Amelioration of Diabetic Neuropathy by TAT-Mediated Enhanced Delivery of Metallothionein and SOD

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Because diabetic neuropathy (DN) appears to result from oxidative stress in neuronal tissues, antioxidant treatment should counteract the condition. Metallothionein (MT) and superoxide dismutase (SOD) are free-radical scavengers, but their ability to cross biological membranes is limited. Applying cell penetrating peptide technologies, we made Tat-MT and Tat-SOD constructs and tested their ability to protect PC12 cells, as surrogates of peripheral nerve cells, from various forms of oxidative damage. Tat-MT and Tat-SOD were successfully delivered to PC12 cells, and the intracellular activities of MT and SOD increased in line with the amount of protein delivered. These agents inhibited cellular damage and apoptotic signaling caused by three different types of injuries (high glucose, hypoxia, and advanced glycation end product injury). We also examined transduction of Tat-MT and Tat-SOD into Otsuka Long-Evans Tokushima fatty rats. A single ip injection of Tat-MT and Tat-SOD resulted in increased radical scavenging activity and decreased apoptosis, by inhibiting nuclear factor \( \text{NF-} \kappa \text{B} \) and MAPK signaling. Continuous treatment resulted in improved myelination of sciatic nerves and delayed the clinical development of DN. We conclude that effective delivery of a combination antioxidant treatment may facilitate the repair of damage in patients with DN. (*Endocrinology 153: 81–91, 2012*)

Diabetic neuropathy (DN) is a serious complication in 60–70% of diabetic patients (both type 2 and type 1) (1). However, the mechanisms responsible for this complication are unclear. Several pathways contribute to the development of DN, including increased activation of the polyol pathway, oxidative stress, advanced glycation end product (AGE) formation, nerve hypoxia/ischemia, activation of protein kinase C, and reduction of nerve growth factor (2–7). As the central integrating mechanism, overproduction of reactive oxygen species (ROS) in peripheral nerves leads to oxidative stress, mitochondrial dysfunction, neuronal damage, and finally apoptosis (1, 3, 4, 8). Oxidative stress results in an increase in substrate for AGE and in precursors of glycoxidation and lipoxidation products and accelerates the free-radical formation that may be accompanied by or caused by a deficiency of antioxidant and detoxification pathways. Oxidative stress occurs in various tissues under diabetic conditions and is involved in the development of diabetic complications (9). However, how increased oxidative stress affecting downstream signaling mechanism may result in the development of DN and whether antioxidant supplementation can help the damaging neurons to regenerate and enable the patients with DN to overcome the devastating life-threatening situation has not been thoroughly studied yet.

Metallothionein (MT) is a low molecular weight intracellular protein that contains 20 cysteine residues. It is an antioxidant that is very efficient in scavenging various free...
radicals but has limited ability to cross lipid bilayers (10). MT has the potential to protect cells and tissues against diabetes and diabetic complications due to its antiapoptotic and antioxidant effects. Superoxide dismutase (SOD), a ubiquitously expressed enzyme, which also has limited membrane permeability, consists of 153 amino acids and protects cellular organelles from oxidative stress by dismuting superoxides produced by oxidative phosphorylation in mitochondria (11). If one were able to circumvent the limitations of membrane permeability and deliver MT and SOD efficiently, these antioxidants might be able to prevent and treat DN. Recently, several small regions of proteins, such as that from the HIV-1 Tat protein, called protein transduction domains, have been developed to allow the delivery of exogenous proteins into living cells (10, 12, 13). Using this protein transduction technology, we found that Tat-MT and Tat-SOD were efficiently transduced into neuronal cells in vitro across the lipid barrier and protected cells against glucotoxicity, AGE toxicity, and ischemia-induced destruction. We also found that injected Tat-MT and Tat-SOD could be delivered to neural tissue in vivo, where they improved the DN in Otsuka Long-Evans Tokushima fatty (OLETF) rats.

