The Involvement of Calcitonin Gene-Related Peptide and Toll-Like Receptor 4 in the Development of Diabetic Sensory Neuropathy
Yan-Ping Zhang, Jin-Feng Yang, Yue Yuan, Karla Gomez, Shan-Shan Mei, Yiliam Rodriguez, and Keith A. Candiotti

ABSTRACT

Background: This study was designed to investigate the course and mechanism of diabetes-induced sensory abnormalities in a type 1 diabetic mouse model. Dynamic neuropeptide calcitonin gene-related peptide (CGRP) expression and involvement of toll-like receptor 4 (TLR4) were explored.

Methods: C57BL/6J mice were divided in two groups, a normal control group and a diabetes mellitus (DM) group which was induced with streptozotocin (STZ). Thermal and mechanical nociceptive behavioral techniques were utilized to measure the development of sensitivities. Mice lumbar L4-L6 spinal cord and dorsal root ganglia (DRG) were taken to evaluate the molecular mechanisms of diabetic neuropathy (DN) by using immunocytochemistry and reverse transcription polymerase chain reaction (RT-PCR) methods.

Results: DM mice developed both thermal and mechanical hypersensitivities. Thermal sensitivity returned to "normal" levels at 6 weeks as the mice then gradually developed thermal hypoalgesia in the late stages of DM. However, mechanical allodynia remained from 2 to 10 weeks post-DM, and mice later developed mechanical hypoalgesia. CGRP expression levels in DRG and the dorsal horn of the spinal cord showed a transient increase post-DM, followed by a significant decrease. In DM mice, TLR4 expression level increased in the dorsal horn of the spinal cord and co-localized with microglia and astrocytes in the spinal cord. The TLR4 specific inhibitor, TAK242, alleviated the symptoms of DM-induced pain.

Conclusions: Our results demonstrated that hyperglycemia-induced diabetic neuropathic behavior includes early-stage hypersensitivity and late-stage hyposensitivity. CGRP and TLR4 pathways may play a role in early-stage of diabetic neuropathic pain.

Glucose control, in the treatment of hyperglycemia, a component of the metabolic syndrome, has only a marginal effect on preventing diabetic neuropathy (DN), suggesting that other factors may be driving nerve injury. DN is a secondary consequence of longstanding diabetes mellitus (DM). Neuropathy frequently results in clinically significant morbidities, such as pain, loss of sensation, foot ulcers, gangrene and amputations (1). Sensory neurons appear particularly vulnerable to elevated glucose levels that occur in DM and as a result, are damaged (2). The dynamic progression of DN on the sensory nerves represents a window through which the level of nerve injury can be diagnosed, and the options of therapy can be evaluated. The progression of sensory neuron damage often results in the loss of sensation in the feet, and insensi-
tivity to injury can lead to foot ulcerations and amputation as a complication. However, our knowledge of the molecular mechanisms related to functional changes in the sensory neurons as a result of DM is limited. In this study, we set out to investigate the progressive course of diabetic-induced hypersensitivity leading to hyposensitivity in a type 1 diabetic animal model.

Calcitonin gene-related peptide (CGRP) is a neuropeptide that is widely distributed in the peripheral and central nervous systems (3). CGRP release potentiates nociceptive signaling and increases pain-related behaviors (4, 5). It is also well known that CGRP plays an important role in peripheral and central sensitization (6). Some studies have reported decreased levels of CGRP in the presence of DM. Levels of CGRP mRNA in the L4 and L5 dorsal root ganglia (DRG) of streptozotocin (STZ)-induced diabetic rats were noted to decrease 6 weeks post-DM (7, 8) as were reductions in substance P and CGRP-like immunoreactivity in the sciatic nerve and DRG. Although CGRP expression has been investigated at several different time points in diabetic models, the corresponding expression levels in the early-stages of diabetic neuropathic pain and in late-stages of diabetic hyposensitivity have not been reported.

