Enhanced Wind-Up of the C-Fiber-Mediated Nociceptive Flexor Reflex Movement Following Painful Diabetic Neuropathy in Mice

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Abstract. We examined wind-up of the nociceptive flexor withdrawal responses in diabetic mice that had developed tactile allodynia after treatment with streptozotocin (STZ). In control and STZ-treated mice, simultaneous activation of Aδ- and C-fibers by electrical stimuli at C-fiber intensity delivered to the ventral aspect of the toe elicited a biphasic withdrawal reflex composed of short- and long-latency movements of the ipsilateral hind paw that were respectively mediated by activation of Aδ- and C-fibers. There were no significant differences between control and diabetic mice in the activation threshold of each reflex movement or the amplitude of reflexes elicited by various stimulus intensities. However, a repetitive conditioning stimulus (CS) elicited significantly greater wind-up of the C-fiber-mediated movement and early saturation of wind-up in diabetic mice. In both control and diabetic mice, the CS elicited no or occasionally slight wind-up of the Aδ-fiber-mediated movement. Moreover, post-CS facilitation, which reflects the prolonged excitability increase, was observed in both Aδ-fiber- and C-fiber-mediated movements of control mice, whereas significant post-CS facilitation was only obtained in the C-fiber-mediated movement of diabetic mice, which may reflect supraspinal descending influences. Such changes in the excitability of spinal neurons in diabetic mice may represent some aspect of painful diabetic neuropathy.

Keywords: diabetes, allodynia, flexor reflex, wind-up, C-fiber

Introduction

Diabetes is frequently associated with painful polyneuropathies; approximately 20 – 30% of diabetic patients develop pain that is extremely difficult to treat (1, 2). Although morphological changes and reduced conduction velocity of the sensorimotor nerves have been demonstrated in animal models of diabetes (3, 4), the etiology of painful diabetic polyneuropathy remains poorly understood.

Animals with streptozotocin (STZ)-induced diabetes exhibit thermal and mechanical hyperalgesia, tactile allodynia, and enhanced flinching behavior during phase Q (quiescent) and 2 of the formalin test (5 – 7). Moreover, electrophysiological studies have revealed enhanced excitability of spinal dorsal horn neurons in response to mechanical stimulation in animals with experimental diabetic neuropathy (8, 9).

Enhanced sensitivity of nociceptive spinal dorsal horn neurons to peripheral sensory input, termed central sensitization, underlies hyperalgesia and allodynia following trauma, inflammation, or peripheral nerve injury. Wind-up, a frequency-dependent increase in the excitability of spinal neurons elicited by electrical stimulation of unmyelinated afferent C-fibers (10), is likely to represent some features of central sensitization (11). Wind-up has been described in dorsal horn neurons (12), field potentials of the dorsal horn (13), and nociceptive withdrawal reflexes (12, 14 – 17). As well as that in the normal condition, wind-up in the sensitized condition has been well investigated using animals with inflammation, in which withdrawal reflexes exhibit an enhanced (14) or reduced (18) wind-up depending on supraspinal influences. However, there have been few studies of wind-up in animal models of neuropathic pain.
In our previous study, we reported a new method that enables us to evaluate myelinated Aδ-fiber- and unmyelinated C-fiber-mediated flexor reflexes simultaneously (19). In the study presented here, we assessed wind-up of this biphasic withdrawal reflex movement in diabetic mice to examine the excitability of the spinal cord during allodynia.

Materials and Methods

Animals and induction of diabetes

All the experimental protocols were approved by the Animal Care and Use Committee of Nagoya City University and were conducted in accordance with the guidelines of the National Institutes of Health and The Japanese Pharmacological Society.

Experiments were performed on adult male ICR mice that were 6 – 7-week-old and weighed 31 – 42 g at the beginning of the study. After a 20 – 24-h fast, the mice were injected with STZ (200 mg/kg, i.p.) dissolved in saline. Saline-injected age-matched mice were employed as a non-diabetic control group. All animals were housed in solid floored cages with a deep layer of paper chips that was changed daily. A 12:12 h light-dark cycle was used, and the animals were allowed free access to sufficient food and water. The blood glucose level was measured 1 day before and 7 or 8 days after STZ injection by measuring the glucose concentration in a blood sample obtained by tail prick using an Accu-Chek blood glucose monitoring system (Roche Diagnostics, Indianapolis, IN, USA). The mice were defined as diabetic when their blood glucose concentration exceeded 350 mg/dl.

