Prophylactic and Antinociceptive Effects of Coenzyme Q10 on Diabetic Neuropathic Pain in a Mouse Model of Type 1 Diabetes

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ABSTRACT

Background: Oxidative stress is a key factor implicated in the development of diabetic neuropathy. This study evaluates the prophylactic and antinociceptive effects of the antioxidant coenzyme Q10 (CoQ10) on diabetes-induced neuropathic pain in a diabetic mouse model.

Methods: Total 56 mice with type 1 diabetes induced by streptozotocin were used, 20 normal mice were used as control. Mechanical and thermal nociceptive behavioral assays were applied to evaluate diabetic neuropathic pain. Tissue lipid peroxidation, immunohistochemistry, reverse transcription, and polymerase chain reaction were used to evaluate the molecular mechanisms of CoQ10. Data are presented as mean ± SEM.

Results: CoQ10 administration was associated with reduced loss of body weight compared with nontreated diabetic mice, without affecting blood glucose levels. Low dose and long-term administration of CoQ10 prevented the development of neuropathic pain. Treatment with CoQ10 produced a significant dose-dependent inhibition of mechanical allodynia and thermal hyperalgesia in diabetic mice. Dorsal root ganglia, sciatic nerve, and spinal cord tissues from diabetic mice demonstrated increased lipid peroxidation that was reduced by CoQ10 treatment. CoQ10 administration was also noted to reduce the proinflammatory factors in the peripheral and central nervous system.

Conclusions: The results of this study support the hypothesis that hyperglycemia induced neuronal oxidative damage and reactive inflammation may be pathogenic in diabetic neuropathic pain. CoQ10 may be protective by inhibiting oxidative stress and reducing inflammation by down-regulating proinflammatory factors. These results suggest that CoQ10 administration may represent a low-risk, high-reward strategy for preventing or treating diabetic neuropathy.

What We Already Know about This Topic

- Diabetic neuropathic pain continues to be a problem for many patients
- Coenzyme Q10 has been studied in trials of diabetic neuropathy patients; reduction in neuropathy symptoms and improved conduction velocity have been shown

What This Article Tells Us That Is New

- Coenzyme Q10 treatment reduced the development allodynia and oxidative stress, indicating similarities to the human and murine models. Future studies could advance our understanding of antioxidative treatments that prevent the development of diabetic neuropathic pain

NEUROPATHIC pain is common in diabetic patients but its pathogenesis is not well understood. A large number of agents are currently used to manage diabetic neuropathic pain,[1,2] although only two, pregabalin[3] and duloxetine,[4] are specifically approved for this indication. These drugs, as well as others, have shown treatment efficacy in diabetic neuropathic pain but only treat the symptoms and not the cause and have significant side effects.[5,6] Thus, none of the current treatments for diabetic neuropathic pain prevent or arrest the development of diabetic neuropathy.

Brownlee[7] hypothesized that hyperglycemia-induced overproduction of superoxide activates the pathways of cell injury. One of these pathways has been demonstrated in the dorsal root ganglion (DRG), where superoxide induced–oxidative stress is severe and has been reported to lead to

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oxidative injury of DRG neurons.[8] Mitochondria, "cellular power plants," appear to be the specific target of oxidative damage, leading to impaired mitochondrial function manifested clinically as sensory neuropathy.[8,9]

Evidence supports the concept that inflammatory responses often accompany neuropathic pain development.[10] The proinflammatory cascade is complex, involving many mediators and modulators. Nuclear factor (NF)-κB is a major player in regulating the inflammatory response and nociceptor sensitivity after peripheral nerve injury.[11] Activated NF-κB translocates to the nucleus, initiating transcription of mediators involved in the pathogenesis of neuropathic pain.[12–14] Chemokines such as monocyte chemoattractant protein 1 (CCL2) and C-X-C motif chemokine 10 (CXCL10) also appear to be important modulators of pain *via* neuronal-glial interactions after peripheral or central nervous system injury.[13–15]

Coenzyme Q10 (CoQ10) is an endogenously synthesized compound that acts as an electron carrier in the mitochondrial respiratory chain.[16,17] In addition to its unique role in mitochondria, CoQ10 functions as a potent antioxidant, scavenging free radicals and inhibiting lipid peroxidation.[18,19] CoQ10 exists in a completely oxidized form and a completely reduced form, enabling it to perform its functions in the electron transport chain and as an antioxidant. CoQ10 has been tested in clinical trials and preclinical studies and was found beneficial in several medical conditions that have relevance for diabetes, including diabetes itself,[20] high blood pressure,[20,21] congestive heart failure, and endothelial dysfunction.[22] CoQ10 has been tested in animal models of diabetic neuropathy and was found to be effective in restoring and improving nerve conduction velocity in diabetes-induced peripheral nerve injury.[23] However, there appears to be no current study evaluating the potential therapeutic effect of CoQ10 on diabetes-induced neuropathic pain.

The objective of the current study is to investigate the effects of: (1) hyperglycemia-induced oxidative stress with the expression of NF-κB, CCL2, and CXCL10 in the nervous system of a type 1 diabetes mellitus (DM) mouse model; (2) CoQ10 treatment on attenuating oxidative stress, ameliorating neuropathic pain, and preventing diabetic neuropathic pain progression.

Materials and Methods

Animal Preparation

All experiments were approved by the Animal Care and Use Committee of the University of Miami (Miami, FL). Mature 15-week-old adult male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in groups of five in plastic cages with soft bedding and free access to food and water under a 12h/12h light–dark cycle (dark cycle: 7:00 PM–7:00 AM). All animals were acclimated in their cages for 1 week before experiments were performed. DM was induced by a single intraperitoneal injection of streptozotocin 200 mg/kg (Sigma, St. Louis, MO). The onset of DM, after the streptozotocin injection, was confirmed by blood glucose levels greater than 300 mg/dl. Blood samples were all taken from the tail vein. Animal body weights were monitored weekly during the experimental period. For this study only mice that developed DM (90% of streptozotocin injected mice) were used as diabetic mice.