Our recently published study demonstrated that Coenzyme Q10 (CoQ10) is effective in attenuating diabetic neuropathic pain in animal models of type 1 and type 2 diabetes (9, 10). Our studies also revealed that treatment with CoQ10 decreased the up-regulation of Toll-like receptor 4 (TLR4) and the downstream proinflammatory factors, such as chemokine (C-C motif) ligand 2 (CCL2) and tumor necrosis factor alpha (TNFα), which occurred in both the peripheral and central nervous system tissues in DM animals. These results suggest that TLR4 and its related downstream factors may participate in the development of DN. In this study, we evaluated the expression and involvement of TLR4 and related factors in the process of diabetic neuropathic pain.

**MATERIALS AND METHODS**

**Animal Preparation**

All experiments were carried out following the guidelines and protocols approved by the University of Miami Animal Care and Use Committee. Ten-week-old male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and were used for this study. All mice were housed in groups of five, in plastic cages with soft bedding and free access to food and water under a 12 hours/12 hours light-dark cycle (dark cycle: 7:00 pm-7:00 am). All animals were acclimated in their cages for 1-2 weeks before experiments were performed. DM was induced through a single intraperitoneal injection of STZ 200 mg/kg (Sigma, St. Louis, MO, USA). The onset of DM was confirmed by measuring blood glucose levels from the tail vein beginning 2 days after STZ injection. Mice were considered to have DM if blood glucose levels were above 300 mg/dl. Age-matched, non-diabetic mice were used as controls for each test and sample collection.

**Testing of Diabetic Neuropathic Behaviors**

Diabetic neuropathic behavior, hypersensitivity or hyposensitivity, was confirmed through thermal and mechanical sensory tests once a week for 24 weeks.

**Thermal Sensory Test**

An Ugo Basile Plantar Test apparatus (Biological Research Apparatus, Comerio, Italy) was used to measure paw withdrawal latency time in unrestrained mice. To determine this time, the mouse was placed in an acrylic restrainer on a thermal stimulator. The hind paws made contact with a 1/4'' 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 withdrawal latency time was determined for the left and right hind paw, with a 5-minute inter-trial interval. A cutoff of 20-second was used. Four trials were performed in each animal to establish withdrawal latency.

**Mechanical Sensory Test**

The mechanical sensory test was conducted with a Touch-Test Sensory Evaluator (von Frey filaments, North Coast Medical, Inc., Wood Dale, IL, USA). Filaments were used to assess the mechanical sensitivity on each day of testing, con-
ducted weekly. For each assessment, the mouse was placed on a wire mesh platform and was covered with a transparent glass container; a period of 30 minutes 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 seconds of the application of the filament was then followed by the application of the thinner filament (or the thicker one if the response was negative). The paw withdrawal threshold was measured five times and was expressed as the tolerance level in grams.

**Immunohistochemistry and Image Quantification**

At two time points—4 weeks and 24 weeks post-DM, age-matched control mice and diabetic mice were sacrificed with an over-dose of nembutal and were then exsanguinated by decapitation. L4-L6 DRG and the related lumbar spinal cord were removed. Half of the tissues were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) overnight and cryoprotected in 0.1 M PBS containing 20% sucrose. Samples were embedded in optimal cutting temperature embedding medium (Sakura Finetek, USA) and stored at -80°C. Frozen samples were sectioned by cryostat into 15 μm-thick sections. Sections were incubated overnight at 4°C with the primary antibodies: anti-CGRP (Sigma-Aldrich, St. Louis, MO, USA), anti-protein gene product (PGP)9.5 (marker of free nerve fibers; from AbD Serotec, Oxford, UK), anti-TLR4 (Abcam, MA, USA), anti-CD11 (marker of microglia; from AbD Serotec, Oxford, UK), and anti-glial filament acidic protein (GFAP) (marker of astrocytes; DakoCytomation, Denmark) followed by secondary species-specific fluorescent antibodies (Jackson ImmunoResearch Lab, Inc. West Grove, PA, USA) or a biotin-conjugated secondary antibody for 1 hour at 22°C. Immunolabeling of biotin-conjugated staining were detected by the streptavidin-horse radish peroxidase method and visualized after diaminobenzidine (DAB) incubation (Vector, Burlingame, CA, USA).

