vascular effects. Together, these findings provide additional evidence of a vasculogenic etiology of diabetic neuropathy, documenting statin-induced angiogenesis and vasodilatation of the vasa nervorum, and suggest potential direct neurotrophic effects of statins.

Methods

Reagents

Rosuvastatin was supplied by AstraZeneca UK. Rosuvastatin is a hydrophilic inhibitor of HMG-CoA reductase.23,24

Animals

All protocols were approved by St Elizabeth’s Institutional Animal Care and Use Committee. In all experiments, investigators performing the follow-up examinations were blinded to the treatment administered. Male C57BLKS/J-1m+/Leprdb homozygous (db/db) mice (Jackson Laboratories, Bar Harbor, Maine) aged 8 to 12 weeks were used. Age-matched heterozygotes (db/hm), a nonpenetrant genotype (Jackson Laboratories), were used as the control animals. Mice were treated with daily subcutaneous injection of rosvastatin (1 mg/kg) or saline as a vehicle. Serum glucose and total cholesterol levels were measured with the use of an instant check meter (Roche) at days 0 and 28 after each treatment.

Neurophysiological Measurements

Sciatic nerve conduction velocity was measured with standard orthodromic surface recording techniques and a Teca TD-10 (Oxford Instruments) portable recording system in all mice at baseline (before treatment) and then at 2 and 4 weeks after treatment, as described previously.25 Briefly, motor nerve conduction velocity (MCV) was calculated by dividing the distance between stimulating electrodes by the average latency difference between the peaks of the compound muscle action potentials evoked from 2 sites (sciatic notch and ankle). Sensory nerve conduction velocity (SCV) was calculated by dividing the distance between stimulating and recording electrodes by the latency of the signal from the stimulation artifact to the onset of the peak signal. For each nerve, maximal velocities were determined bilaterally. All measured data from both sides were averaged.

Tail-Flick Testing

This behavioral test examined the response of each mouse to a thermal noxious stimulus administered to its tail with a variation of the tail immersion test. This measure was chosen because of the role that small fiber dorsal root ganglia sensory neurons play in pain transmission. The animals were loosely restrained with their tails immersed in a beaker of water to a depth of ~2 cm. The beaker was uniformly heated at a rate of ~2°C per minute beginning at 38°C. A magnetic stirring bar was used to maintain a uniform temperature. The temperature at which each animal flicked its tail out of the water was recorded to the nearest 0.5°C.

Assessment of Vascularity in Sciatic Nerve

Vascularity of sciatic nerves from both nondiabetic and diabetic mice was assessed by in situ fluorescent staining with the use of the endothelial cell–specific marker FITC-conjugated BS1-lectin (Vector Laboratories). After anesthesia, BS1-lectin (0.1 mg per mouse) was injected systemically by direct cardiac puncture. Ten minutes later, the animals were euthanized, and sciatic nerves were harvested and fixed in 2% paraformaldehyde for 2 hours. After fixation, nerves were either whole mounted for longitudinal analysis or embedded in OCT compound for frozen cross section. Samples were analyzed with the use of a computer-assisted Nikon fluorescence microscope with a digital camera (ECLIPS TE200, Nikon Inc.). The number of vessels was counted in 10 randomly selected cross sections under fluorescent microscope (×20), and vessel cross-sectional areas were measured by NIH Image (version 1.62) in the same sections. Because BS1-lectin binds to vascular endothelium, the fluorescence labeling that occurs by intracardiac injection of the FITC-conjugated BS1-lectin is restricted to vascular structures. Vessels are identified as small circular/punctate areas of fluorescence in the cross-sectional images and were counted in 10 randomly selected cross sections under the fluorescent microscope (×20). Vessel cross-sectional area, which reflects vessel size, was measured with the use of NIH Image (version 1.62) in the same nerve cross sections.

