Elevated Protein Carbonylation, and Misfolding in Sciatic Nerve from \(db/db\) and \(Sod1^{-/-}\) Mice: Plausible Link between Oxidative Stress and Demyelination

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Abstract

Diabetic peripheral polyneuropathy is associated with decrements in motor/sensory neuron myelination, nerve conduction and muscle function; however, the mechanisms of reduced myelination in diabetes are poorly understood. Chronic elevation of oxidative stress may be one of the potential determinants for demyelination as lipids and proteins are important structural constituents of myelin and highly susceptible to oxidation. The goal of the current study was to determine whether there is a link between protein oxidation/misfolding and demyelination. We chose two distinct models to test our hypothesis: 1) the leptin receptor deficient mouse (\(db/db\)) model of diabetic polyneuropathy and 2) superoxide dismutase 1 knockout (\(Sod1^{-/-}\)) mouse model of in vivo oxidative stress. Both experimental models displayed a significant decrement in nerve conduction, increase in tail distal motor latency as well as reduced myelin thickness and fiber/axon diameter. Further biochemical studies demonstrated that oxidative stress is likely to be a potential key player in the demyelination process as both models exhibited significant elevation in protein carbonylation and alterations in protein conformation. Since peripheral myelin protein 22 (PMP22) is a key component of myelin sheath and has been found mutated and aggregated in several peripheral neuropathies, we predicted that an increase in carbonylation and aggregation of PMP22 may be associated with demyelination in \(db/db\) mice. Indeed, PMP22 was found to be carbonylated and aggregated in sciatic nerves of \(db/db\) mice. Sequence-driven hydropathy plot analysis and in vitro oxidation-induced aggregation of purified PMP22 protein supported the premise for oxidation-dependent aggregation of PMP22 in \(db/db\) mice. Collectively, these data strongly suggest for the first time that oxidation-mediated protein misfolding and aggregation of key myelin proteins may be linked to demyelination and reduced nerve conduction in peripheral neuropathies.

Introduction

Diabetic peripheral polyneuropathy is a consequence of elevated blood glucose leading to pain and dysfunction of lower extremities and potentially loss of limb. In humans, the pathologic changes include neuronal/Schwann cell dysfunction, axonal degeneration and chronic motor/sensory neuron demyelination [1]. Reduced nerve conduction velocity (NCV) is also observed in diabetic mice [2] and in human type II diabetics [3]. Considerable evidence exists to suggest that oxidative stress may play a critical role in reduction of sciatic nerve conduction and alteration of sciatic/myelin morphology and function in diabetes [4,5,6]; for example, rodent models of type I and II diabetes show increase in oxidative stress and/or damage, e.g. superoxide production, nitrotyrosine and 4-hydroxy nonenal [7,8,9,10]. However, direct evidence for this hypothesis is still lacking in the literature.

Myelin membrane is produced by Schwann cells and it is rich in cholesterol, lipid and protein [11]. In particular, peripheral myelin membrane has heterogeneous kinds of myelin basic proteins and glycoproteins. Therefore, it is likely that chronic elevation of
oxidative stress will modify and alter the structure of myelin proteins and have negative impacts on myelin structure and nerve function in peripheral neuropathies. Among the various oxidative modifications of proteins, carbonylation is a critical and irrepairable modification that has been shown to be elevated during aging, diabetes and in neurodegeneration [12,13,14,15,16,17,18]. Diabetic subjects are known to have elevated glucose-, and lipoxidation-derived carbonyl stress [18,19,20,21,22,23], however, it remains unknown if carboxylation of sciatic nerve/myelin proteins are elevated in peripheral neuropathies. Therefore, we asked (i) does oxidative stress have an effect on carbonylation and misfolding of sciatic nerve proteins in peripheral neuropathy, and (ii) is the carboxylation of sciatic nerve/myelin proteins associated with loss in nerve conduction and myelination in peripheral neuropathy? We chose two distinct models to address the questions, the well-characterized leptin receptor deficient (dbdb) mouse model of diabetic polyneuropathy [2,24], and mice lacking the superoxide dismutase 1 (Sod1) gene (Sod1<sup>−/−</sup>) as a model of in vivo oxidative stress [25,26,27,28]. Sod1 is an important cytosolic antioxidant enzyme that detoxifies harmful superoxide radicals. Previous studies from our group have shown that Sod1<sup>−/−</sup> mice exhibit significant increase in in vivo oxidative stress and deficits in neuromuscular function with age [25,26,27,28]. However, it remains unknown if the chronic elevation of oxidative stress in Sod1<sup>−/−</sup> mice results in alterations in myelin structure and reduced nerve conduction and whether these phenotypes are linked to increase in sciatic nerve protein carbonylation and misfolding.