Diabetic Neuropathic Pain Measurement

Neuropathic pain was confirmed through mechanical allodynia and thermal hyperalgesia. The mechanical and thermal tests were performed as previously described.[14]

Mechanical allodynia test: Briefly, the mechanical allodynia test was conducted with a Touch-Test Sensory Evaluator (von Frey filaments, North Coast Medical, Inc., Wood Dale, IL). Filaments were used to assess the degree of allodynia. For each assessment, the mouse was placed on a wire-mesh platform and was covered with a transparent glass container; a period of 30 min was allowed for habituation. Five measurements were taken for each animal randomly starting at the left or right paw. The observation of a positive response (paw lifting, "shaking," or licking) within 5 s of the application of the filament was then followed by the application of a thinner filament (or a thicker one if the response was negative). The paw withdrawal threshold was measured five times and expressed as tolerance level in grams.

Thermal hyperalgesia test: An Ugo Basile Plantar Test apparatus (Biological Research Apparatus, Comerio, Italy) was used to measure the paw withdrawal latency time in unrestrained mice. To determine paw withdrawal latency to radiant heat, the mouse was placed in an acrylic restrainer on a thermal stimulator. The hind paws made contact with a ¼"-thick glass plate that was maintained at room temperature. A light source that produced radiant heat was focused below the glass onto the plantar surface of one hind paw. The paw withdrawal latency was determined for the left and right hind paws, with a 5-min intertrial interval. A cutoff of 20 s was used. Four trials were performed in each animal to establish the mean paw withdrawal latency ± SEM.

CoQ10 Treatment

CoQ10 (Sigma-Aldrich) was dissolved in olive oil (Sigma-Aldrich) at three different concentrations: (1) 15 mg/ml for the dose of 50 mg/kg/day; (2) 30 mg/ml for the dose of 100 mg/kg; and (3) 45 mg/ml for the dose of 150 mg/kg. These doses represent the equivalent human doses of 4 mg/kg, 8 mg/kg, and 12 mg/kg, respectively, based on body surface area.[24] The CoQ10 solution was prewarmed to 37°C and then injected intraperitoneally once daily at a volume of 100 μl/30 g of body weight.

Prophylactic Treatment Protocol

To investigate the prophylactic effect of CoQ10 on the development of diabetic neuropathic pain, three groups of mice were used: G1—normal mice (n = 6), administered vehicle only; G2—diabetic mice (n = 10) administered vehicle

only; G3—diabetic mice (n = 10) administered 50 mg/kg of CoQ10. All treatments were started the day after the confirmation of DM and given daily for 24 days followed by testing of mechanical sensitivity.

Neuropathic Pain Treatment Protocol

To investigate the antinociceptive effects of CoQ10 on diabetic neuropathic pain, four groups of mice were used: G4—normal mice (n = 8), administered 150 mg/kg of CoQ10; G5—DM mice (n = 12), administered vehicle only; G6— DM mice (n = 12), administered 100 mg/kg of CoQ10; and G7— DM mice (n = 12), administered 150 mg/kg of CoQ10. All mice in G5, G6, and G7 had demonstrated DM for 2 weeks and had confirmed neuropathic pain behavior before CoQ10 administration.

Lipid Peroxidation Measurement

Thiobarbituric acid reactive substances measurement was used to evaluate lipid peroxidation associated with the onset of diabetes and the antioxidative effects of CoQ10 in serum, DRG, sciatic nerve, and spinal cord. The basic principle of thiobarbituric acid reactive substances assay is that malonaldehyde, a secondary decomposition product of polyunsaturated fatty acids with three or more double bonds, reacts with thiobarbituric acid to form a stable pink chromophore with maximal absorbance at 532 nm. For this assay, tissues were immediately cooled in ice and homogenized in 1.15% KCl diluted 1:5 (w/v) containing 1 mM phenyl methyl sulphonyl fluoride. The homogenates were centrifuged at 1500*g* for 20 min. Samples of supernatant fluid obtained were frozen at −70°C for further measurements. In the assay, trichloroacetic acid (10%) was added to the homogenate. This mixture was then centrifuged (5 min, 1000*g*). The sample was extracted and thiobarbituric acid (0.67%) was added to the reaction medium. The samples and standards were placed in a water bath (100°C, 15 min). Malonaldehyde, an intermediate product of lipoperoxidation, was determined by the absorbance at 535 nm and the results are reported as μM/mg protein.

Immunohistochemistry and Image Quantification

Normal mice, mice with diabetic pain, and diabetic mice treated with CoQ10 were sacrificed *via* an overdose of nembutal and were then decapitated. L4-L5 DRG were removed, fixed in 4% paraformaldehyde in phosphate buffered saline (pH 7.4) overnight, cryoprotected in 0.1 M phosphate buffered saline containing 20% sucrose and sectioned by cryostat into 15-μm thick sections. Sections were incubated overnight at 4°C with the primary antibodies: anti-4-Hydroxy-2-nonenal (HNE; Abcam, Cambridge, MA), anti-p65 (Millipore, Billerica, MA), anti-CCL2 (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA), or anti-CXCL10 (BioVision, Mountain View, CA), followed by secondary species-specific fluorescent antibodies (Jackson ImmunoResearch Lab, Inc. West Grove, PA) or biotinylated secondary antibody (Vector Lab, Burlingame, CA) for 1 hr at 22°C. To ensure the specificity of each primary antibody, there was one section in each set of stains where the primary antibody was replaced by the diluent of the antibody so as to exclude nonspecific background staining. In the anti-HNE staining, preabsorption control was performed by preincubating with a 10:1 dilution of HNE and an anti-HNE-antibody to ensure that the antibody was binding specifically to HNE in the tissue. For image quantification for the DRG sections, two sections were randomly selected from each sample and images were taken with a high-resolution digital camera and analyzed with NIH Image J software (developed by the U.S. National Institutes of Health, Bethesda, MD). Tissue sections from four to six animals per experimental condition were analyzed. The proportion of positive cells of p65 (the activated marker of NF-κB), CCL2, or CXCL10 was determined by positive neuron profiles divided by the total neuronal profiles on each section of DRG. The researcher conducting the image analysis was blinded to the group from which the tissue was taken, to avoid any possible observation bias.