Double-Fluorescent Immunohistochemistry

Sciatic nerves were isolated from mice at the time of euthanasia and prepared for frozen sections. Frozen tissue sections (4 μm in thickness) were air dried and fixed with 100% acetone for 5 minutes at −20°C. Nonspecific protein binding was blocked with 10% normal horse serum. Sections were incubated overnight at 4°C with a rabbit polyclonal antibody against endothelial nitric oxide synthase (eNOS) (1:250), neuronal nitric oxide synthase (nNOS) (1:200), and VEGF (1:500) (Santa Cruz Biotechnology, Santa Cruz, Calif) diluted in 2% goat serum/PBS, followed by 30 minutes of incubation with a secondary antibody, Cy3-conjugated anti-rabbit IgG (1:500) (Jackson ImmunoResearch). Normal rabbit IgG was used as a negative control. After they were washed with PBS, sections were incubated with second primary antibodies biotinylated isoleucin B4 (ILB4) (1:100, Vector Laboratories) for detection of endothelial cells or goat polyclonal anti-S100 (1:100, Santa Cruz) for detection of Schwann cells at 4°C overnight, followed by FITC-conjugated streptavidin (1:500) or Cy2-conjugated anti-goat IgG (1:500) (Jackson ImmunoResearch) as a secondary antibody, respectively. Sections were counterstained with DAPI (1:5000) and mounted in aqueous mounting medium.

Immunocytochemistry for BrdU

Cells were fixed with 4% paraformaldehyde for 5 minutes in 4-well chamber slides (Nalge Nunc). After they were washed with PBS, slides were incubated with 2N HCl for 20 minutes at 37°C followed by incubation with 100 mmol/L sodium borate buffer for 20 minutes, and nonspecific protein binding was blocked with 10% normal goat serum. Sections were incubated with biotinylated sheep anti-BrdU antibody (1:100, BIODESIGN) at 4°C overnight, followed by rhodamine-conjugated streptavidin (1:500, Jackson ImmunoResearch) as a secondary antibody. Nuclei were stained with DAPI (1:5000), and slides were mounted in aqueous mounting medium. Proliferation activity was evaluated under fluorescent microscopy as an average percentage of BrdU-positive cells in 5 randomly selected high-powered fields per well.

nNOS Inhibition Study

Mice were treated with a daily subcutaneous injection of rosvastatin (1 mg/kg) or rosuvastatin (1 mg/kg) plus selective nNOS inhibitor 1,2-trifluoromethylphenyl imidazoline (TRIM) (50 mg/kg) (Sigma). MCV and SCV were measured at 2 and 4 weeks after the treatment. The assessment of vascularity in sciatic nerve with FITC-conjugated BS1-lectin was performed at the time of euthanasia, as described above.

Cell Culture

Primary mouse Schwann cells were obtained from sciatic nerve by explant method as described previously.26 Cells were seeded on 10-cm plastic culture dishes coated with rat type I collagen (300 μg/mL) (Sigma) and cultured in DMEM F-12 (GIBCO) supplemented with 20% fetal bovine serum (FBS), 10 ng/mL progesterone, 5 μg/mL transferrin, 5 μg/mL insulin, 10 μg/mL putrescine (Sigma), B27 supplement, 25 mmol/L HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO). Cultured Schwann cells were identified on the basis of cell soma and nuclear morphology, and the purity was also confirmed by immunocytochemical labeling for cytoplasmic S100 protein and glial fibrillary acidic protein to be >95% (data not shown). Mouse Schwann cell line MSCP was a
kind gift from Dr Jean-Jaques Hauw (Raymond Escourroul Neuro-pathology Laboratory, Paris, France). MSC80s were cultured and maintained in DMEM (GIBCO) supplemented with 4 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FCS (GIBCO).

Primary human adult dermal microvascular endothelial cells (HMVECs), obtained from BioWhitaker, were cultured in endothe-lial basal medium supplemented with 5% FBS, 0.1 mg/mL bovine brain extract, 10 ng/mL epidermal growth factor, 0.5 mg/mL hydrocortisone, and 100 μg/mL gentamicin (Cambrex). All the cells were maintained at 37°C and 5% CO₂.

**Proliferation Assay**

The number of viable Schwann cells was determined with a validated nonradioactive cell proliferation assay kit (CellTiter 96; Promega). Cells were seeded in 96-well plates at 80% confluence (1×10⁴ cells per well) and cultured in 0.5% FBS medium for 48 hours. Then rosuvastatin was added at concentrations from 0.0001 to 10 μmol/L in 20% FBS medium. Twenty percent FBS medium alone was used as a control. After 48 hours, 15 μL of dye solution was added per well, and cells were incubated for 4 hours before measurement of absorbance at 570 nm. The proliferation activity of HMVECs was evaluated by Brdu incorporation as described previously.²³ Cells were seeded in human fibroinectin-coated (50 μg/mL at 37°C for 1 hour) 4-well Laboratory-Tek chamber slides (Nalge Nunc) at a density of 1×10⁴ cells per well and cultured in 0.5% FBS medium for 24 hours. Then rosuvastatin was added at concentrations from 0.01 to 10 μmol/L in 5% FBS medium. Five percent FBS medium was used as a control. After 18 hours of exposure to each treatment, BrdU (Sigma) was added to each well at a concentration of 10 μmol/L, and its incorporation into the cells was determined after 6 hours. Immunocytochemistry for BrdU was performed as described above. BrdU-positive and total cells in each well were counted in 10 high-powered fields (×40), and the percentage of BrdU-positive cells was calculated.