All immunohistological stained slides were captured via digital images using a high-resolution digital microscope camera with an 8-bit acquisition of color. Identical camera and microscope settings were used throughout the capturing of images and analysis of the slides. To estimate the density of free nerve endings in the epidermis, images of randomly selected areas from the footpad skin were taken at 20× magnification and the number of immune-positive nerve fibers in the epidermis within each image area was counted. The average number of PGP9.5-positive fibers per 200 μm length of epidermis was then calculated for each animal. In reference to counting TLR4-positive cells, anti-TLR4 positive cells were counted in a fixed area of the dorsal horn of the lumbar spinal cord. During cell counting, the images were magnified to help identify cells. Four to six different animals from each group and three sections from each animal were included in image analysis. The researcher conducting the image analysis was blind to which group the tissue was from.

**RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR) of the CGRP, TLR4, CCL2 and TNFα mRNA**

The other half of the collected tissues were freshly frozen in dry ice and stored at -80°C. The levels of CGRP, TLR4, CCL2 and TNFα mRNA were evaluated by RT-PCR in the DRG and spinal cord tissues. Extraction of total RNA was carried out with TRIzol (Invitrogen, Grand Island, NY, USA) according to manufacturer's instructions. One μg of RNA was reverse transcribed with 200 U/sample SuperScript II (Invitrogen, Grand Island, NY, USA) and 250 ng/reaction of random primers (Promega, San Luis Obispo, CA, USA). The genes of CGRP, TLR4, CCL2 or TNFα were amplified from 0.1 μg aliquots of cDNA in a standard PCR buffer (50 mM KCl, 1.5 mM MgCl₂, and 10 mM Tris-HCl, pH 8.3) containing 10 pmol of forward and reverse primers, along with 0.5 U/sample of AmpliTaq DNA polymerase (Applied Biosystems, Grand Island, NY, USA). Mouse β-actin was amplified as the internal control for the PCR reaction. The sequences of primer pairs were as follow—CGRP: forward, 5-caccaatgtggcctcaag-3 and reverse, 5-ccgctgaggttagcagag-3; TLR4: forward, 5-gtcgctgctcctcaac-3 and reverse, 5-aggcatttgcccttcaag-3; CCL2: forward, 5-gccgttctgctgctggtc-3 and reverse, 5-agcctggctgctgctggtc-3; TNFα: forward, 5-cctttccctctcctcaac-3 and reverse, 5-ccccttctgtcctgtcctg-3.
Involvement of CGRP and TLR4 in DM Pain Progression

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Treatment of TAK242

TLR4 involvement in diabetic neuropathic pain was evaluated by treating animals with the TLR4 specific inhibitor, TAK242, after diabetic mice developed neuropathic pain. Three groups of animals were used: G1, age-matched control mice receiving the vehicle of TAK242 (translipid) intravenously; G2, DM mice treated as in G1; G3, DM mice treated with 2 mg/kg of TAK242 (InvivoGen, San Diego, CA, USA).

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM) and analyzed using Prism 4 software (GraphPad Software Inc., San Diego, CA, USA). The behavior test data was analyzed with two-way analysis of variance (ANOVA) with two-repeated factors followed by Tukey’s multiple comparison test. Comparison between two groups was assessed by unpaired, two-tailed Student t-test. P values of less than 0.05 were designated as statistically significant.

RESULTS

The Progression of Diabetic Neuropathic Behavior in Long-Duration of DM

Two groups of mice were tested during the entire 24-week course of the study: G1, age-matched control mice (N=8) and G2, DM mice that had DM induced at 12 weeks of age (N=15). Animals were monitored to insure no loss of motor sensory function.