Assessment of allodynia

A standardized testing regime was performed to measure tactile allodynia (20) 1 day before and 7 or 8 days after STZ administration. Briefly, mice were placed in individual transparent Perspex cubicles with a wire mesh bottom and allowed to acclimatize for 90 – 120 min. Eight von Frey filaments (North Coast Medical, Morgan Hill, CA, USA), with approximately equal logarithmic incremental bending forces, were chosen (von Frey numbers: 2.36, 2.44, 2.83, 3.22, 3.61, 3.84, 4.08, 4.17; equivalent to 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4 g, respectively). Testing was initiated with the 0.16 g hair, and each hair was pressed perpendicularly against the plantar surface of the left hindpaw until slight buckling was observed (5 s each). Lifting of the paw was recorded as a positive response, and withdrawal thresholds were determined using the up-down method of Dixon (21). If the mouse did not respond, the next stronger hair was applied. If a positive response was obtained, the next weaker hair was applied. This paradigm was continued until four measurements had been obtained after an initial change in the behavior or until four consecutive negative (score of 0.01 g) or five positive scores (score of 1.5 g) had been obtained. Each testing was repeated twice at approximately 20-min intervals, and the mean value was used. In this study, diabetic mice that exhibited a threshold of less than 0.2 g in the von Frey test 7 or 8 days after STZ administration were considered to be developing tactile allodynia and used in the following experiments.

Measurement of the hindlimb withdrawal reflex movement

Measurement of flexor reflex movement was performed 7 or 8 days after the saline or STZ treatment. Mice were anesthetized with urethane (1.6 g/kg, i.p.) and α-chloralose (15 mg/kg, i.p.), and cannulae were inserted into the trachea to maintain respiration.

Mice were placed in the ventral recumbent position on a plastic board, and the hindlimbs were suspended through the holes in the board (Fig. 1A). The left hind
toe was connected to a force-displacement transducer (SB-1T; Nihon Kohden, Tokyo), and a pair of bipolar electrodes was inserted subcutaneously, approximately 3 mm apart, into the ventral aspect of the interphalangeal skin zone. Electrical stimuli (a train of 5 pulses, 1-ms duration, 200 Hz) were applied every 30 s, and stimulus intensity was fixed at 15 V before and after a repetitive conditioning stimulus (CS). The output of the transducer was amplified and recorded on a recorder (RTA-1200, Nihon Kohden). Stable baseline reflex responses were recorded for at least 10 min before the CS. The CS consisted of 20 electrical stimuli delivered at 1 Hz with stimulus intensity at 30 V. Thirty seconds after the last CS, the reflex response was again measured and recorded continuously for 20 min. Each of 20 withdrawal reflexes during the CS was expressed as a percentage of the reflex response elicited by the first CS. The withdrawal responses recorded after the CS were expressed as percentages of the response obtained during 1 min before the CS. The rectal temperature was maintained at 36.0 ± 0.5°C by radiant heat.

**Drugs**

Urethane was purchased from Sigma (St. Louis, MO, USA). α-Chloralose was from Tokyo Kasei (Tokyo), and streptozotocin (STZ) was from Calbiochem (La Jolla, CA, USA).

**Statistical analyses**

All data were expressed as the mean ± S.E.M. Student’s t-test was used to compare data between the saline and diabetic groups. Test for the slope of linear regression during the first 5 stimuli of the CS was used to assess the appearance of wind-up. Differences at P<0.05 (two-tailed) were considered to be significant.

**Results**

**Blood glucose concentrations and tactile alldynia after STZ administration**

Changes in blood glucose concentrations and threshold in the von Frey test were studied for 3 weeks after STZ (n = 13) and saline (n = 9) treatment in some mice. Most of the STZ-treated mice were hyperglycemic at 3 days after STZ administration (corresponding to the time of behavioral testing), and blood glucose concentrations remained elevated for at least 3 weeks and often exceeded the upper detection limit (600 mg/dl) of the blood glucometer.

In agreement with previous studies on changes in nociceptive thresholds in rats (5, 6) and mice (7) after STZ treatment, STZ-treated mice exhibited a reduction of the threshold in the von Frey test, which was obvious at 7 days after STZ administration [0.37 ± 0.04 g for saline-injected control mice (n = 9) and 0.23 ± 0.03 g for STZ-injected mice (n = 13), P<0.05], and remained allodynic for at least 3 weeks (Fig. 2). Of 13 STZ-treated mice, 8 exhibited a threshold below 0.2 g at 7 days after STZ administration. The body weight of diabetic mice was significantly reduced 7 days after STZ treatment [37.4 ± 0.8 g for saline-injected control mice (n = 9) and 31.9 ± 0.6 g for STZ-treated mice (n = 13), P<0.01].