**Migration Assay**

Schwann cell and HMVEC migrations were evaluated with a modified Boyden’s chamber assay as described previously.²³ Briefly, the polycarbonate filter (8-μm pore size) (Poretics) was placed between upper and lower chambers. Cells were preincubated in DMEM-F-12 or endothelial basal medium for 30 minutes with rosuvastatin at concentrations from 0.0001 to 10 μmol/L. Cell suspensions (1×10⁴ cells per well) were placed in the upper chamber, and the lower chamber was filled with medium containing human recombinant nerve growth factor (100 ng/mL) or VEGF (50 ng/mL) (R&D Systems). The chamber was incubated for 4 hours at 37°C and 5% CO₂. Migration activity was evaluated as the mean number of migrated cells in 5 high-powered fields (×40) per chamber.

**Dominant Negative Akt and Myristoylated Forms of Akt/Adenovirus Infection**

Cultured MSC80s were transduced with an adenoviral construct encoding dominant negative Akt (dn-Akt) or myristoylated Akt (myr-Akt) at a multiplicity of infection of 500 for 3 hours in DMEM with supplement. On the following day, cells were used for Western blot analysis.

**Western Blot Analysis**

MSC80s were cultured with the following conditions for 48 hours: mannitol (25 mmol/L), glucose (5 and 25 mmol/L), high glucose (25 mmol/L) in the presence of rosuvastatin ranging from 0.001 to 10 μmol/L, and high glucose (25 mmol/L) with rosuvastatin (1 μmol/L) in the presence of the phosphatidylinositol 3-kinase (PI3K)/Akt inhibitor LY294002 (10 μmol/L). Cells were lysed in 100 μL SDS sample buffer (20% SDS, 5% β-mercaptoethanol, 10% glycerol in 50 mmol/L Tris-HCl, pH 7.4) per 35-mm culture dish. Protein extracts were separated by 7.5% SDS-PAGE and transferred to a 0.2-μm PVDF membrane (Bio-Rad). The membranes were blocked with 10% nonfat dry milk and 0.2% Tween-20 in PBS, pH 7.4, then immunoblotted overnight at 4°C with rabbit polyclonal antibodies against mouse nNOS (1:1000; BD Pharmingen), phospho-Akt/Akt (1:1000; Cell Signaling), and a goat polyclonal antibody against α-actin (1:1000, Santa Cruz). Blots were washed with 0.2% Tween-20 in PBS and incubated for 45 minutes with horseradish peroxidase–linked goat antibody against rabbit IgG (1:5000; Santa Cruz). Immunoreactive bands were visualized with ECL reagent (Amersham). Densitometric analyses for the blots were performed with the use of NIH Image software.

**Measurement for NO Production in Schwann Cells**

MSC80s were cultured on 24-well plates at 70% to 80% confluence under low-glucose (5 mmol/L) and high-glucose (25 mmol/L) conditions with or without rosuvastatin (1 and 10 μmol/L) for 48 hours. Culture medium was collected for the measurement of nitrate/nitrite. NO production was evaluated by its final metabolite nitrate/nitrite. The concentration of nitrite was measured as an OD value at 540 nm with the use of a Nitrate/Nitrite Colorimetric Assay Kit (Cayman) according to the manufacturer’s instructions.

**Statistical Analysis**

All results are presented as mean±SEM. Statistical comparisons between 2 groups were performed by Student t test, and ANOVA was performed for serial analysis. Probability values <0.05 were considered statistically significant. All in vitro experiments were repeated at least in triplicate and analyzed.