It is well established that oxidative modification of proteins and their subsequent misfolding can lead to their aggregation, which may have an important role in the pathology of several diseases including neurodegenerative diseases [29,30,31]. With respect to the peripheral nervous system, it has been shown that peripheral myelinating protein 22 (PMP22), a key myelin protein, undergoes mutation and aggregation in multiple mouse models of human neuropathies [32,33,34,35]. In fact, aggregation of PMP22 has been proposed to be causal for the demyelination phenotype in the J trembler mouse model of human peripheral neuropathy [32,35]. However, it remains unknown if the decrements in nerve conduction and myelination in diabetic peripheral neuropathy is linked to carboxylation and aggregation of PMP22.

In this study, we report that reduced nerve conduction and altered myelin morphology in dbdb and Sod1<sup>−/−</sup> mice are associated with increases in carboxylation and misfolding of sciatic nerve proteins. We further show that PMP22 undergoes carboxylation and aggregation in sciatic nerves of dbdb mice, a finding that is supported by our in vitro oxidation assay with tert-butyl hydroperoxide (tBHP) and purified PMP22. Our results reveal for the first time that sciatic nerve/myelin proteins are potential targets for carboxylation and aggregation, which may have important implications for loss in myelin integrity and nerve conduction in peripheral neuropathies.
Results

Alterations in nerve conduction and myelin structure in dbdb and Sod1−/− mice

Dbdb mice have been previously shown to have reduced sciatic NCV and increased tail distal motor latency (tdml) [2]. In the current study, we wanted to determine whether oxidative stress is associated with reduced sciatic NCV and increased tdml. We observed a 1.32±0.04 (p<0.001) fold reduction in sciatic NCV by 4 months of age in dbdb mice (Figure 1A). 6-mo-old Sod1−/− mice also demonstrated a 1.36±0.07 (p<0.01) fold reduction in sciatic NCV (Figure 1B). The dbdb mice and Sod1−/− mice exhibited a 1.41±0.04 fold (p<0.001) and 1.12±0.02 fold (p<0.05) increase in tdml, respectively (Figure 1C&D).

Since demyelination is closely linked to reduced nerve conduction [2] and oxidative stress is associated with reduced sciatic NCV and increased tdml as shown in Figure 1A-D, we measured axon diameter, nerve fiber diameter and myelin thickness in 5-mo-old dbm and dbdb mice, and 6 and 20 mo-old Sod1−/− mice in toluidine blue stained sciatic nerve thick sections. Dbdb mice exhibited reduced axon, nerve fiber diameter and reduced myelin thickness when compared to comparable sized axons from sections obtained from the sciatic notch in dbm mice (Figure 2 A&B, marked by asterisks). Moreover, a significant reduction in axon diameter/area (p<0.05), fiber diameter/area (p<0.001), myelin thickness/area (p<0.001) and increase in G-ratio (axon diameter/fiber diameter) (p<0.001, Figure 2C) was also observed in these sections. Reductions in axon diameter, fiber diameter and myelin thickness in dbdb mice are consistent with previous studies [2]. In comparison, the Sod1−/− mice exhibited reduced axon and fiber diameters compared to age-matched wild-type mice at 6 months of age (Figure 2D&E), which was confirmed by quantification of axon diameter/area (p<0.001) and fiber diameter/area (p<0.001). The myelin area was reduced but the results did not reach statistical significance (p=0.16) (Figure 2F). However, 20 month Sod1−/− mice showed reductions in myelin thickness/area and fiber diameter/area (Figure 2G&H). Quantification of nerve morphology demonstrated a significant reduction in fiber diameter/area (p<0.001), and myelin thickness/area (p<0.001) with concomitant increase in G-ratio (p<0.001), by 20 months of age (Figure 2). These results strongly suggest that oxidative stress is closely associated with alterations in peripheral nerve myelin morphology and function.