Protein Extraction and Enzyme-linked Immunosorbent Assay

For the CXCL10 and CCL2 enzyme-linked immunosorbent assay (ELISA), spinal cord tissues were homogenized on ice in sterile phosphate buffered saline (pH 7.5) with a protease inhibitor cocktail (Sigma-Aldrich) and centrifuged at 11,000 rpm for 30 min at 4°C. Protein content of the supernatant was quantified using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). The CXCL10 and CCL2 ELISA were performed using a CXCL10 ELISA kit (mouse CXCL10 Immunoassay, R&D Systems, Minneapolis, MN) or a CCL2 ELISA kit (CCL2 [MCP-1] ELISA Ready-SET, eBioscience, San Diego, CA).

RNA Isolation and Reverse Transcription and Polymerase Chain Reaction of TLR4, CCL2, and CXCL10 mRNA of the Spinal Cord

The mRNA level of CCL2 or CXCL10 in the spinal cord tissue was evaluated by reverse transcription and polymerase chain reaction (RT-PCR). Extraction of total RNA was carried out with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA (0.5 μg) was reverse-transcribed with 200 U/sample SuperScript II (Invitrogen) and 250 ng/reaction of random primers (Promega, Madison, WI). Real-time PCR was performed using the TAQurate green real-time PCR master mix (Epicentre Biotechnologies, Madison, WI) with specific primers following the manufacturer's instructions, and data were collected on the Rotor-Gene 2000 (Corbett Research, Mortlake, Australia). The measurements revealed the mRNA activity for each of these factors in control and experimental mice tissue.

Statistical Analysis

Data are presented as mean ± SEM and analyzed using Prism 4 software (GraphPad Software Inc., San Diego CA). The

Table 1. Effects of CoQ10 on Glucose and Body Weight over the 24-Day Post DM

Group Type	Body Weight (g)	Blood Glucose Level (mg/dl)
G1(n = 8): normal + vehicle	29.00 ± 0.42*	113 ± 7.43†
G2 (n = 10): DM + CoQ10 (50 mg/kg)	26.60 ± 0.0.55‡	310 ± 25.33
G3 (n = 10): DM + vehicle	22.00 ± 0.54	323 ± 40.09

The body weights of normal mice treated with vehicle were significantly greater than DM mice (G1 *vs.* G2 or G3, * $P < 0.01$). CoQ10-treated mice had significantly higher body weights than vehicle-treated DM mice (G2 *vs.* G3, ‡ $P < 0.01$). Final blood glucose concentrations were comparably increased in both groups of diabetic mice. CoQ10 did not affect blood glucose concentrations (G1 *vs.* G2 and G3 † $P < 0.001$, G2 *vs.* G3 $P > 0.05$).

CoQ10 = coenzyme Q10; DM = diabetes mellitus.

data for mechanical allodynia test were analyzed with two-way analysis of variance with two-repeated factors followed by Tukey multiple comparison test. Unpaired comparisons were assessed by two-tailed Student *t* test for thermal hyperalgesia test, lipid peroxidation level analysis, image analysis, or RT-PCR analysis. On the basis of our previous studies, we set up a sample size of 8–12 for behavioral testing and 6 for ELISA, image analysis or RT-PCR. *P* values of less than 0.05 were considered statistically significant and are presented along with the data.

Results

Effects of CoQ10 on Animal Body Weights and Blood Glucose

When compared with normal mice, the weight of DM mice decreased over the 24-day experimental period. CoQ10 treatment, however, increased the body weight of type 1 diabetic mice toward that of the normal control group and was greater when compared with diabetic mice receiving vehicle only. CoQ10 did not have any effects on blood glucose levels between the two groups of DM mice (table 1). All data were collected after 24 days of CoQ10 treatment. Administration of CoQ10 in normal control mice did not show any effects on body weight or blood glucose level.

Prophylactic Effects of CoQ10 on Diabetic Neuropathic Pain Development

Three groups of mice were used in this portion of the study: G1—normal mice as naïve controls (n = 8) vehicle administrated; G2—DM mice (n = 10) vehicle administrated; and G3—DM mice (n = 10) administrated 50 mg/kg/day of CoQ10 for 24 days starting on the second day of DM. Mechanical thresholds were measured at regular 4-day intervals after DM was confirmed (fig. 1). Diabetic mice treated with vehicle developed significant mechanical allodynia after 12 days of DM (G1 *vs.* G2, $P < 0.001$). However, in the DM mice treated with CoQ10 the onset of neuropathic pain was significantly delayed and its severity was decreased compared with that in DM mice treated with vehicle only. The mechanical thresholds of DM mice treated with CoQ10 were significantly higher than those treated with vehicle only (G2 *vs.* G3, $P < 0.05$ or $P < 0.01$ on corresponding time points).

Two-way ANOVA showed a significant interaction between group and time ($P < 0.0001$), and significant main effect for group or time (both $P < 0.0001$).