**Results**

**Nerve Conduction Velocities and Characterization of Mice**

db/db mice develop a severe peripheral neuropathy at 8 weeks, as described previously.²⁸ Electrophysiological recordings in db/db mice documented significant slowing of MCV and SCV (MCV=29.1±1.2 m/s and SCV=24.6±1.4 m/s) compared with those of control mice (MCV=46.7±2.4 m/s and SCV=52.5±1.5 m/s; P<0.01 for both MCV and SCV). Placebo-injected db/db mice showed persistent, stable neuropathy 4 weeks after initiation of treatment (MCV=27.0±0.5 m/s and SCV=29.6±1.0 m/s), whereas rosuvastatin-injected db/db mice demonstrated nearly complete recovery of MCV and SCV back to levels of nondia-betic mice. Specifically, MCV and SCV in rosuvastatin-injected db/db mice were 47.2±1.6 and 44.7±1.8 m/s, and those in nondiabetic mice were 49.1±2.1 and 47.3±2.3 m/s, respectively (nondiabetic mice versus rosuvastatin-injected diabetic mice; P=NS) (Figure 1a and 1b). Tail-flick testing, a measure of the function of small fiber dorsal root ganglia sensory neurons, was also performed 4 weeks after treatment. In saline-injected db/db mice, tail-flick temperatures were significantly increased to 46.1±0.2°C (P<0.001 versus nondiabetic mice). In contrast, tail-flick temperatures recorded in rosuvastatin-injected db/db mice did not differ significantly from those of age-matched nondiabetic control animals (44.6±0.3°C in rosuvastatin-injected mice versus 44.9±0.3°C in control mice; P=NS) (Figure 1c). Blood glucose and serum total cholesterol levels before and after treatment in db/db mice were not significantly different in rosuvastatin-injected versus saline-injected mice (Table).

**Vascularity of Vasa Nervorum in Sciatic Nerve**

To investigate the potential role of microvascular pathology in the development of diabetic neuropathy, we performed in
Rosuvastatin (ROS) treatment reverses nerve conduction slowing and sensory deficit in diabetic mice. Nondiabetic mice were used as controls (control; n=10), and db/db mice were randomly assigned to saline injection (saline; n=10) or rosuvastatin injection (ROS; n=10). Data are expressed as mean±SEM. Electrophysiological parameters MCV (a) and SCV (b) were performed before and 2 and 4 weeks after the initiation of treatment. *P<0.01, **P<0.0001, saline vs rosuvastatin. Tail-flick testing, to assess sensory function, was performed 4 weeks after initiation of treatment (c). P=NS, *P<0.01 compared with nondiabetic control.

statu fluorescent imaging of the vasa nervorum by in vivo perfusion of FITC-labeled BS1-lectin. In the nerves of saline-injected db/db mice, the vascularity of vasa nervorum was markedly reduced (Figure 2b and 2e). Cross sections from these nerves disclosed a significant reduction not only in the number (capillary density) but also in the size (cross-sectional area) of vasa nervorum compared with that of nondiabetic mice (16.4±0.8 versus 24.0±1.6 vessels per cross section [P<0.01] and 53.0±2.8 versus 79.0±4.7 μm² per vessel [P<0.01], respectively; n=8) (Figure 2g and 2h).

nNOS, eNOS, and VEGF Expression in Sciatic Nerve

Prior investigations have established the roles of eNOS and VEGF signaling in the angiogenic response to ischemia. To investigate the contribution of these molecules in the recovery of vasa nervorum, we performed fluorescent immunohistochemistry for not only eNOS and VEGF but also nNOS expression in vasa nervorum in diabetic mice. There were marked differences of nNOS (Figure 3a), eNOS (Figure 3b), and VEGF (Figure 3c) expression between saline-injected db/db mice and rosuvastatin-injected db/db mice. The expression of nNOS was most notably decreased in saline-injected db/db mice in comparison to rosuvastatin-injected db/db mice. In contrast, the expression of nNOS in rosuvastatin-injected db/db mice was similar to that seen in nondiabetic mice (data not shown).

TRIM Partly Reversed the Effect of Rosuvastatin on Nerve Dysfunction

To further investigate whether NO production is essential for rosuvastatin-mediated improvement in diabetic neuropathy, we inhibited nNOS by coadministration of a selective nNOS inhibitor, TRIM, with rosuvastatin. In rosuvastatin-injected db/db mice, significant restoration of MCV and SCV was observed at 2 and 4 weeks after treatment (MCV=37.3±0.9 and 47.2±1.6 m/s for 2 and 4 weeks versus 26.4±1.3 m/s at week 0; SCV=38.2±2.1 and 44.7±1.8 m/s for 2 and 4 weeks versus 22.7±1.3 at week 0, respectively; P<0.01 for both MCV and SCV; n=10 in each group). In contrast, rosuvastatin-injected mice receiving TRIM did not demonstrate the full extent of neurological recovery seen in rosuvastatin-injected mice in which nNOS function was unimpaired (Figure 4a and 4b). Nerve conduction velocity measurements in rosuvastatin- versus rosuvastatin+TRIM-treated diabetic mice revealed significant reduction in the beneficial effect of rosuvastatin on nerve function resulting from inhibition of nNOS (2 weeks [SCV, 38.2±2.1 for rosuvastatin versus 32.7±1.3 m/s for rosuvastatin+TRIM; P<0.01] [Figure 4b] and 4 weeks [MCV, 47.2±1.6 for rosuvastatin versus 35.8±1.3 m/s for rosuvastatin+TRIM; SCV, 44.7±1.8 for rosuvastatin versus 34.9±0.9 m/s for rosuvastatin+TRIM; P<0.01] [Figure 4a and 4b]). Inhibition of nNOS also prevented the normalization of tail-lick temperature by rosuvastatin (rosuvastatin 44.6±0.3°C versus