Protein carbonylation and misfolding in dbdb and Sod1−/− mice

Our findings on impaired nerve conduction and reduced myelin thickness in dbdb and Sod1−/− mice strongly suggest that oxidative stress might be the common contributor for these changes. In fact, other studies have indicated that oxidative stress might be the causative factor for nerve fiber abnormalities in diabetes [4,5]. We have previously shown that oxidative stress has major impact to the structure and function of proteins and enzymes [36]. Therefore, we investigated if protein oxidation and misfolding are selectively elevated in both the experimental models. Protein carbonylation is one of the common oxidative modifications detected and studied in aging [14,37,38,39] and in various pathophysiological conditions including diabetes [40]. In this study, we asked if protein carbonylation has any negative impact to structure of proteins in sciatic nerves of dbdb and Sod1−/− mice. We measured protein carbonylation and exposure of hydrophobic domain of sciatic nerve proteins utilizing two distinct fluorescence-based technologies developed by our group [14,41]. We found a significant increase in the overall level of protein carbonyls in the cytosolic fraction of both dbdb mice (1.7±0.2 fold, p<0.01, Figure 5A) and Sod1−/− mice (1.5±0.1 fold, p<0.05, Figure 3B). This intriguing observation led us to examine if the protein carbonylation level is also elevated in the detergent-soluble fraction as it has been often found that protein oxidation, including protein carbonylation induces protein aggregation [42,43,44]. Therefore, we measured protein carbonylation in the detergent-soluble protein fraction isolated from the sciatic nerve of dbdb and Sod1−/− mice. As we predicted, protein carbonyls were elevated 1.30±0.10 fold in dbdb mice (p<0.05, Figure 5C) and 1.24±0.08 fold (p<0.05, Figure 3D) in Sod1−/− mice compared to their respective controls.

Since both cytosolic and detergent-soluble fractions exhibited elevation in protein carbonylation, we next asked whether the global state of protein conformation is affected in these experimental models by measuring protein hydrophobicity using a BisANS photolabeling approach. There was a 1.8±0.1 (p<0.001) fold increase in global exposure of hydrophobic pockets in sciatic nerves of dbdb mice (Figure 4A) and a 1.26±0.03 fold increase (p<0.05, Figure 4B) in Sod1−/− mice.

PMP22 is carbonylated and aggregated in dbdb sciatic nerves

Because we found a global increase in cytosolic and detergent-soluble sciatic nerve protein carbons in dbdb mice and increase in global exposure of hydrophobic pockets, we next investigated if peripheral myelin proteins undergo carbonylation and aggregation in diabetic neuropathy. We chose PMP22 to test our hypothesis as it is one of the abundant proteins (2–5%) in peripheral myelin and have previously shown that oxidative stress might be the common contributor for these changes. In fact, other studies have indicated that oxidative stress might be the causative factor for nerve fiber abnormalities in diabetes [4,5]. We have previously shown that oxidative stress has major impact to the structure and function of proteins and enzymes [36]. Therefore, we investigated if protein oxidation and misfolding are selectively elevated in both the experimental models. Protein carbonylation is one of the common oxidative modifications detected and studied in aging [14,37,38,39] and in various pathophysiological conditions including diabetes [40]. In this study, we asked if protein carbonylation has any negative impact to structure of proteins in sciatic nerves of dbdb and Sod1−/− mice. We measured protein carbonylation and exposure of hydrophobic domain of sciatic nerve proteins utilizing two distinct fluorescence-based technologies developed by our group [14,41]. We found a significant increase in the overall level of protein carbonyls in the cytosolic fraction of both dbdb mice (1.7±0.2 fold, p<0.01, Figure 5A) and Sod1−/− mice (1.5±0.1 fold, p<0.05, Figure 3B). This intriguing observation led us to examine if the protein carbonylation level is also elevated in the detergent-soluble fraction as it has been often found that protein oxidation, including protein carbonylation induces protein aggregation [42,43,44]. Therefore, we measured protein carbonylation in the detergent-soluble protein fraction isolated from the sciatic nerve of dbdb and Sod1−/− mice. As we predicted, protein carbonyls were elevated 1.30±0.10 fold in dbdb mice (p<0.05, Figure 5C) and 1.24±0.08 fold (p<0.05, Figure 3D) in Sod1−/− mice compared to their respective controls.