Figure 1 showed the progression of diabetic neuropathic sensory properties as demonstrated by thermal and mechanical testing over the course of 24 weeks post-DM. At 2 weeks post-DM, DM mice developed both thermal and mechanical hypersensitivities (G1 vs. G2, P<0.05 in thermal test, P<0.001 in mechanical test). However, thermal sensitivity returned to a "normal level" around 6 weeks post-DM. The mice then gradually developed thermal hyposensitivity compared to age-matched controls at 8 weeks post-DM. The mice then gradually developed thermal hyposensitivity compared to age-matched controls at 8 weeks post-DM (G1 vs. G2, P<0.01 or 0.001 on corresponding time points). Mechanical allodynia was retained for a longer period, from 2 weeks until 8-10 weeks post-DM (G1 vs. G2, P<0.001). It was then observed that DM mice developed hyposensitivity after 16 weeks post-DM, compared to age-matched controls (G1 vs. G2, P<0.05 or 0.01 on corresponding time points). These results indicated that DM animals developed diabetic neuropathic pain in the early stage of DM and then developed a loss of sensation to thermal and mechanical stimuli in the late stages of DM.

Two-way ANOVA showed a significant interaction between groups and time points (P<0.01), and significant main effect for group or time (both P<0.001).
The Expression of CGRP in DRG and the Dorsal Horn of the Spinal Cord

CGRP positive neurons in DRG and the CGRP expression levels in the dorsal horn of the L4-L6 spinal cord were studied in age-matched control mice and DM mice that were 4 weeks and 24 weeks post-DM. As shown in figure 2, CGRP expression levels corresponded to a transient increase at 4 weeks post-DM and significantly decreased in the late stages of DM. The results of real time RT-PCR revealed the same pattern of mRNA levels of CGRP in DRG and spinal cord (Figure 2B and D).

Epidermis Innervations

In order to evaluate the mechanisms of diabetic neuropathic sensory properties, the innervation of the hind paw foot pad skin epidermis was quantified. Both peptidergic and non-peptidergic epidermal nerve fibers were marked by antibody PGP9.5. Figure 2E showed the immunohistochemical staining and figure 2F demonstrated the quantification results of PGP9.5-immunoreactive nerve fibers in the epidermis. The results indicated that the number of free fiber endings were decreased at 4 weeks and the loss of fibers reached significance at 24 weeks post-DM (compared to age-matched controls, ***P<0.001, Student t-test).

TLR4 Expression in DM and Its Involvement in Diabetic Neuropathic Pain

The level of expression of TLR4 in the dorsal horn of the spinal cord (L4-L6) was evaluated by immunohistochemical staining. The mRNA levels of TLR4, CCL2 and TNFα in the dorsal horn of the L4-L6 spinal cord were analyzed using real time RT-PCR. Immunohistochemical results demonstrated that TLR4 positive cells in the dorsal horn of the spinal cord were significantly higher in DM mice than those in normal mice. Figure 3A showed images of the dorsal horn of the spinal cord. Figure 3B demonstrated the quantification results, which showed significantly more TLR4 positive cells in the dorsal horn of DM mice than those in control mice (for an age-matched group: P<0.05 or 0.01, Student t-test). In the spinal cord, TLR4 co-localized with microglia and astrocytes in DM mice (Figure 3C).

Quantification of RT-PCR indicated that mRNA levels of TLR4, CCL2 and TNFα were significantly increased in the early stage of DM (4 weeks post-DM) in the DRG and spinal cord. TLR4 and CCL2 remained at higher levels in the spinal cord, however, CCL2 in DRG and TNFα in DRG and the spinal cord declined in the later stage of DM (24 weeks post-DM) (Figure 4).