### Blood Glucose and Serum Total Cholesterol Levels

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<th>Glucose, mg/dL</th>
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<td></td>
<td>0 Weeks</td>
<td>4 Weeks</td>
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<td>db/m mice (control; n=5)</td>
<td>168±23.5</td>
<td>168.3±28.7</td>
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Values are mean±SEM. Statistical comparisons between 0 and 4 weeks were made by Student t test.
versus 79.0/H11006 H11006/versus rosuvastatin

vascularity in both the number (capillary density) and the size also show significant reversal of the improvement in nerve function induced by rosuvastatin. Quantitative analyses dependent NO signaling is required for a portion of the recovery (Figure 4e and 4h). These findings indicate that nNOS-

4d and 4g) compared with that in the rosuvastatin-injected group (a and d). Bar=200 μm. Vascularity was quantified in tissue cross sections of mouse sciatic nerve. Before euthanasia at 4 weeks after treatment, mice were perfused with FITC-conjugated BS1-lectin to visualize vasa nervorum. Ten cross sections per frozen sample were randomly selected from each specimen, and the number (capillary density) (g) and size (cross-sectional vessel area) (h) of vessels per cross section were quantified and averaged. Data are expressed as mean±SEM (n=5 per study group). *P<0.01 compared with control.

Figure 2. Rosuvastatin (ROS) treatment restores vasa nervorum in diabetic mice. Representative fluorescence photomicrographs show longitudinal views of whole-mounted mouse sciatic nerves (a, b, and c) and their respective cross sections (d, e, and f) 4 weeks after treatment. The network of vasa nervorum is markedly reduced in the diabetic saline injection group (b and e). The vascularity of vasa nervorum in the rosuvastatin group (c and f) appears well preserved, and the number and size of visible vessels in the cross sections appear similar to those of nondiabetic controls (a and d). Bar=200 μm. Vascularity was quantified in tissue cross sections of mouse sciatic nerve. Before euthanasia at 4 weeks after treatment, mice were perfused with FITC-conjugated BS1-lectin to visualize vasa nervorum. Ten cross sections per frozen sample were randomly selected from each specimen, and the number (capillary density) (g) and size (cross-sectional vessel area) (h) of vessels per cross section were quantified and averaged. Data are expressed as mean±SEM (n=5 per study group). *P<0.01 compared with control.

Effect of Rosuvastatin on HMVEC Proliferation and Migration
To determine whether the direct action of rosuvastatin on the vasa nervorum includes direct modulation of endothelial cell phenotype, we investigated the effect of rosuvastatin on HMVEC proliferation and migration at concentrations from 0.001 to 10 μmol/L. Rosuvastatin significantly promoted proliferative activity of HMVECs from 60% to 28% at concentrations from 0.001 to 0.1 μmol/L (Figure 5a). In contrast, rosuvastatin significantly promoted VEGF-induced HMVEC migration by 30% at lower concentrations (0.001 μmol/L) and inhibited migration by 40% and 50% at higher concentrations (1 and 10 μmol/L, respectively) (Figure 5b).

Rosuvastatin Directly Modulates Schwann Cell Proliferation and Migration
To determine whether the effect of rosuvastatin on the nerve recovery might also be the result of a direct neurotrophic effect, we investigated the effect of rosuvastatin on Schwann cell proliferation and migration at concentrations from 0.001 to 10 μmol/L. Rosuvastatin significantly promoted proliferative activity of Schwann cells by up to 86% at concentrations of 0.1 μmol/L (Figure 5c). Rosuvastatin also enhanced nerve growth factor–induced Schwann cell migration significantly by 25% to 62% at concentrations from 0.01 to 10 μmol/L in a dose-dependent manner (Figure 5d).