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Discussion

In humans, the diabetic etiology includes neuronal dysfunction, axonal degeneration, Schwann cell dysfunction, and chronic motor/sensory neuron demyelination with increasing disease severity [1]. It has been reported that Schwann cell death [45], neuronal cell death as observed in streptozotocin-induced diabetes [46], and damage to motor/sensory neurons [46] are associated with diabetic neuropathy and reduced nerve function. All of these changes are likely contributors to loss of muscle mass, limb pain and dysfunction of the lower extremities in dbdb mouse model of peripheral neuropathy. Chronic oxidative stress is believed to be one of the leading underlying mechanisms of reduced myelination in diabetic neuropathy. Indeed, recent studies have shown that reduction in oxidative stress may improve sciatic nerve/myelin morphology and function in diabetic neuropathy [4,5]. It has also been well documented that oxidative stress plays a key role in initiating misfolding and aggregation of proteins and enzymes [36,41,42,43,44,47]. However, it is unknown whether imbalanced structural states of proteins (protein oxidation, misfolding and aggregation) are linked to reduced nerve conduction and myelin thickness in peripheral neuropathies, in part, due to the lack of sensitive technologies to measure these changes. In this study, we report for the first time that sciatic nerve proteins of dbdb and Sod1−/− mice undergo oxidation (protein carbonylation) and alteration in protein conformation which may be closely linked to decrement in nerve conduction and altered myelin morphology.

The novelty of this study is the use of two mouse models to determine the role of oxidative stress in peripheral neuropathy (i) the well-characterized dbdb mouse model of diabetic peripheral neuropathy that is known to be associated with oxidative stress/damage [2,24] and (ii) the Sod1−/− mouse model of in vivo oxidative stress which exhibits age-associated deficits in neuro-muscular function [23,26,27,28]. We found significant changes in protein carbonylation and misfolding in diabetic mice which correlated well with reductions in sciatic NCV and myelin thickness in dbdb and Sod1−/− mice. These findings suggest that oxidative stress plays a key role in the pathogenesis of diabetic neuropathy and may provide a potential target for therapeutic intervention.

### Figure 2

Reductions in myelin and neuron structure in dbdb and Sod1−/− mice. Thick sections of sciatic nerves were sectioned and visualized at 100x (A) non-diabetic dbm mouse and (B) dbdb mice. (C) Quantification of axon diameter/area, nerve fiber diameter/area, myelin area/thickness and g ratio (axon diameter/fiber diameter) were tabulated from thick sections of dbdb and control mice (n = 3). Thick sections of (D) 6-mo-old control and (E) 6-mo-old Sod1−/− mice are shown. (F) Quantification was performed similarly to that shown in 2C. Thick sections of (G) 20-mo-old control and (H) 20-mo-old Sod1−/− mice are presented. (I) Quantification was performed similarly to that in 2C and results expressed as mean ± SEM of n = 3 mice/group. Results were analyzed by two-tailed t-test (*p<0.05 by two-tailed t-test).

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Figure 3. Increase in sciatic nerve protein carbonyls in dbdb and Sod1−/− mice. Total sciatic nerve cytosolic protein bound carbonyls in (A) dbdb and (B) Sod1−/− mice are presented. Total protein bound detergent-soluble protein carbonyls in (C) dbdb and (D) Sod1−/− mice are shown. Results are expressed as mean ± SEM (n = 6; *p < 0.05 by two-tailed t-test).

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Figure 4. Increase in hydrophobic domain of protein exposure in dbdb and Sod1−/− mice. Total BisANS labeling of sciatic nerve homogenates for (A) dbdb and (B) Sod1−/− mice are presented. Results are expressed as mean ± SEM (n = 6; *p < 0.05 by two-tailed t-test).