The Antinociceptive Effects of CoQ10 Treatment on Diabetic Neuropathic Pain

To examine the antinociceptive effects of CoQ10 on diabetic neuropathic pain, mice were injected intraperitoneally with CoQ10 or vehicle once daily at 24-hr intervals for 3 days. Four groups of mice were used in the mechanical test: G4—normal mice treated with 150 mg/kg of CoQ10 (n = 8); G5—mice after 21 days of DM with neuropathic pain treated with vehicle (n = 12); G6— mice after 21 days of DM with neuropathic pain treated with 100 mg/kg of CoQ10 (n = 12); and G7— mice after 21 days of DM with neuropathic pain treated with 150 mg/kg of CoQ10 (n = 12).

Administration of CoQ10 or vehicle was continued for 3 days at 24-hr intervals and was then terminated. Threshold measures were taken every 24 hr before the daily dose

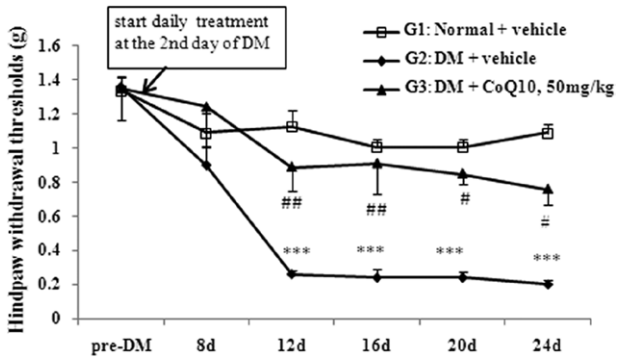

Fig. 1. Prophylactic effect of CoQ10 on the development of diabetic mechanical allodynia. The data represent mean mechanical thresholds as assessed by hind paw withdrawal thresholds (g) using a von Frey test. The X-axis represents the timeline of experiment. DM mice showed a significant decrease in hind paw withdrawal thresholds (G1 *vs.* G2: ***$P <$ 0.001). The onset of mechanical allodynia was significantly delayed and the severity decreased in the DM mice that received CoQ10 (50 mg/kg/day, 24 days) *vs.* DM mice received vehicle (G3 *vs.* G2: ##$P < 0.01$ or #$P < 0.05$; two-way ANOVA followed by Tukey multiple comparisons). CoQ10 = coenzyme Q10; DM = diabetes mellitus.

administration of CoQ10 or vehicle and then every 24 hr for the next 3 days when no agent was administered to the mice.

The mechanical threshold was found to be significantly lower in DM mice as compared with non-DM control mice. The results of the administration of CoQ10 to DM mice suggested a dose-dependent, statistically significant increase in mechanical thresholds ($P < 0.01$ or $P < 0.001$ for both doses of 100 mg/kg and 150 mg/kg of CoQ10 *vs.* DM mice treated by vehicle alone); however, CoQ10 did not alter mechanical nociception in normal mice (fig. 2A). The effect of CoQ10 was transient and disappeared in 3 days after the administration period had ended and all the thresholds returned to preadministration levels.