**Withdrawal reflex of diabetic mice**

As we had demonstrated in our previous study (19), electrical stimuli (a train of 5 pulses, 1-ms duration, 200 Hz, 15 V) delivered to the ventral aspect of the toe of saline-treated control mice elicited a biphasic withdrawal reflex that was composed of short- and long-latency movements of the ipsilateral hind paw (Fig. 1B). Diabetic mice also exhibited a similar biphasic withdrawal reflex. Stimulus-response recruitment curves, generated by gradually increasing the stimulus intensity from 0.5 to 40 V, revealed that there were no significant differences in the amplitude and development of Aδ-fiber- and C-fiber-mediated flexor reflexes between control and diabetic mice (Fig. 3). The amplitude of each movement was dependent upon stimulus intensity. The Aδ-fiber-mediated movement increased progressively at lower stimulus strength and became almost stable between 15 – 30 V, whereas the C-fiber-mediated movement increased gradually up to 30 V and became almost stable between 30 – 40 V. The activation thresholds of the Aδ-fiber- and C-fiber-mediated withdrawal reflexes
Fig. 3. Stimulus-response recruitment curves of the Aδ-fiber- and C-fiber-mediated flexor reflex. Each point represents the mean ± S.E.M. of 6 separate experiments for control (clear circles) and diabetic (solid circles) mice. Ordinate: amplitude of the flexor reflex per 10-g body weight. Abscissae: stimulus intensity (volts).

Fig. 4. Enhanced wind-up of the C-fiber-mediated withdrawal reflex in diabetic mice. A: A sample wind-up response in a diabetic mouse. The triangles show each electrical stimulus during the CS. B: Averaged wind-up response during the CS obtained from 6 separate experiments for the Aδ-fiber- (left) and C-fiber-mediated (right) withdrawal reflex. Each point represents the mean ± S.E.M. Dashed lines indicate linear regression designated during first 5 stimuli of the CS. Ordinates: amplitude of the flexor reflex (%) normalized to the first response during the CS. Abscissae: stimulus number. The significance of differences between control and diabetic groups, and the significance of the regression slope were determined by Student’s t-test (*P<0.05 and **P<0.01) and test for regression slope (†P<0.05 and ††P<0.01), respectively.
were 3.5 ± 0.4 and 8.8 ± 1.2 V in the controls and 4.5 ± 0.4 and 10.3 ± 1.7 V in the diabetic mice, respectively (n = 6 each). The times to peak of Aδ-fiber- and C-fiber-mediated flexor reflexes were 66.7 ± 3.6 and 207.8 ± 15.3 ms in the controls and 54.8 ± 4.8 and 201.5 ± 5.6 ms in the diabetic mice, respectively (n = 6 each).

**Wind-up during the repetitive conditioning stimulus**

A repetitive conditioning stimulus (CS; 20 stimuli at 1 Hz, 30 V) elicited wind-up of the C-fiber-mediated withdrawal reflex in both control and diabetic mice (Fig. 4), evidenced by the significance of the regression slope obtained from the first 5 responses during the CS ($P < 0.05$ for control and $P < 0.01$ for diabetic mice). The C-fiber-mediated response was facilitated to 208.4 ± 16.0% (wind-up of 108.4 ± 16.0%, n = 6) with the peak at 12 stimuli in control mice during the CS. Notably, the same CS in the diabetic mice resulted in pronounced facilitation to 349.0 ± 35.4% (wind-up of 249.0 ± 35.4%, n = 6) which peaked at 9 stimuli. In contrast, the simultaneously recorded Aδ-fiber-mediated withdrawal reflex was slightly facilitated (about 25% on average), but was not considered to exhibit wind-up in both control and diabetic mice (not significant in test for the regression slope), and there were no differences in these two groups.

**Post-CS withdrawal reflex facilitation**

After the termination of the CS, the withdrawal reflex elicited at 15 V was temporarily facilitated, reflecting an increased excitability of the spinal cord. The amplitude of the facilitated withdrawal reflex was higher than that elicited at the last CS and declined to the pre-CS baseline.
within 10 min after the CS (Fig. 5A).

After the CS, there were no significant differences in the facilitation of the C-fiber-mediated withdrawal reflex between control and diabetic mice (255.2 ± 57.7% for control vs 264.1 ± 54.0% for diabetic mice, n = 6 each). However, the facilitation of the Aδ-fiber-mediated withdrawal reflex was smaller in diabetic mice than in control mice (Fig. 5B; 85.3 ± 18.0% for control vs 25.2 ± 18.7% for diabetic mice, n = 6, P<0.05).