Materials and Methods

Cloning, expression, and purification of fusion proteins

To express the basic domain (amino acids 49–57) of HIV-1 Tat as a fusion with MT, we inserted a Tat oligonucleotide and the sequence of human MT-1 into the pRSET bacterial expression vector (Invitrogen, Carlsbad, CA), as described previously (10). Tat-MT was expressed in Escherichia coli BL21 (DE3) pLysS (Novagen, Madison, WI) and purified with a Ni²⁺-nitrilotriacetic acid Sepharose column (QIAGEN, Valencia, CA). The salts in the purified 6-His-tagged protein were removed by PD10 column chromatography (Amersham, Buckinghamshire, UK). We expressed and purified Tat-green fluorescent protein (GFP) and Tat-SOD similarly (14). Each of these expression products contained a sequence encoding six histidine residues and Tat (YGRKKRRQRRR). MT, SOD, and GFP constructs were also generated without Tat. All constructs were verified by DNA sequencing.

Cell culture and cell damage

PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla and was used as a substitute for peripheral neuronal cells. The cells were grown and maintained in DMEM (Invitrogen) containing 100 μg/ml of streptomycin and 100 U/ml of penicillin supplemented with 10% fetal bovine serum. They were maintained in six-well plates in a humidified atmosphere at 37°C with 95%-saturated O₂ and 5% CO₂ and subcultured using 0.25% trypsin/edTA (GIBCO Laboratories, Grand Island, NY) when they were approximately 80% confluent. To transduce the each of the Tat fusion protein into cultured PC12 cells, 1 μM Tat-GFP, MT, or Tat-MT was added to the culture medium for 1 h.

To measure high glucose-, hypoxia-, and AGE-induced cell death, cells were plated in six-well plates in serum-free DMEM and exposed to each type of damage. To examine the ability of Tat-MT and Tat-SOD to protect against high glucose-induced apoptosis, glucotoxicity was induced by exposing the PC12 cells to high glucose (30 mM) DMEM for 10 d together with 1 μM Tat fusion protein. Control cells were exposed to low glucose (5.5 mM) DMEM for 10 d. To induce acute and severely hypoxic condition, the cells were incubated for 24 h at 37 °C in 95% N₂/5% CO₂ in a hypoxia chamber with 1 μM of the Tat fusion protein. Control cells were exposed under normoxia. To examine the capacity of Tat-MT and Tat-SOD to protect against AGE-induced apoptosis, the PC12 cells were exposed to 400 μg/ml of AGE in low glucose with 1 μM of the Tat fusion protein. Control cells were exposed to 400 μg/ml of BSA. The production of AGE has been described previously (15).

Cell viability assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, an index of cell viability and cell growth, is based on the ability of viable cells to reduce MTT from a yellow, water-soluble dye to a dark blue, insoluble formazan product. Cells were seeded in multiwell micro plates at 10,000 cells per well for 24 h and then incubated in the presence or absence of damage-causing conditions together with Tat-MT (and/or Tat-SOD). MTT (0.5 mg/ml) dye was added to each well at 37 °C for 4 h. Supernatants were discarded and dimethylsulfoxide added. After incubating at room temperature for 30 min, absorbance was measured at 570 nm using a micro plate reader (Molecular Devices, Sunnyvale, CA). Cells incubated with culture medium alone were included as control (representing 100% viability) in all experiments.

To assess the cell viability independently, PC12 cells were also grown on multiwell micro plate for trypan blue exclusion. At predetermined times, cells were stained with 0.4% trypan blue and examined microscopically in a hemocytometer to determine the total number of cells and the number of dead cells, i.e., those retaining trypan blue. Four representative high-power fields were selected and counted, and the mean was calculated.

Measurement of ROS using nitroblue tetrazolium (NBT)

Superoxide generation was detected by the NBT assay. NBT (yellow water soluble) is reduced by superoxide to formazan-NBT (dark-blue and water insoluble); 1 mg/ml of NBT (Sigma, St. Louis, MO) was added to each well at 37°C for 30 min. The resulting formazan was dissolved in KOH and dimethylsulfoxide, and the absorbance was immediately measured at 630 nm with a microplate reader (Molecular Devices).