In order to evaluate the involvement of TLR4 in diabetic neuropathic pain, the TLR4 specific...
inhibitor, TAK242 was used in DM mice after 4 weeks of DM. DM mice were confirmed to have behaviors consistent with diabetic neuropathic pain prior to treatment. Three groups of animals were used: G1, normal mice receiving the vehicle of TAK242 (translipid), intravenously (N=8); G2, 4 weeks post-DM mice treated as in G1 (N=8) and G3, 4 weeks post-DM mice treated with 2 mg/kg of TAK242 (N=8). Figure 5A and B demonstrates that TAK242 alleviated the symptoms of diabetic neuropathic pain as tested by thermal hyperalgesia and mechanical allodynia (compared to DM mice treated with vehicle, \(P<0.001\) in post-treatment 3, 24 and 48 hours, ANOVA), implicating the involvement of TLR4 in the development of diabetic neuropathic pain.

To evaluate whether increased TLR4 in the dorsal horn cells of the spinal cord in the later stage of DM were involved in hyposensitivity, the effects of administration of TAK242 at a later stage of DM (18 weeks post-DM) were tested. Our results demonstrated that TAK242 did not affect the hyposensitivity of DM mice (Figure 5C and D) in thermal or mechanical sensory. Additionally, TAK242 had no obvious effects on normal age-matched animals.

**DISCUSSION**

**The Dynamic Course of Neuropathic Behavior in DM**

The development of DN is a dynamic progression from hypersensitivity to hyposensitivity. Although the clinical symptoms of DN are different in each case, awareness and treatment in the early stages may prevent subsequent hyposensitivity and maintain sensory nerve function for a longer period of time. Our current study systematically explored the development of hypersensitivity (hyperalgesia) and eventual hyposensitivity (hypoalgesia) in a type 1 diabetic animal model. It is interesting to note that the thermal hyperalgesia only appeared for a very short period, 2-4 weeks post-DM, after which the mice gradually developed thermal hypoalgesia. However, mechanical hyperalgesia was sustained longer, lasting until 10 weeks post-DM and then developed into mechanical hypoalgesia at 16 to 24 weeks post-DM. Thermal hyperalgesia has been clinically described in early DM while thermal hypoalgesia is typically present in advanced DM (11). Mechanical hyperalgesia in diabetic animals appears to be equivalent to pain noted from pressure in humans with early DN. Mechanical hypoalgesia corresponds to the loss of sensitivity to noxious mechanical stimuli in advanced DN (12). Hypersensitivities may result from the injury of peripheral nerves and the primary neurons in the DRG while hyposensitivities may result from the further degeneration and loss of peripheral sensory fibers and neurons.

Smaller sensory fibers, including unmyelinated and lightly myelinated fibers, transmit pain and temperature sensation. Caselli et al. (13) demonstrated that damage to small-fiber is an early event in the natural history of DN. Shun et al. (14) indicated that small-fiber sensory neuropathy presents with reduced intraepithelial nerve fiber densities, which is consistent with our present findings. Our findings also indicated that further reduction of intraepithelial nerve fibers is correlated with elevation of thermal and
mechanical thresholds in the later stage of DM. Previous studies in the histological assessment of nerve fiber morphology have indicated that the relative proportion of Aδ and Aβ-fibers is reduced in diabetic skin-nerve preparations when compared with non-diabetic control mice (15). In clinic reports, quantitative immunohistochemical assessments of cutaneous innervation have established that human diabetics experiencing decreased tactile sensation concomitantly have significantly reduced dermal and epidermal innervation (14, 16, 17). Combining these findings with our present results, we postulated that chronic DM damages the distal ends of sensory axons and suppresses axon regeneration, ultimately leading to chronic denervation of cutaneous tissues.