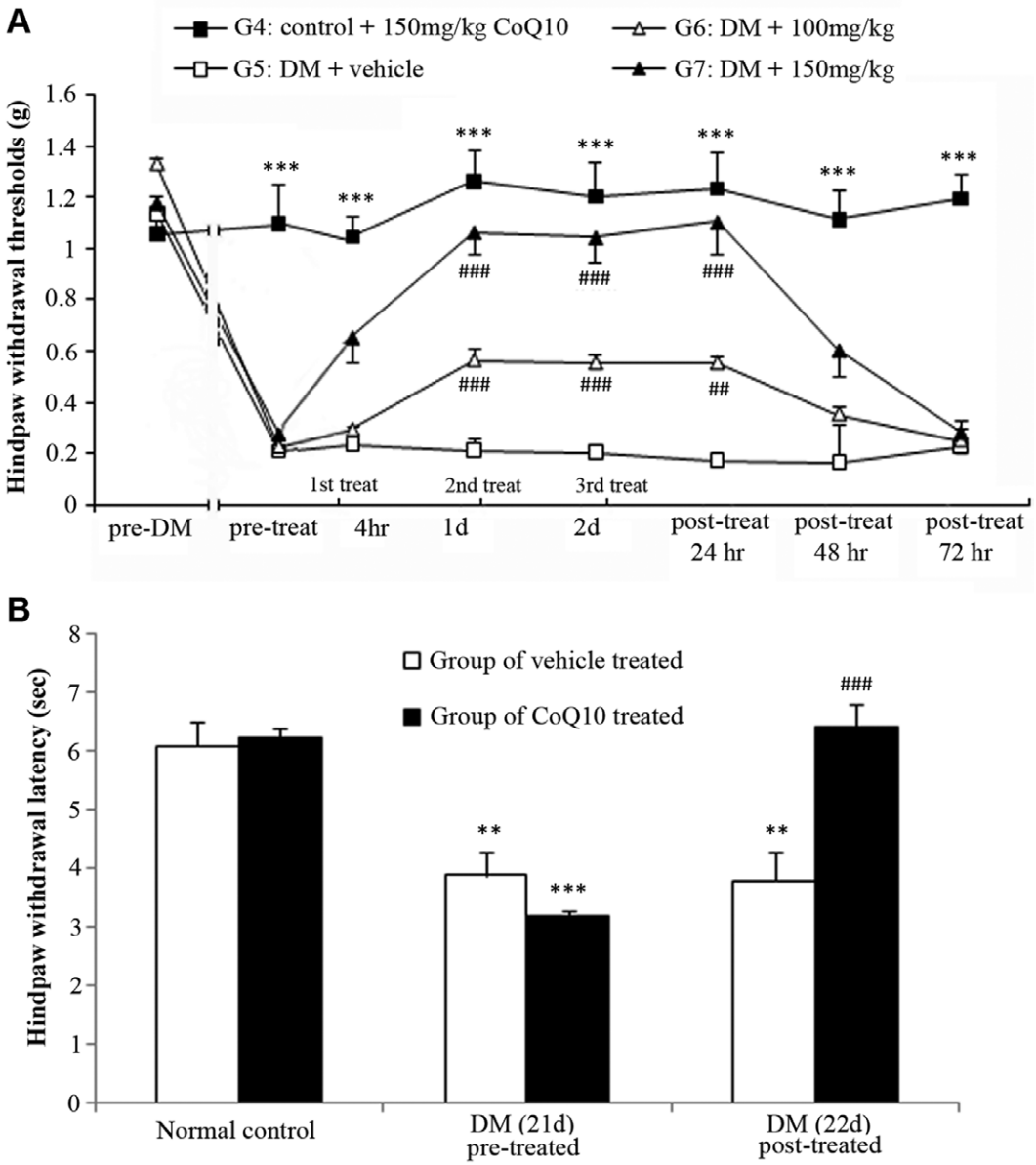

Fig. 2. (*A*) The antinociceptive effects of CoQ10 on diabetes-induced mechanical allodynia. The data represent mean mechanical thresholds as assessed by hind paw withdrawal thresholds (g) using a von Frey test after three consecutive daily treatments with vehicle or CoQ10. Normal control mice (G4) were treated with 150 mg/kg of CoQ10, which showed no change in withdrawal thresholds. DM mice treated with vehicle (G5) showed a significant reduction of withdrawal thresholds which is consistent with increased neuropathic pain behavior (G4 *vs.* G5: ***$P < 0.001$). DM mice treated with 100 mg/kg (G6) or 150 mg/kg (G7) showed a significant improvement in mechanical neuropathic pain compared with DM mice treated with vehicle (G6 or G7 *vs.* G5: ##$P < 0.01$, ###$P < 0.001$; two-way ANOVA followed by Tukey multiple comparisons). Data are shown and the mean ± SE. (*B*) The effect of CoQ10 on thermal hyperalgesia. The data indicate that 21 days post-DM, mice developed thermal hyperalgesia. CoQ10 (150 mg/kg) treatment for 24 hr completely reversed thermal hypersensitivity. DM (21 days) *vs.* normal control: ** $P = 0.0085$ or *** $P < 0.0001$. DM (21 days) treated with CoQ10 *vs.* DM (21 days) treated with vehicle or pretreated: ### $P = 0.0003$ (Student *t* test). Data are shown and the mean ± SE. CoQ10 = coenzyme Q10; DM = diabetes mellitus.