Western blotting

Whole-cell extracts were prepared by incubation in lysis buffer (150 mM NaCl, 50 mM Tris-Cl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 1 mM NaF, 1% Nonidet P-40, 0.25% deoxycholic acid, and 1 μg/ml of leupeptin) for 20 min on ice, followed by a 15-min centrifugation at 1500 rpm. Extracts were mixed with loading buffer and electrophoresed on 8–12% acrylamide gels. Proteins were transferred onto polyvi-
nylidene fluoride membranes in transfer buffer for 1 h at 120 V. After blocking for 1 h in Tris-buffered saline with Tween 20 (TBST) [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween 20] with 5% skim milk, membranes were incubated overnight at 4°C with one of the following antibodies: anti-HIS probe, anti-MT, anticytochrome c, anti-CHOP (GADD 153), antinuclear factor κB (NF-κB) (p65), and anti-α-tubulin from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-Bcl-2, anti-Bax, anti-cleaved caspase-3, anti-phospho-eIF2α, anti-phospho/p44/42 MAPK, anti-phospho/p44/42 MAPK (Thr202/Tyr204), anti-phospho MAPK, anti-phospho-p38 MAPK (Thr180/Tyr182), anti-c-Jun N-terminal kinase (JNK), and antiphospho-JNK (Thr183/Tyr185) from Cell Signaling Technology (Beverly, MA); and ATF6 from Lab-Prontier (Seoul, Korea). After extensive washing in TBST, membranes were incubated with secondary antibody conjugated to horseradish peroxidase diluted 1:20,000 (horseradish peroxidase-linked goat antirabbit IgG; Santa Cruz Biotechnology, Inc.) was applied for 1 h followed by incubation with fluoro-nyl cyanine dyes (Cy2, Cy3, and Cy5; Molecular Probes, Eugene, OR). The nucleotides were detected by an Odyssey imaging system (LI-CORE, Madison, WI) and the ratio of signal intensity was quantified by ImageJ software (19).

Animals

Male OLETF and Long-Evans Tokushima Otsuka (LETO) rats were kindly supplied by the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan). All the rats were kept under light-controlled conditions (12:12 h) and humidity (35±5%) with a 12-h light, 12-h dark cycle. They were housed two per plastic cage and fed standard rat chow. We first evaluated the ability of single injection of each Tat fusion protein to enter into the neurons (sciatic nerve and hippocampus) of OLETF rats at 20 wk of age (4 d after the transduction), after the diagnosis of diabetes mellitus was confirmed. Then, because Tat-MT and Tat-SOD in combination was superior to any antioxidant alone in increasing cell viability in vitro, all diabetic OLETF rats were randomly divided into two groups: the Tat-GFP-treated group and the Tat-MT-Tat-SOD combination-treated group (n = 10 for each group). The former were injected ip every 3 d with 3 mg/kg of Tat-GFP, and the latter with 3 mg/kg of Tat-MT and 3 mg/kg of Tat-SOD. The fusion protein treatment continued for 16 wk. All animals were given 30% sucrose solution as drinking water to accelerate the development of diabetic complications. The clinical characteristics between two groups at baseline and follow-up are shown in Supplemental Table 1, published on The Endocrine Society’s Journals Online website at http://endo.endojournals.org. All animal procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Hanyang University Animal Laboratory.

Measurement of caudal motor nerve conduction velocity (NCV)

NCV was measured in the tail using the ring electrodes (16). The proximal stimulating cathode was placed 2 cm distal to the base of the tail, and the distal cathode was placed 6 cm distal. Compound muscle action potentials were obtained by recording at 10 cm distal (active electrode) and 13 cm distal (indifferent electrode) to the base of the tail. NCV was calculated by dividing the distance between the two stimulating point by the onset latency differences. Measurements were performed in each rat just before (0 wk) and at 16 wk after the beginning of the transduction.