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thickness and was in agreement with previous studies in dbdb mice [2] and human type II diabetes [3]. Sod1<sup>−/−</sup> mice exhibited all of the physiological changes observed in dbdb mice (with the exception of myelin thickness) at 6 months of age and biochemical changes (elevation of protein carbonylation and alteration in protein surface hydrophobicity), however, the changes at this age were not as robust as that observed in 5-mo-old dbdb mice. It is interesting to note that Sod1<sup>−/−</sup> mice also exhibited a reduction in sciatic NCV and a modest increase in tdmI, which was also less robust than that observed in dbdb mice. There was clearly an age-related effect in Sod1<sup>−/−</sup> mice as myelin thickness was significantly reduced by 20 months of age which affirms that oxidative stress is indeed one of the critical determinants for demyelination in peripheral neuropathies. We postulate that the subtle effects on myelin thickness observed in Sod1<sup>−/−</sup> mice compared to dbdb mice at 6 months of age may be due to a lesser degree of oxidative damage and misfolding in these mice. As both sugar aldehydes (arabinose, glyoxal, methyl glyoxal, glycoaldehyde, and 3-deox-
Results are expressed as mean ± SEM (n = 6; *: p < 0.05 by two-tailed t-test).

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Figure 6. In vitro assessment of oxidative stress-induced aggregation of PMP22 protein. Purified PMP22 protein was subjected to in vitro oxidation with increasing concentrations of tert-butyl hydroperoxide. SDS-PAGE was performed on oxidized PMP22 protein from both the soluble and detergent-soluble protein fractions. Results are expressed as mean ± SEM (n = 6; *: p < 0.05 by two-tailed t-test).

Phosphatase and protease cocktail was purchased from Thermoscientific, Rockford, IL. PMP22 polyclonal antibody was purchased from Abcam (ab61220, Abcam, Cambridge, MA). Purified PMP22 was purchased from Novus Biologicals (H00005376, Littleton, CO). BisANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonyl acid, dipotassium salt) and FTC (Fluorescein-5-thiosemicarbazide) were purchased from Invitrogen (Grand Island, NY).

Materials and Methods

Materials

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Ethics Statement

This study was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health’s recommendations. All procedures were approved and performed in accordance with the Committee on the Ethics of Animal Experiments (Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio (UTHSCSA)) under protocol 10003-34-01-A and 00080z as well as the Audie L. Murphy Veterans Hospital in San Antonio using protocol 0503-002. All experiments were performed to minimize pain and discomfort.

Animals

a) Diabetic mice homozygous for leptin receptor mutant (LepR<sup>b<sup>+/−</sup></sub>) Dock7m-J (dbdb)) and their heterozygous controls (LepR<sup>b<sup>+/+</sup></sub>) Dock7m-J (dbm)) in a C57BL/KS-J background were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) (Protocol, 10003-34-01-A). All mice were fed an ad libitum normal chow diet. Experiments were performed in 5-month-old dbm and dbdb mice that exhibit significant deficits in sciatic nerve conduction velocity, increases in latencies and alterations in myelin morphology [2]. b) Sod1<sup>−/−</sup> mice (on a C57BL/6 background) were used as a model of in vivo oxidative stress as described previously (protocol, 00080z and 0503-062) [23,50]. 6-month-old Sod1<sup>−/−</sup> and their wild-type (WT) littermates were used for the biochemical studies. 6-month- and 20-month- Sod1<sup>−/−</sup> and their WT littermates were used for morphological assessments.
Nerve Conduction Velocity and latency

Mice were anesthetized with isoflurane and maintained at 34°C with a heating lamp. All experiments were performed with a Nicolet Viking Quest portable EMG apparatus (CareFusion, San Diego, CA, USA) as described previously [51]. Subdermal needle electrodes were cleaned with 70% alcohol between animals. Supramaximal stimulation was delivered with 0.2 millisecond electrical impulses for all experiments. Electrodes were inserted 3 cm apart and the latency of the tail distal motor action potential was measured by proximal to distal stimulation. Sciatic NCV was measured by stimulating proximal ankle electrodes with current and the latency for response at the dorsal digits divided by the distance traveled was measured. Then the stimulating electrodes were placed at the sciatic notch and the latency to the ankle was measured, subtracted from the initial foot ankle latency and divided by the notch to the ankle to obtain values for sciatic NCV.