Rosuvastatin Preserves NO Production Downregulated by High Glucose in Schwann Cells
To confirm that rosuvastatin upregulates not only nNOS expression under high-glucose conditions but also promotes NO production in Schwann cells, we evaluated the actual NO production by measuring its final metabolite nitrate/nitrite in culture medium. Rosuvastatin significantly preserved NO production by Schwann cells despite high-glucose conditions (Figure 5e).

Effect of Rosuvastatin on Downregulated nNOS Expression in Schwann Cells by High Glucose
The expression of nNOS in MSC80 was reduced by 60% in high-glucose conditions. In contrast, the same concentration of osmotic substance, mannitol, did not affect nNOS expression (Figure 6a). Rosuvastatin significantly preserved nNOS expression at doses ranging from 0.1 to 10 μmol/L despite high-glucose conditions. Phosphorylated Akt expression was also evaluated and coincided with nNOS expression, showing a decrease in high-glucose conditions with restoration of phospho-Akt expression by increasing concentrations of rosuvastatin (Figure 6a). To provide further evidence of the association between Akt and nNOS, we performed Western blot for nNOS using a PI3K inhibitor and dn-Akt adenoviral vector. Rosuvastatin restored nNOS expression in high-glucose conditions. However, both a PI3K inhibitor and dn-Akt adenoviral transduction reversed the effect of rosuvastatin on nNOS expression. On the other hand, overexpression of myr-Akt by adenoviral transduction resulted in preservation of nNOS expression despite high-glucose conditions. The expression of phosphorylated Akt was also consistent with that of nNOS. These data indicate that

rosuvastatin+TRIM 45.7±0.2°C, *P<0.01; saline 46.1±0.2°C versus rosuvastatin+TRIM 45.7±0.2°C, *P=NS) (Figure 4c). The vascularity of vasa nervorum documented by BS1-lectin in rosuvastatin+TRIM-treated db/db mice was also less developed (Figure 4f and 4i) as well as saline-injected db/db mice (Figure 4d and 4g) compared with that in the rosuvastatin-injected group (Figure 4e and 4h). These findings indicate that nNOS-dependent NO signaling is required for a portion of the recovery in nerve function induced by rosuvastatin. Quantitative analyses also show significant reversal of the improvement in nerve vascularity in both the number (capillary density) and the size (cross-sectional vessel area) in the rosuvastatin+TRIM group compared with the rosuvastatin group (15.7±1.4 versus 24.0±1.6 vessels per cross section [P<0.01] and 50.4±2.9 versus 79.0±4.7 μm² per vessel [P<0.01], respectively).
Figure 3. Representative photomicrographs of fluorescent immunohistochemistry for nNOS (a), eNOS (b), and VEGF (c) in sciatic nerves in nondiabetic and diabetic mice 4 weeks after treatment. Reduced expression of nNOS, eNOS, and VEGF, identified by red fluorescence, was observed in saline injection group (saline) compared with the rosvastatin injection group (ROS) and nondiabetic control. Schwann cells and capillaries were identified by specific markers S100 and ILB4 (green), respectively. Merged images indicate colocalization of S100 and nNOS, ILB4 and eNOS, and ILB4 and VEGF (orange). Bar=100 μm.
Rosuvastatin acts via PI3K to phosphorylate Akt, which in turn mediates the effect of rosuvastatin on nNOS (Figure 6b).

Discussion

This is the first study to investigate the effect of HMG-CoA reductase inhibitors on diabetic peripheral neuropathy. The data demonstrate lipid-independent effects of the HMG-CoA reductase inhibitor rosuvastatin on the recovery of vasa nervorum and nerve function in diabetic neuropathy. Moreover, these data indicate that HMG-CoA reductase inhibitors may have direct neurotrophic effects.