Histological staining

Morphology was assessed 16 wk after the beginning of the treatment with the fusion proteins. Sciatic nerve samples were fixed in 2.5% glutaraldehyde in 0.025 M cacodylate buffer overnight, followed by washing repeatedly with 0.015 M cacodylate and glucose-buffered cacodylate, progressive dehydration with alcohols and propylenoxide, staining with osmium tetroxide, and placing in epon. Semiultra thin (1 μm) sections of sciatic nerves were cut on an ultramicrotome and stained with 0.5% toluidine blue. The proportion of abnormal axons was counted in given areas (frame size, 35 × 35 μm) with a magnification of ×400. Ultrathin sections (70 nm) were observed with a transmission electron microscope, and the proportion of abnormal axon myelin was counted again in the same area at a magnification of ×5000 and ×10,000.

Statistical analysis

Data are presented as means ± SEM. Differences in mean values were tested by Student’s t test using SPSS for Windows software for statistical analysis (version 18.0; SPSS, Chicago, IL). Electrophysiological measurements and morphometric data were analyzed by one-way ANOVA and Tukey’s post hoc test. P values of less than 0.05 were considered statistically significant.

Results

Transduction of Tat-MT and Tat-SOD into PC12 cells

Tat-MT, Tat-SOD, and Tat-GFP fusion protein were expressed in E. coli and purified by Ni-NTA column chro-
matography. To investigate whether Tat-MT and Tat-SOD fusion proteins could be transduced into PC12 cells, we added the fusion proteins to the culture medium for 1 h and examined the intracellular expression of histidine by Western blotting or immunohistochemistry and confocal microscopy. As shown in Fig. 1, Tat-GFP, Tat-MT, and Tat-SOD were successfully transduced into the PC12 cells, whereas control MT was not. The expression of the Tat fusion proteins persisted as long as 4 d after transduction (data not shown). The intracellular delivery of Tat-MT into PC12 cells was confirmed by staining with MT antibody. Fluorescence was detected in both cytoplasm and nucleus.

Protective effects of Tat-MT and Tat-SOD against high glucose-, hypoxia-, and ROS-induced cell death

To determine whether the transduced fusion proteins functioned properly within the cells, we tested their effect on cell viability after injuries induced by high glucose, hypoxia, or direct ROS source (AGE). As shown in Fig. 2, oxidative stress induced by high glucose, hypoxia, or AGE increased mitochondrial cytochrome c release, caspase activation, and the Bax/Bcl-2 (or Bax/Bcl-xL) protein ratio, and all these effects were reduced by Tat-MT and Tat-SOD. As little as 1 μM Tat-MT or Tat-SOD almost completely prevented mitochondria-mediated apoptosis (Fig. 3). Next, we investigated the activation of proapoptotic ER stress markers (CHOP, ATF6, and phospho-eIF2α) by the different types of injury. High glucose, hypoxia, and AGE caused dramatic increases in the expression of CHOP, ATF6, and phospho-eIF2α levels, whereas Tat-MT, Tat-SOD, or Tat-MT and Tat-SOD in combination attenuated these changes (Fig 3). We also found that ROS produced by the different insults activated NF-κB, whereas Tat-MT, Tat-SOD, or Tat-MT and Tat-SOD in combination inhibited this activation (Fig. 3). We further investigated the regulatory effect of Tat-MT and Tat-SOD on the activity of MAPK, such as p38, ERK, and JNK protein kinase. ROS produced by the various damaging conditions increased phosphorylation of p38, ERK, and JNK, and Tat-MT, Tat-SOD, or Tat-MT and Tat-SOD in combination nearly abolished this phosphorylation. Collectively, these results indicate that Tat-MT and Tat-SOD effectively reduce ROS-induced apoptosis in PC12 cells by decreasing mitochondrial-mediated apoptosis and ER stress and downstream signaling in response to the different kinds of injuries.
Effects of *in vivo*-transduced antioxidants on diabetes-induced electrophysiological changes