Thick sections and imaging

A 1-2 cm segment of sciatic nerve at the sciatic notch for all sectioning was fixed in 4% paraformaldehyde (PFA) and was switched to buffer containing PBS with 4% PFA and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide and finally in 1% uranyl acetate. Sections were cut at 1–2 μm in thickness and then stained with the following solution of toluidine blue (1 g of toluidine blue, 1 g of borax and 100 mL of water). Using a 0.2 μl filtered syringe filled with prepared toluidine dye 1 drop was applied to thick sections. Slides were placed at 180 degrees on a hot plate for 10 seconds. Samples were washed with water and allowed to dry and then covered with cover slips. Sectioning of sciatic nerves was performed by the UTHSCSA electron microscopy core (San Antonio, TX) and visualized using Nikon Eclipse TE2000-U fluorescence microscope (Nikon Inc.) at 40- and 100X magnification. Axon and fiber diameters/areas were quantified utilizing Roper Scientific software and analyzed as described earlier [2,52]. The approximate circumference was quantified to determine the area and diameter of both axons and axon plus myelin (fiber). Myelin diameter and area was obtained from the subtraction of axon diameter and area from fiber diameter and area (Fiber-axon = myelin). The myelin thickness was determined by dividing the myelin diameter by 2. G-ratios were determined as the axon/ fiber diameter [2,52]. Greater than 200 nerve fibers were measured for each individual animal and as described earlier [2,52].

Measurement of protein carbonyls

Sciatic nerve protein extracts were made by sonication in 20 mM potassium phosphate buffer, pH 6.0 with 0.5 mM MgCl₂, and 1 mM EDTA as previously described [14]. Homogenates were centrifuged at 100,000x g for 1 hour to obtain the cytosolic fraction. Pellets obtained after centrifugation were resuspended by sonication in P3 buffer (2% SDS, 0.5% NP40, 0.5% deoxycholate, pH 6.0) and centrifuged at 100,000x g for 20 minutes to obtain the detergent soluble fraction. Both the fractions were labeled with FTC to measure global level of protein carbonyls in cytosol and detergent soluble fraction as previously described [14]. Samples were loaded onto 4–15% gels and visualized utilizing the Typhoon 9400 (Amersham, Piscataway, NJ, USA) with excitation at 532 nm and emission with a 526 SP emission filter. Total carbonylated proteins were analyzed against the abundance of the protein with Sypro Ruby staining [14] and quantified using Un-Scan-it software (Silk Scientific, Orem, Utah, USA).

Measurement of protein surface hydrophobicity

Sciatic nerves were homogenized in 50 mM tris buffer, pH 7.4, followed by photo-labeling the protein surface hydrophobic domain with BisANS (0.1 mM) under UV light-exposure as previously described [36,41]. Thereafter, equal amounts of BisANS-labeled proteins were loaded onto SDS-PAGE and visualized on an Alpha Innotech FluorChem HD2 camera utilizing UV transillumination. The level of incorporation of BisANS was measured as described in protein carbonyls and normalized to Coomassie protein stain [36,41].

Determination of hydrophobicity based on primary sequence

Primary sequence for PMP22 was obtained from known sequences on Pubmed protein search for mouse and analyzed for hydrophobicity utilizing Kyte-Doolittle hydrophathy plots as described previously [53].

Immunoprecipitation

FTC-labeled cytosolic and detergent soluble protein fractions were incubated with PMP22 polyclonal antibody (Abcam, Cambridge, MA, Prod# ab61220) in KEI buffer overnight at 4°C. After overnight incubation, 25 μL of protein A bead (Pierce, Prod# 20366) was added and incubated with rotation for 2 hours at 4°C. Samples were then centrifuged at 16000x g for 1 minute. Pellet was washed three times with 500 μL of KEI buffer plus 0.5 M NaCl and then two times with 500 μL of 50 mM Tris. The pellet was dried and 4x loading buffer and 4 mM dithiothreitol were added to the beads. Total carbonyls and protein were measured by SDS-PAGE and quantified as described in the carbonyl assay section.