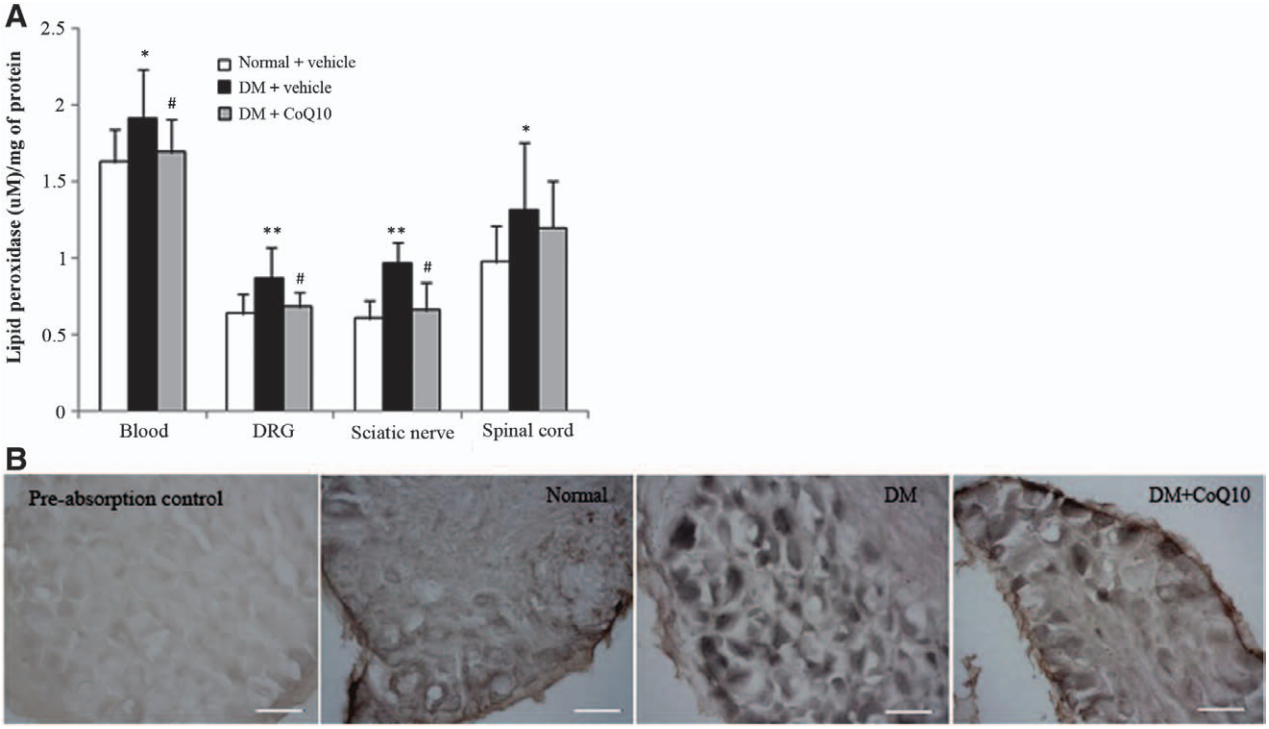

Fig. 3. (*A*) The antioxidative (lipid peroxidation) effect of CoQ10 on tissue in DM mice. The Y-axis indicates the lipid peroxidation level in the thiobarbituric acid reactive substances assay (mean ± SEM). The data demonstrate that CoQ10 (150 mg/kg, administered for 3 days) decreases lipid peroxidation in serum, DRG, and sciatic nerves from DM mice. Normal + vehicle (n = 6) *vs.* DM + vehicle (n = 6): $^{**}P < 0.01$ ($P = 0.0022$ for DRG, $P = 0.0013$ for sciatic nerve), $^{*}P < 0.05$ ($P = 0.0390$ for blood, $P = 0.0281$ for spinal cord). DM + vehicle *vs.* DM + CoQ10 (n = 6): $\#P < 0.05$ ($P = 0.016$ for DRG, $P = 0.0214$ for sciatic nerve). (Student *t* test). (*B*) DRG images of anti-HNE. The images indicate that the staining intensity of HNE, representing the level of HNE production, it is weakest in the DRG of the normal mouse, strongest in the DRG of the DM mouse, and weaker in the DRG of the DM mouse treated with CoQ10. Image of the preabsorption control demonstrates the dim background staining when the primary antibody was preabsorbed by HNE solution. Bar = 100 μm. CoQ10 = coenzyme Q10; DM = diabetes mellitus; DRG = dorsal root ganglia; HNE = 4-Hydroxy-2-nonenal.

Two-way ANOVA showed a significant interaction between group and time ($P < 0.0001$), and significant main effect for group and time (both $P < 0.0001$).

Thermal hyperalgesia was tested on the normal control mice and the mice with mechanical allodynia after 21 days of DM. DM mice developed thermal hyperalgesia after having hyperglycemia for 21 days. The thermal hyperalgesia behavior returned to baseline level in 24 hr (fig. 2B) after a single treatment of CoQ10 (150 mg/kg).

The Antioxidative (Lipid Peroxidation) Effects of CoQ10 on Blood Serum, Dorsal Root Ganglia, Sciatic Nerve, and Spinal Cord Tissues

To investigate the antioxidant activity of CoQ10 in diabetic mice, we measured lipid peroxidation in serum and lysates from dorsal root ganglia, sciatic nerves, and spinal cord by measuring the lipid peroxidation with the thiobarbituric acid reactive substances assay. Hyperglycemia was noted to induce an increase in lipid peroxide levels. Mice treated with CoQ10 (3 days 150 mg/kg, administered daily) had a decreased production of lipid peroxide (fig. 3A). This suggests that treatment with CoQ10 is capable of blocking or producing an antioxidant response in these tissues, especially the dorsal root ganglia and sciatic nerve in mice with DM, the major site of sensation transduction in the peripheral nerve system.

HNE is a major product of endogenous lipid peroxidation. Tissues such as neural tissue with polyunsaturated fatty acids may produce HNE as a result of free radical attack. We therefore, used antibodies to HNE to further visualize the HNE and CoQ10 antioxidative effect in the tissues of the DRG. In figure 3B, the image of "preabsorption control" confirmed the specificity of the antibody by showing the dim background staining when anti-HNE was preabsorbed. Images of normal mice DRG, DM mice DRG, and DM mice treated with CoQ10 DRG show the visible differences in HNE staining for the different conditions. This reveals that the signal intensity of HNE in the DRG of the DM mouse is increased compared with that in the DRG of a normal mouse or DM mouse-treated with CoQ10.

The Expression of Proinflammatory Factors in the DRG

To investigate the potential molecular mechanisms underlying the improved functional outcomes associated with CoQ10 treatment in diabetic mice, we evaluated the expression of NF-κB, along with the chemokines CCL2 and CXCL10; all are known to be up-regulated after