In our in vivo studies, the development of diabetic neuropathy is related to loss of vasa nervorum responsible for perfusion of peripheral nerves. Nerve function correlated with morphological observations of the vasa nervorum in the affected nerves of the diabetic mice. The overall restoration of the number and cross-sectional area of vasa nervorum in db/db mice to a pattern similar to that of nondiabetic mice was observed after rosuvastatin administration. The coincidence of restoration of vasa nervorum accompanied by functional nerve recovery has now been documented in diabetic animal models with the use of 3 distinct angiogenic agents, VEGF, sonic hedgehog, and now rosuvastatin. In the present study the recovery of vasa nervorum was mainly associated with recovery of nNOS/NO production to nondiabetic levels. Moreover, we found that codadministration of rosuvastatin and an nNOS specific inhibitor, TRIM, partially reversed the effect of rosuvastatin. These results suggest that NO production through nNOS might play an important role in the regeneration of vasa nervorum. Multiple prior reports have provided evidence of a link between statins, eNOS, and angiogenesis and that rosuvastatin enhances release of NO from the rat aortic vascular endothelium. Weis et al have reported that cerivastatin increases endothelial VEGF release and modulates VEGF receptor-2 expression in endothelial cells. As indicated in these studies, the evidence that statins regulate eNOS and VEGF in endothelial cells has already been shown. However, no reports have shown that statin also regulate nNOS, which is mainly expressed in neuronal tissue and has the potential to produce more NO than eNOS. Our in vitro data suggest that rosuvastatin directly upregulates nNOS/NO in Schwann cells via the PI3K/Akt signaling pathway. Together with these prior findings, our data support a vascular mechanism for at least part of the effect of rosuvastatin on nerve function.

Figure 4. Rosuvastatin (ROS)-induced restoration of nerve dysfunction is nNOS dependent. To determine whether rosuvastatin-induced recovery of nerve function required nNOS, we used the nNOS inhibitor TRIM and assessed MCV, SCV, and tail-flick temperature in diabetic mice. db/db mice were randomly assigned to saline control, rosuvastatin injection (n=5), or rosuvastatin injection + TRIM administration (n=5). Sciatic nerve conduction measurements were performed at the time of treatment (0 week) and then at 2 and 4 weeks. Tail-flick testing was performed at 4 weeks after treatment. Data are expressed as mean±SEM. a and b, P=NS, *P<0.01 compared with saline. c, P=NS, *P<0.01 compared with nondiabetic control. P=NS, saline vs rosuvastatin + TRIM. d to i, Fluorescence photomicrographs of representative longitudinal views of whole-mounted mouse sciatic nerves (d to f) and their respective cross sections (g to i) 4 weeks after treatment. Total network of vasa nervorum in rosuvastatin injection group was well developed and preserved (e and h). In contrast, vascularity of vasa nervorum in the rosuvastatin + TRIM group (f and i) was reduced and appears similar to that in saline injection group (d and g). Bar=200 µm. j and k, Quantitative analyses of nerve vascularity (j, capillary density; k, cross-sectional vessel area) in the rosuvastatin + TRIM group compared with the rosuvastatin group in 10 randomly selected cross sections per nerve. Data are expressed as mean±SEM (n=5 per study group). P=NS, *P<0.01 compared with control.
of the documented statin-mediated restoration of nerve function.

Prior reports about the effect of statins on angiogenesis have been conflicting between a stance of promotion\textsuperscript{14,15,32} and inhibition\textsuperscript{33,34}. These discrepant data may result from the different cell types used or may be attributed to the different statin concentrations. Indeed, recent data indicate that statins have biphasic effects on angiogenesis\textsuperscript{30,31}. In our in vitro studies, we used a wide range of concentrations of rosvastatin to permit identification of a dose-response effect\textsuperscript{30}. Rosuvastatin promoted HMVEC proliferation at low concentrations (0.0001 to 0.1 \(\mu\text{mol/L}\)). However, a dose-dependent inhibitory effect of rosvastatin on HMVEC migration was
observed at concentrations from 0.1 to 10 μmol/L. On the other hand, rosuvastatin dose-dependently promoted both Schwann cell proliferation and migration at concentrations from 0.0001 to 10 μmol/L. The interpretation of these in vitro findings, specifically attempting to use these data to explain the in vivo observations, is complicated by several factors. First, the cell culture results are observations made in monoculture and therefore lack the dynamic environment of the intact organism. Second, the concentrations used, which bracket the serum concentrations in patients, may not perfectly reflect the microenvironment of the nerve. With these caveats, however, the in vitro data demonstrate direct effects of statins on neural elements. Indeed, in vivo proliferation in the nerve assessed by the BrdU labeling technique indicated that statin slightly promoted Schwann cell proliferative activity for at least 4 weeks after initiation of treatment (data not shown). These results may explain why TRIM incompletely reversed electrophysiological recovery induced by rosuvastatin. The restoration of vasa nervorum resulting from rosuvastatin treatment was almost completely reversed by administration of TRIM, as demonstrated by in situ fluorescent imaging of whole-mounted explants of sciatic nerve (Figure 4). These data suggest that rosuvastatin may have not only a vasodilating effect on the vasa nervorum but also direct neurotrophic effects on the sciatic nerve itself. Administration of TRIM to diabetic mice, without rosuvastatin, was not performed in this study because our goal was to evaluate the effect of nNOS antagonism on the salutary effects of rosuvastatin, as demonstrated by in situ fluorescent imaging of whole-mounted explants of sciatic nerve (Figure 4). These data suggest that rosuvastatin may have not only a vasodilating effect on the vasa nervorum but also direct neurotrophic effects on the sciatic nerve itself. Administration of TRIM to diabetic mice, without rosuvastatin, was not performed in this study because our goal was to evaluate the effect of nNOS antagonism on the salutary effects of rosuvastatin rather than in the native diabetic state. Nevertheless, one could expect further worsening of nerve function by inhibiting nNOS expression, which is already reduced in diabetic mice, because nNOS has been shown to play a role as a neuroprotective factor as well as a NO producer. The data also suggest that statins could act by upregulating nNOS expression via Akt activation in Schwann cells. The in vivo data show that rosuvastatin resulted in recovery of both vessel number and size in diabetic mice up to the nondiabetic level. It is tempting to conclude, on the basis of this association, that statin-induced angiogenesis led to nerve recovery; however, further study will be needed to provide definitive evidence of the sequence of events induced by statin administration that led to restoration of nerve function.