Measurement of PMP22 aggregates

Sciatic nerves were homogenized in phosphate buffer, pH 6.0, as described in protein carboxyl measurement section and centrifuged at 100,000x for 1 hour. Resultant pellets were resuspended by sonication in P3 buffer (2% SDS, 0.5% NP40, 0.5% deoxycholate, pH 6.0) and centrifuged for 20 minutes at 100,000x g to obtain the detergent soluble fraction. One tenth of a μg of protein was used to quantify the total increase in PMP22 in the detergent soluble fraction by western blot using the PMP22 polyclonal antibody. Blots were visualized and scanned on a Typhoon 9400 followed by TMB colorimetric assay (Vector Laboratories, Burlingham, CA) and a Alpha Innotech FluorChem HD2 camera was used to capture the image. Western blot image for the high molecular weight aggregates (75 Kd–250 kd) were quantified.

In vitro oxidation of PMP22

Purified PMP22 was incubated with varying concentrations of tBHP (0, 50, and 100μM) at 37°C for 2 hr. The soluble and pellet fractions of PMP22 were obtained with centrifugation at 100,000x g. The pellet was resuspended in P3 buffer to obtain the detergent-soluble fraction. Soluble and detergent-soluble fractions of PMP22 were run on SDS-PAGE followed by Coomassie stain to quantify PMP22 protein loading. The ratio of soluble to detergent-soluble fraction of PMP22 was quantified.

Statistical analysis

Results are expressed as mean ± SEM. Significant differences were established utilizing student’s t-test and by analysis of the variance with tukey posthoc analysis (ANOVA) utilizing Graph Pad Prism software (La Jolla, CA).
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References