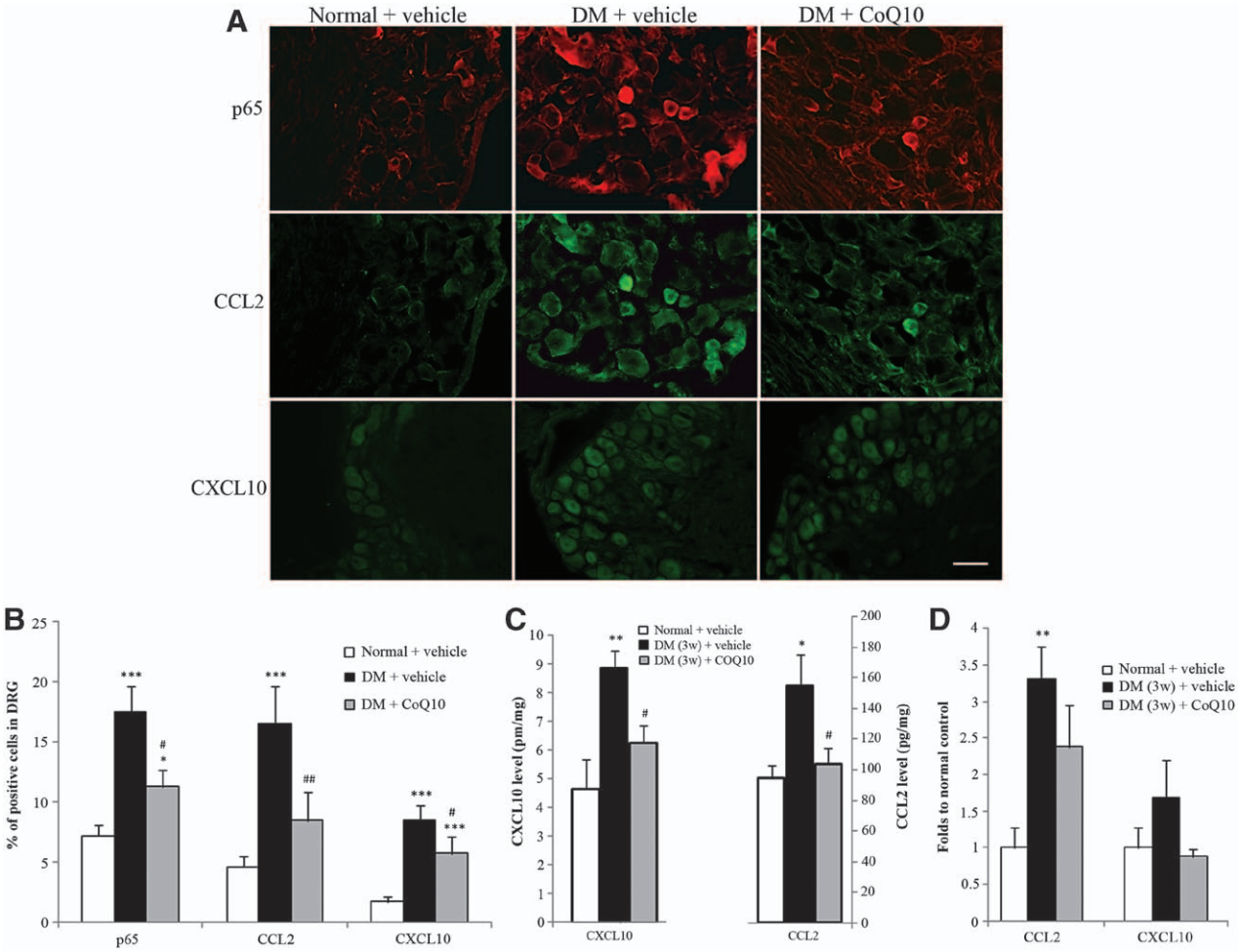

Fig. 4. (*A*) Immunohistological staining of p65 (the activated marker of nuclear factor-κB), CCL2 and CXCL10 in the DRGs. DRG images from normal mice, mice 23 days after the onset of DM treated with vehicle, and mice 23 days after the onset of DM treated with CoQ10 are shown in three separate columns. The number of immunopositive cells of p65, CCL2, and CXCL10 were all increased in DM mice, but decreased after CoQ10 treatment (150 mg/kg, administered for 3 days). Scale bar = 100 μm. (*B*) Positive cells quantification of p65, CCL2, and CXCL10 in DRG. Results are expressed as percent ± SEM of total neurons. *** $P \leq 0.0001$ or * $P = 0.0194$ compared with corresponding normal mice that received only vehicle. ## $P = 0.0073$ or # $P = 0.0210$ for p65, $P = 0.0268$ for CXCL10 compared with corresponding DM mice with neuropathic pain that received vehicle (Student *t* test). (*C*) Protein level of CCL2 and CXCL10 in spinal cord by enzyme-linked immunosorbent assay. Six mice samples per group were analyzed with the results showing: * $P = 0.0122$ or ** $P = 0.0061$ compared with normal control, # $P = 0.0231$ for CXCL10, $P = 0.0374$ for CCL2 compared between DM + CoQ10 (150 mg/kg, after 24 hr of treatment) and DM + vehicle (Student *t* test). (*D*) Gene expression of CCL2 and CXCL10 in spinal cord. For each gene, results are expressed as folds of normal control ± SEM after normalization to β-actin. Six animal samples per group were analyzed, ** $P = 0.0061$ compared with normal control (Student *t* test). DM + CoQ10 (150 mg/kg, after 24 hr of treatment) is considered the group representing CoQ10 treatment. CCL2 = monocyte chemoattractant protein 1; CoQ10 = coenzyme Q10; CXCL10 = C-X-C motif chemokine 10; DM = diabetes mellitus; DRG = dorsal root ganglia.

peripheral nerve injury and are associated with neuropathic pain behavior. In the normal control mice DRG, levels of p65—the activated marker of NF-κB, CCL2 and CXCL10—were found to be low. In contrast, in the DRG of DM mice with neuropathic pain that were administered vehicle only, all three proinflammatory factors increased. These factors, which were elevated in the DM mice, were reduced by CoQ10 treatment (150 mg/kg, administered for 3 days), but still remained higher compared with normal mice treated with only vehicle. Figure 4A shows the visible difference in the DRGs of three types of samples using immunohistochemistry staining of p65, CCL2, and CXCL10. Figure 4B shows the quantification of immunostained neurons in DRG.

The protein levels of CCL2 and CXCL10 in the lumbar spinal cord samples were measured by ELISA, and figure 4C demonstrates that levels of CCL2 and CXCL10 are increased in the spinal cord of DM mice; however, CoQ10 treatment decreased the protein levels of CCL2 and CXCL10.

The mRNA levels of CCL2 or CXCL10 in the spinal cord were analyzed using real-time RT-PCR. Figure 4D shows that tissue from DM mice have higher mRNA levels of CCL2

and CXCL10 than tissues from normal or DM mice administered CoQ10 (150 mg/kg, administered for 3 days).

Both ELISA and real-time RT-PCR results demonstrates that hyperglycemia not only induces lipid peroxidation but also increases the expression of CCL2 and CXCL10 in the nervous tissue of spinal cord. Furthermore, CoQ10 administration attenuated the increase in these proinflammatory factors.

Discussion

Oxidative stress results from cells or tissues failing to detoxify free radicals produced during metabolic activity. Diabetes is characterized by chronic hyperglycemia, which produces dysregulation of cellular metabolism. Hyperglycemia directly stimulates activation of the polyol pathway, mitochondria, protein kinase C, and nicotinamide adenine dinucleotide phosphate oxidase all resulting in the production of free radicals, a molecular signature of oxidative stress.[25,26] Neural tissue is especially vulnerable to oxidative attack due to its relatively low antioxidant capacity, high consumption of oxygen, and high content of polyunsaturated fatty acids.[27] It is generally believed that oxidative stress is the key pathological process inducing nerve damage in diabetes.[9,25,26,28,29] In addition, mitochondrial DNA is particularly sensitive to oxidative damage, resulting in impaired energy regulation, which is critically important in high-energy–requiring neurons.[30,31] Therefore, a higher than normal level of lipid peroxidation may be considered a potential sign of neural injury.