Certain pieces of prior data are consistent with our present findings. For example, Cameron et al showed improvement in nerve function in a streptozotocin-induced model of diabetes after administration of rosuvastatin. The same authors documented the impact of rosuvastatin on NO-dependent function in aorta and corpus cavernosum of diabetic mice. Finally, and perhaps most importantly, Fried et al studied patients with diabetes and showed a potential attenuating effect of statin therapy on the advent of neuropathy in diabetic patients. In contrast, there is a significant body of literature suggesting a positive relationship between statin use and the onset of neuropathy. Together these prior studies and the present data suggest that additional study is required to achieve comprehensive understanding of both the mechanisms of diabetic neuropathy and the potential therapeutic impact of statins.

In summary, rosuvastatin, a new HMG-CoA reductase inhibitor, has a favorable effect on diabetic neuropathy that is independent of its cholesterol-lowering effect and that is associated with restoration of vasa nervorum. The effect of rosuvastatin on vasa nervorum appears to be mediated via an NO-dependent pathway. Moreover, we found that rosuvastatin restored downregulated nNOS expression in Schwann cells under high-glucose conditions at least in part via a PI3K/Akt signaling pathway. Rosuvastatin also has a direct neurotrophic effect, promoting proliferation and migration activity of Schwann cells.

In the clinical setting, statins generally have been shown to reduce cardiovascular events in association with reducing lipid levels. In addition, however, recent data have revealed that cardioprotective effects are extended to populations of patients without significant lipid abnormalities. Our observations suggest an additional lipid-independent activity of statins that may be of therapeutic relevance.

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References


Peripheral polyneuropathy occurs in 50% of patients with long-standing diabetes. The sensory deficits that characterize this condition are a major factor in the development of skin ulcerations that contribute to a major source of morbidity in diabetic patients. Despite the frequency of diabetic neuropathy, its pathophysiology remains incompletely characterized, and, as a result, no uniform effective therapy has been developed for preventing or reversing this condition. Among the prevailing hypotheses for the pathophysiology of diabetic neuropathy, attrition of the vasa nervorum recently has gained momentum as a result of clinical and experimental studies. Tesfaye et al (N Engl J Med. 2005;352:341–350) recently reported the strong association between vascular risk factors and the advent of neuropathy in people with diabetes, who also exhibit reduced circulating levels of endothelial progenitor cells, and our laboratory has shown in multiple models that nerve dysfunction in diabetes is preceded by loss of the vasa and that the process can be prevented or reversed by angiogenic factors. In diabetic patients treated with vascular endothelial growth factor gene therapy, improvements in nerve function have been observed. The stenoses have been shown to act as angiogenic factors by multiple potential mechanisms. In the present study, we show that stenoses may reverse or prevent the onset of diabetic neuropathy by preserving the integrity of the vasa nervorum. The data suggest that this effect is mediated in part by a previously unrecognized salutary effect of stenoses on neuronal nitric oxide synthase expression by neural elements. These findings may add to the growing list of indications for statin administration early in the course of diabetes, but of course this recommendation awaits confirmation in clinical trials.
Neuronal Nitric Oxide Synthase Mediates Statin-Induced Restoration of Vasa Nervorum and Reversal of Diabetic Neuropathy
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