1. Thomas PK (1997) Clinical features and investigation of diabetic somatic peripheral neuropathy. Clin Neuropath 4: 341–345.
2. Wang L, Chopra M, Nealad A, Liu Z, Bole M, et al. (2011) Phosphodiesterase-5 is a therapeutic target for peripheral neuropathy in diabetic mice. Neuroscience 193: 399–410.
3. Padua L, Stalberg E, Calandrino P, Mascogtii G, Pazzaglia C, et al. (2011) Single-fiber conduction velocity test allows earlier detection of abnormalities in diabetes. Muscle Nerve 43: 652–656.
4. Obronska IG, Pacher P, Szabo C, Zengyeder Z, Hirooka H, et al. (2003) Aldose reductase inhibition counteracts oxidative-nitrosative stress and poly(ADP-ribose) polymerase activation in tissue sites for diabetes complications. Diabetes 54: 234–242.
5. Gurpinar T, Ekberci N, Harazdin NU, Barat T, Tarakci F, et al. (2011) Statin treatment reduces oxidative stress-associated apoptosis of sciatic nerve in diabetic mellitus. Biochimie 86: 373–378.
6. Vincent AM, Callaghan BC, Smith AL, Feldman EL (2011) Diabetic neuropathy: cellular mechanisms as therapeutic targets. Nat Rev Neurol 7: 573–583.
7. Vareniki I, Pavlov IA, Drel VR, Lysozhubov VV, Ilnytska O, et al. (2007) Nitrative stress and peripheral diabetic neuropathy in leptin-deficient (ob/ob) mice. Exp Neurol 205: 425–436.
8. Obronska IG, Ilnytska O, Lysozhubov VV, Pavlov IA, Mashitrik N, et al. (2007) High-fat diet induced neuropathy of pre-diabetes and obesity: effects of "healthy" diet and aldose reductase inhibition. Diabetes 56: 2598–2608.
9. Ilnytska O, Lysozhubov VV, Stevyn J, Drel VR, Mashitrik N, et al. (2006) Poly(ADP-ribose) polymerase inhibition alleviates experimental diabetic sensory neuropathy. Diabetes 55: 1696–1698.
10. Coppeny LJ, Davidson EP, Rinehart TW, Gellett JS, Oltman CL, et al. (2006) Detection of protein carbonyls in aging liver tissue: A fluorescence-based proteomic approach. Mech Ageing Dev 127: 849–861.
11. Garbay B, Heape AM, Sargueil F, Cassagne C (2000) Myelin synthesis in the peripheral nervous system. Prog Neurobiol 61: 267–304.
12. Shaw PJ, Ircse PG, Fautko G, Manle D (1995) Oxidative damage to protein in sporadic motor neuron disease spinal cord. Ann Neurol 38: 691–695.
13. Reed TT, Pierce WM, Markelsbury WR, Butterfield DA (2005) Proteinic identification of HNE-bound proteins in early Alzheimer disease: Insights into the role of lipid peroxidation in the progression of AD. Brain Res 1274: 66–76.
14. Chaudhuri AR, de Waal EM, Pierce A, Van Remmen H, Ward WF, et al. (2006) Detection of protein carbonyls in aging liver tissue: A fluorescence-based proteomic approach. Mech Ageing Dev 127: 849–861.
15. Garbay B, Heape AM, Sargueil F, Cassagne C (2000) Myelin synthesis in the peripheral nervous system. Prog Neurobiol 61: 267–304.
16. Butterfield DA, Periahgi M, Sultana R (2006) Oxidative stress in Alzheimer's disease brain contribute to neuronal death. Neurobiol Aging 23: 635–646.
17. Miyata T, Kurokawa T, Van Ypersele De Stroo C (2000) Advanced glycation and lipoxidation end products: role of reactive carbfonyl compounds generated during carbohydrase and lipid metabolism. J Am Soc Nephrol 11: 1744–1752.
18. Pillon NJ, Groz M, Veiga RE, Soulieres L, Lagarde M, et al. (2012) The lipid peroxidation by-product 4-hydroxy-2-nonenal (4-HNE) induces insulin resistance in skeletal muscle through both carbfonyl and oxidative stress. Endocrinology 153: 2099–2111.
19. Wei Q, Ren X, Jiang Y, Jin H, Liu N, et al. (2013) Advanced glycation end products accelerate rat vascular calcification through RAGE/oxidative stress. BMC Cardiovasc Disc 13: 13.
20. Vouyan P, Brown KL, Cheryakin S, Hudson B (2013) Site-specific AGE modifications in the extracellular matrix: a role for glyoxal in protein damage in diabetes. Clin Chem Lab Med 1: 7–7.
21. Grigbby J, Betts B, Vidiot-Kochan E, Cullert R, Tsin A (2012) A possible role of acriderin in diabetic retinopathy: involvement of a VEGF/TGFbeta signaling pathway of the retinal pigment epithilum in hyperglycemia. Curr Eye Res 37: 1045–1053.
22. Shirasaki N, Rosenbluth J (1991) Structural abnormalities in freeze-fractured sciatic nerve fibers. J Neurocytol 20: 373–384.
23. Muller FL, Song W, Liu Y, Chaudhuri A, Pieke-Dahl S, et al. (2006) Absence of CuZn superoxide dismutase leads to elevated oxidative stress and acceleration of age-dependent skeletal muscle atrophy. Free Radic Biol Med 40: 1993-2004.
24. Shirasaki N, Rosenbluth J (1991) Structural abnormalities in freeze-fractured sciatic nerve fibers. J Neurocytol 20: 373–384.
49. Yamada K, Sato J, Oku H, Katakai R (2003) Conformation of the transmembrane domains in peripheral myelin protein 22. Part 1. Solution-phase synthesis and circular dichroism study of protected 17-residue partial peptides in the first putative transmembrane domain. J Pept Res 62: 78–87.

50. Elchuri S, Oberley TD, Qi W, Eisenstein RS, Jackson Roberts L, et al. (2005) CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. Oncogene 24: 367–380.

51. Oh SS, Hayes JM, Sims-Robinson C, Sullivan KA, Feldman EL (2010) The effects of anesthesia on measures of nerve conduction velocity in male C57Bl6/J mice. Neurosci Lett 483: 127–131.

52. Sherman DL, Kroh M, Wu LM, Grove M, Nave KA, et al. (2012) Arrest of myelination and reduced axon growth when Schwann cells lack mTOR. J Neurosci 32: 1817–1825.

53. Juretic D, Zucic D, Lucic B, Trinajstic N (1998) Preference functions for prediction of membrane-buried helices in integral membrane proteins. Comput Chem 22: 279–294.