We examined whether the DN that developed in nearly all the OLETF rats could be ameliorated by continuous treatment with Tat fusion proteins. We first evaluated the ability of the Tat fusion proteins to enter neuronal tissues (sciatic nerve and hippocampus). OLETF rats were injected with 3 mg/kg of Tat fusion proteins with a single ip injection at the age of 20 wk. OLETF tissues dissected at 4 d after injection of Tat-GFP, Tat-SOD, or Tat-MT showed that *in vivo* transduction of Tat fusion proteins into neuronal tissues did occur (Fig. 4, A and B). To investigate the effect of Tat-MT and Tat-SOD in combination on development of DN, we treated rats ip with 3 mg/kg of either Tat-GFP or Tat-MT and Tat-SOD in combination every 3 d for 16 wk from the age of 20 wk. At 0 wk of transduction (baseline), there was no difference in NCV in the three groups (LETO, 40.0 ± 1.6 m/sec; Tat-GFP, 39.4 ± 1.7 m/sec; Tat-MT and Tat-SOD in combination, 39.5 ± 1.5 m/sec) (Fig. 4C). However, after 16 wk of Tat fusion protein transduction, tail NCV decreased less in the antioxidant-treated group than in the Tat-GFP group (*P* < 0.05) (Fig. 4, C and D). These results indicated that Tat-MT and Tat-SOD are able to provide some protection against the development of DN.

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**FIG. 3.** Influence of fusion proteins on mitochondrial apoptosis, NF-κB activation, ER stress, and MAPK activation after various injuries. PC12 cells were transduced with Tat-GFP, Tat-MT, Tat-SOD, or a combination of Tat-MT and Tat-SOD and subsequently transferred to DMEM with high glucose (30 mM) for 10 d, under acute and severe hypoxia for 24 h, or, in low glucose (5.6 mM) and AGE2 (400 μg/ml) (ROS injury) for 24 h. A and D, Cells in the control group were incubated in low glucose DMEM (5.6 mM). Tat-GFP was used as control construct. B and E, To induce hypoxia-mediated injury, the cells were incubated for 24 h at 37°C in 95% N2/5% CO2 in a hypoxia chamber with either Tat-GFP, Tat-MT, Tat-SOD, or combination of Tat-MT and Tat-SOD. Control cells were exposed under normoxia (95% air/5% CO2) condition. C and F, To examine the effect of Tat-MT and Tat-SOD to protect ROS-induced apoptosis, the PC12 cells were exposed to 400 μg/ml of AGE under low glucose with either of Tat-GFP, Tat-MT, Tat-SOD, or Tat-MT and Tat-SOD in combination. Control cells are exposed to 400 μg/ml of BSA. BSA was used as the AGE-negative control. A–C, Expression of mitochondrial apoptosis (Bcl-2, Bax, caspase-3, and cytochrome c release) and NF-κB activation (cytoplasmic and nuclear p65) were studied by Western blot analysis of the cell or subcellular extracts after each injuries. D–F, Protein-matched cell extracts were probed for ER stress markers (CHOP, ATF6, and phospho-eIF2α) using the respective antibodies, and α-tubulin served as loading control. Cell extracts were also prepared and analyzed for MAPK activation by immunoblotting using phospho-specific antibodies against MAPK proteins (p38, ERK, and JNK). Western blottings were quantified by densitometry and are expressed as fold changes relative to control. These data are representative of three experiments performed on different days. LG, Low glucose (control); HG, high glucose; TGFP, Tat-GFP; TMT, Tat-MT; TSOD, Tat-SOD; TMTS, Tat-MT-Tat-SOD combination; Con, control; Hypo, hypoxia. *, P < 0.05; †, P < 0.01; and #, P < 0.001 vs. Tat-GFP group.
stained with toluidine blue. At 16 wk after transduction, toluidine blue-stained axons of the sciatic nerves of the LETO rats appeared normal, whereas many of the axons of the OLETF rats were abnormal, and darkly stained debris was found (Fig. 5A). The myelinated axons of the OLETF rats appeared qualitatively less organized when compared with nerves from the LETO group. Interestingly, the overall pattern of myelination improved much in the Tat-MT and Tat-SOD treatment group, although axons with abnormal myelin structure were still present in some degree compared with the Tat-GFP group. The nerves in the Tat-MT and Tat-SOD group appeared to be similar to the LETO. The myelin sheaths of axons of antioxidant-treated rats had fewer abnormalities than those of the Tat-GFP group (P < 0.05) (Fig. 5C). Immunoblotting of OLETF nerve tissues exposed to Tat-MT and Tat-SOD revealed less evidences of apoptosis, ER stress, and phosphorylation of JNK (Fig. 5D). Taken together, these data help to correlate the changes in physiological phenotypes by Tat-MT and Tat-SOD treatment with structural changes in peripheral nerve.