**The Relationship of Neuropathic Behavior with CGRP Expression Level**

Considerable evidence indicates that the release of the neuropeptide CGRP from sensory nerve terminals in peripheral tissue plays a key role in neurogenic inflammation, whereas the release from terminals in the dorsal horn of the spinal cord modulates pain transmission (18). In the DRG, CGRP is synthesized predominantly in small neurons, receiving signals through the peripheral C- and Aδ- afferents (19). A recent study reported that CGRP expression was significantly increased in the spinal cord dorsal horn after 4 weeks of STZ-induced DM in rats (20). However, decreased levels of CGRP mRNA in the DRG and the sciatic nerves of STZ-induced DM rats at week 6 and 12 have also been previously reported (21, 22). These different results, whether increased or decreased levels of CGRP in DN, appear to depend on the time course of the study. Our results showed that CGRP expression was increased in the early stages of DM, which coincided with neuropathic pain behavior in this period and the increased CGRP expression may be involved in the thermal and mechanical hypersensitivity seen in the early stages of DM. The reduction of CGRP in the DRG or peripheral nerves in the late stages of DM may be associated with degeneration, or even loss of the peptidergic primary sensory neurons in DRG, resulting from damage from chronic hyperglycemia. Our results were consistent with clinical reports indicating that CGRP concentrations in the cerebrospinal fluid of diabetic patients with painless neuropathy were lower than those in patients with painful neuropathy (23).

**TLR4 Involved in Diabetic Neuropathy**

Our recent studies indicated that CoQ10 treatment not only alleviated diabetic neuropathic pain in type 1 and type 2 DM models (9, 10), but also decreased the up-regulation of TLR4 and its downstream proinflammatory factors--NF-κB, CCL2 and mitogen-activated protein kinase (MAPK). These results suggested that all of these proinflammatory factors may participate in the development of diabetic neuropathic pain. In our current experiments, we demonstrated that up-regulated TLR4 co-localizes in microglia and astrocytes in the spinal cord of DM mice at 4 weeks post DM (Figure 3A, B and C) and administration of the TLR4 inhibitor,
TAK242, significantly attenuates diabetic neuropathic pain in the early stages of DM. The results would appear to confirm the involvement of TLR4 in the development of diabetic neuropathic pain.

Microglia and astrocyte activation in the spinal cord are observed following peripheral nerve injury or central nerve injury (24-26). Interestingly, the increased TLR4 positive cells (microglia and astrocytes) in DM mice remained at relatively higher levels even in the later stages of DM, when DM animals showed hyposensitivity. Administration of TAK242 in the late stage of DM did not affect hyposensitivity, suggesting that peripheral nerve injury and nerve loss play major roles in the development of DN, early stage neuropathic pain and later stage hyposensitivity. Our results implied that TLR4 positive cells in the spinal cord, induced by hyperglycemia, may participate in the process of DN, from early stage peripheral nerve injury to later stage nerve damage and neuron loss.

Diabetic neuropathic pain shares some histological features and underlying mechanisms with traumatic or nerve injury-induced neuropathy (25). DN displays, however, other distinct features as we have demonstrated here, including decreased sensory input to the spinal cord rather than increased input in the later stages of DM. Consequently, development of central sensitization in DN involves mechanisms that are distinct from traumatic neuropathic pain. In diabetic neuropathic pain, the contribution of spinal cord TLR4-positive cells to central sensitization and pain processes emerges in the early stages. Aside from inflammation in the periphery, hyperglycemia and the resulting production of reactive oxygen species, affects the local microen-
vihon in the spinal cord. All these alterations could trigger resting and sessile microglia to the activated phenotype.

CONCLUSIONS

Our results demonstrated that hyperglycemia-induced diabetic neuropathic behavior includes early-stage hypersensitivity and late-stage hypersensitivity. CGRP may play a role in early-stage diabetic neuropathic pain. The reduction of CGRP in late-stage DM may be linked to the loss and degeneration of primary neurons and peripheral nerve fibers. Hyperglycemia may induce activation of TLR4 positive cells in the spinal cord. In addition, increased TLR4 itself may worsen diabetic neuropathic pain. A TLR4 inhibitor may be considered as a potential treatment for diabetic neuropathic pain in the early stage of DM. Increased TLR4 positive cells in the spinal cord may participate in the process of DN—from early stage peripheral nerve injury to later stage nerve damage and neuron loss.

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