A molecular consequence of neural injury is the up-regulation of proinflammatory cytokines and chemokines, such as NF-κB, CCL2, and CXCL10, which play major roles in regulating reactive inflammation to nerve injury and nociceptive sensory responses.[13,14,32]

Our research investigated the possibility that oxidative damage, accumulating in neural tissues, initiates a reactive neuroinflammatory cascade characterized by increased production of proinflammatory cytokines and chemokines. Our study suggests that in the mouse model of type 1 diabetes, CoQ10 administration has both an antinociceptive and a prophylactic effect on diabetic neuropathic pain. This conclusion is based on the results demonstrating a CoQ10 dose-dependent decrease in mechanical hyperalgesia after diabetic neuropathic pain has developed, along with a delayed onset of neuropathic pain and attenuated severity of pain when CoQ10 is administrated prophylactically in chronic low doses. Treatment with CoQ10 also decreased thermal hyperalgesia induced by hyperglycemia. In the lipid peroxidation studies, our results biochemically and immunohistochemically demonstrated that hyperglycemia induced oxidative stress by significantly increasing lipid peroxide in serum, sciatic nerve, and spinal cord (especially in the dorsal root ganglia) where nociceptor cell bodies are located and nociception is regulated. CoQ10 also produced an antioxidant response and protected neural tissue from oxidative stress.

The current study clearly suggests a vicious pathway whereby hyperglycemia induces membrane lipid peroxidation and overproduction of proinflammatory factors in neural tissue resulting in neuropathic pain. It is becoming clear that neurons express a wide variety of cytokine or chemokine receptors,[15,33] which implies a direct effect of cytokines and chemokines on neurons. Thus, cytokines and chemokines may play a variety of roles in the nervous and immune systems. Our gene expression data demonstrated that hyperglycemia results in increased mRNA levels of CCL2 and CXCL10 in the spinal cord. The immunohistological and ELISA results corroborate these findings showing an increased protein level for CCL2 and CXCL10 in DRG and spinal cord. Importantly, our results demonstrate that the overproduction of the proinflammatory factors NF-κB, CCL2, and CXCL10 is also significantly negated by administration of CoQ10. CoQ10 as a potent antioxidant appears to play a key role in preventing neural tissue damage from oxidative stress and by decreasing the expression of proinflammatory factors attenuates neuropathic pain behavior.

Several studies have addressed the effects of CoQ10 on the complications of diabetes. CoQ10 therapy has been shown to prevent progressive hearing loss and improve blood lactate levels after exercise in patients with maternally inherited DM and deafness.[34] CoQ10 supplementation has also been shown to improve endothelial dysfunction in type 2 diabetic patients, possibly by altering local vascular oxidative stress.[35] Pilot studies have also shown that CoQ10 decreases neurological symptoms in patients with Parkinson and Huntington diseases,[36,37] and when considered with the antinociceptive and preventive effects of CoQ10 on diabetes-induced neuropathic pain, suggests that CoQ10 may play an important role in neuroprotection against diabetic neuropathy and potentially other neurodegenerative disorders. The definitive impact of CoQ10 will need to be tested in diabetic patients with neuropathy and/or at high risk of developing neuropathy.

Striving for superior antioxidative therapies remains essential for the prevention of neuropathy in diabetic patients. The current study clearly demonstrates the potential benefits of CoQ10 as a potent antioxidant and its ability to relieve neuropathic pain in the type 1 diabetic mouse model. In the current study, the doses of CoQ10 used in mice are the equivalent human doses of 4 mg/kg, 8 mg/kg, and 12 mg/kg, respectively, based on body surface area.[24] Published reports concerning safety studies indicate that CoQ10 has low toxicity and does not induce serious adverse effects in humans.[38] The acceptable daily intake (12 mg/kg/day) is calculated from the no-observed-adverse-effect level of 1200 mg/kg/day derived from a 52-week chronic toxicity study in rats, *i.e.*, 720 mg/day for a person weighing 60 kg.[39] The dose range of 30–100 mg/kg of CoQ10 has been trialed in animal models evaluating the CoQ10 effect on antiinflammation, wound healing, and ameliorating cardiovascular risk in metabolic syndrome.[40–42] Our current results would appear to support CoQ10 as a

potentially ideal agent for use in humans. As previously mentioned, pregabalin[3] and duloxetine[4] are specifically approved for painful diabetic neuropathy, and although effective at reducing pain, they only affect the symptoms and do not treat the root cause of the pain. Additionally, both drugs also have well-reported side effects.[5,6] Moreover, the inability to correct the underlying cause of the pain may lead to a progression of diabetic neuropathy and is associated with a worsening of sensory function.[6] Ideally, therapy should be directed at preventing or arresting the progressive loss of nerve function and improving symptoms with minimal side effects. CoQ10 administration may represent a low-risk, high-reward strategy for preventing or treating painful diabetic neuropathy with minimal side effects while targeting the underlying pathology. Although the beneficial effects of CoQ10 on diabetic neuropathic pain in this study is limited to type 1 diabetic animals, similar results from our lab have also been demonstrated in type 2 diabetes animal models (data not shown).

A limitation of this study is the short duration of CoQ10 administration. It is unclear whether a significant benefit will remain with long-term use. Another limitation of our study is the lack of direct evidence of mitochondrial dysfunction and the beneficial effects of CoQ10 on mitochondria preservation. CoQ10 neuroprotection may also be attributed to functional improvement of respiratory chain activity and prevention of neuronal apoptosis. The relevance of these outcomes at the molecular and organelle level needs to be explored further in future studies.

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