**Discussion**

A number of reports have proposed increasing antioxidant enzyme expression in neuronal cells to enhance their re-
FIG. 5. Changes of histology in sciatic nerves after treatment of Tat fusion proteins demonstrated by toluidine blue staining, electron microscopy, and Western blotting. A–C, Toluidine blue-stained semithin sections were made from the sciatic nerves of the rats at 16 wk after the start of transduction (with a magnification of ×400). D–I, Ultrathin sections (70 nm) were observed with a transmission electron microscope at a magnification of ×5000 and ×10,000. J, The proportion of abnormal axons with abnormal myelin structure was counted in given areas (frame size, 35 × 35 μm) with a magnification of ×400. Abnormal axons, abnormal degenerative changes in myelin sheath, and axoplasm consist of myelin sheaths such as splitting, engulfment, and disintegration, with or without degeneration of the axoplasm (arrowheads). K, Protein-matched cell extracts from rat sciatic nerves at 16 wk after transduction were probed for Bcl-2, cleaved caspases (caspase-3 and caspase-9), ER stress markers (CHOP, ATF6, and XBP-1), and phospho-specific MAPK (p38, ERK, and JNK) using the respective antibodies, and α-tubulin served as loading control. L–N, Western blottings were quantified by densitometry and are expressed as fold changes relative to control. These data are representative of three experiments performed on different days. TGFP, Tat-GFP; TMTS, Tat-MT-Tat-SOD combination. *, P < 0.05 vs. Tat-GFP group.
sistance to cytotoxic damage caused by ROS, nitric oxide, and cytokines (16–21). Antioxidant therapy indeed has been extensively explored for the prevention and treatment of DN, but the results have been inconsistent because of factors such as difficulty in maintaining consistent circulating antioxidant levels, inadequate tissue distribution, and lack of suitable exogenous antioxidants (16, 17). α-Lipoic acid was the only consistent antioxidant found to improve the clinical DN, but objective evidences of promoting nerve repair and enhancing functional restoration after nerve damages are lacking (18, 19). Therefore, a strategy to induce an endogenous and nonspecific antioxidant with broad spectrum and to deliver one more efficiently could be an attractive option. MT and SOD are good candidate endogenous free-radical scavengers, albeit limited membrane permeability. The potential of the Tat protein as carrier has been studied in different cell types and various tissues (10, 13, 14, 22–24). In spite of the shortcoming of nonspecificity, HIV-1-derived Tat mediates the translocation of diverse proteins in vitro and in vivo, and the biological functions of the transduced proteins are preserved (10, 13, 14, 22–24). Using this protein transduction technology, we found that an in-frame Tat-MT and Tat-SOD fusion proteins were efficiently transduced into neuronal cells across their lipid membranes and protected them from various types of injuries. Without the aid of Tat delivery, MT and SOD were transduced into the PC12 cells inefficiently. Because the PC12 cell line is not a model of DN, we were keen to clarify that our results in this cell line were related to DN. We, therefore, examined the transduction of Tat-MT and Tat-SOD into OLETF rats in vivo. A single ip injection of Tat-MT and Tat-SOD resulted in delivery of these antioxidants to the sciatic nerve and brain and persisted as long as 4 d after the transduction. Increased radical scavenging activity, decreased apoptosis via the mitochondrial and ER pathways, and less damaged myelination were found in the sciatic nerves after continuous, prolonged (for 16 wk), and periodic (at every 3 d) treatment with Tat-MT and Tat-SOD, which delayed development of DN clinically. The Tat-MT and Tat-SOD treatment increased the resistance to cytotoxic damage caused by various types of injury was also accompanied by activation of the MAPK pathway. It is known that JNK signaling is activated by ER stress (27, 28). Nonetheless, Tat-MT and Tat-SOD treatment increased the resistance of PC12 cells and reduced the activation of ER stress and of the MAPK pathway, and reduced apoptosis. The Bcl-2/Bax ratio, an indicator of apoptotic cells, was significantly decreased, and mitochondrial cytochrome c release was also reduced by the antioxidants, possibly due to stabilization of mitochondrial membrane potential. Cleavage of caspase-9 and caspase-3 was also reduced. All these in vitro changes in apoptosis and signaling pathways were supported and confirmed by the in vivo immunoblotting results of sciatic nerve after a 16-wk treatment of Tat-MT and Tat-SOD (Fig. 5D). Tat-MT and Tat-SOD in combination significantly repressed various ROS-mediated injuries and resultant neuronal damage in vivo and in vitro through inhibiting the activation of redundant signaling pathways and their downstream inflammatory mediators.

We also evaluated the protective effect of Tat-MT and Tat-SOD treatment on the clinical development of DN. Development of DN in OLETF rats was obvious from the decrease in NCV, and this is in accord with a report (29), in which a decline in motor NCV was seen in streptozotocin-induced diabetic rats. We observed a 35% decline in
NCV after treating OLETF rats with Tat-GFP for 16 wk, and this was reduced to a 18% decrease by exposure to the Tat-MT-Tat-SOD combination. Sciatic nerve morphology was examined to determine whether the peripheral nerve pathology correlates with the physiological phenotypes by Tat-MT and Tat-SOD treatment. Because the antioxidants enable the axons to improve their myelin structure, as a whole, the sciatic nerves of OLETF rats exposed to the Tat-MT-Tat-SOD combination displayed less pathophysiological abnormalities than those of the control Tat-GFP-treated rats. Exposure to hyperglycemia for 16 wk in our Tat-GFP-treated diabetic OLETF rats made more degeneration of axons and axons with abnormal myelin structure in particular. These changes, in turn, were represented as more decrement in NCV clinically. Myelin is essential for rapid propagation of nerve impulses. In demyelinating injuries, such as diabetes, the conduction velocity markedly slows (30). Because myelination improved, the Tat-MT and Tat-SOD protected against attenuation of NCV under diabetic condition. By the way, remyelination, the formation of the myelin by Schwann cell proliferation, could be another reason for the protective effect of the Tat-MT and Tat-SOD. We found a few evidences of remyelination. Taken together, our results demonstrated improved conduction velocity by TAT-MT and Tat-SOD treatment in diabetic OLETF rats with otherwise inevitable DN, which might indicate neuroprotective effect or remyelinating effect of antioxidant treatment.

In conclusion, our study demonstrates the protective effect of antioxidants on DN using HIV-1 Tat protein, which rendered antioxidants to transduce into neuronal cells and tissue. The fusion proteins protect PC12 cells against high glucose, hypoxia, AGE injury, and delay DN in type 2 diabetic rat model. The clinical phenotype of the OLETF rats correlated with the structural changes in the peripheral nerve. It is our hope that the successful transduction of Tat-MT and Tat-SOD protein demonstrated above will provide a novel means of protecting neuronal cells and counteracting redundant signaling mechanism activation in patients at risk of developing DN.

Acknowledgments

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This work was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science, and Technology (2010-0010898) and the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (A102065).

Disclosure Summary: The authors have nothing to disclose.

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