Discussion

Wind-up in the sensitized condition has been well investigated using animals with inflammation, and withdrawal reflexes generally exhibit an enhanced wind-up after inflammation (14). However, there have been few studies of wind-up in animal models of neuropathic pain (22 – 24). In particular, to our knowledge, there have been no studies of wind-up during allodynia in diabetic animals. The results of our present study demonstrated enhanced excitability of spinal cord neurons in diabetic neuropathy, evidenced by increased wind-up of the nociceptive withdrawal reflexes.

The STZ-treated mice used in the study of wind-up developed tactile allodynia on the day of behavioral testing (Fig. 2). In contrast, the activation thresholds of the Aδ-fiber- and C-fiber-mediated withdrawal reflexes were almost equivalent in the control and the diabetic mice (Fig.3). Tactile allodynia caused by diabetic neuropathy is attributable to the significantly lowered activation threshold of afferent Aβ- and Aδ- but not C-fibers in response to von Frey filaments, as demonstrated by Khan et al. (25). In contrast, the electrical stimuli to elicit withdrawal flexor reflex movements, which employ Aδ- and C-fibers that may mediate thermal and/or mechanical nociception, are distinct from mechanical stimulation with von Frey filaments to assess tactile allodynia. Our observation is in agreement with various studies that demonstrate profound mechanical hyperalgesia with thermal hypoalgesia or no changes in thermal withdrawal thresholds in diabetic animals (26 – 28). Hence it may depend on the stimulation we employed whether we detect any differences between control and diabetic animals.

Normal (saline-treated) mice exhibited wind-up of the C-fiber-mediated withdrawal reflex movement (Fig. 4), in agreement with studies showing wind-up of nociceptive flexor reflexes recorded as an electromyogram (15 – 17) or a single motor unit (12, 14) in response to noxious stimuli. Diabetic mice exhibited enhanced wind-up of the C-fiber-mediated withdrawal reflex (Fig. 4B). Similar enhancement of wind-up of the nociceptive reflex has been observed in non-spinalized rats with inflammation (14), consistent with our present results with non-spinalized diabetic mice. Several factors including peripheral and central changes in neuronal activity may contribute to the enhanced wind-up observed in diabetic mice. Diabetic animals have been demonstrated to show increased spontaneous activity as well as hyper-responsiveness of afferent Aδ- and C-fibers (25, 29, 30). Subsequently, such tonic excitatory afferent inputs increase glutamate release and remove Mg²⁺ blockade of NMDA receptor channels of neurons in the spinal dorsal horn (26). Such elevated basal excitability of spinal neurons may largely contribute to the enhanced wind-up that exceeded the saturated wind-up level exhibited in control mice. Moreover, the CS is likely to generate inhibitory as well as excitatory influences on spinal nociceptive activity. In inflamed spinalized rats, the inhibitory influence of the CS by recruitment of endogenous opioids could be enhanced, and this may underlie the reduction of wind-up (18). However, functional μ-opioid receptors in the spinal cord, which may play a role in inhibitory influences during the CS, are shown to be impaired in diabetes (9, 31). Thereby, reduction of recruitment of the endogenous inhibitory system during the CS favors the enhancement of wind-up in diabetic mice.

In the present study, Aδ-fiber-mediated withdrawal reflex movements showed only slight facilitation during the CS, and there was no significant difference between control and diabetic mice (Fig. 4B). As many reports in the literature describe, wind-up of the Aδ-fiber-mediated response could be hardly elicited in the normal condition (11). In fact, wind-up of short-latency responses mediated by A-fibers was occasionally evoked by stimulation at supramaximal intensity for C-fibers (32), and Aβ- or A-fibers can acquire the ability to evoke wind-up only in inflamed situations (11). Although similar CS parameters as we employed here (with C-fiber strength at 1 Hz) are usually used to elicit wind-up, other CS parameters could elicit wind-up of Aδ-fiber-mediated withdrawal reflex in diabetic mice. Alternatively, different factors may contribute to the generation of wind-up in inflamed and diabetic conditions.

Although post-CS facilitation of withdrawal reflexes, which reflects the prolonged excitability increase (16, 17, 33), was observed in both control and diabetic mice, the Aδ-fiber-mediated withdrawal reflex in diabetic mice revealed reduced facilitation compared with control mice after the CS (Fig. 5B). In non-spinalized animals, wind-up is strongly modulated by supraspinal descending influences. The descending inhibitory mechanism counteracts the progressive increase in the withdrawal reflex, leading to the habituation of wind-up (15).
Apart from the long-lasting descending inhibitory control that continues even after cessation of the CS in non-spinalized, halothane-anesthetized rats (15), the inhibitory control after the CS is likely to be less effective in non-spinalized, urethane-anesthetized mice, which was evidenced by the existence of post-CS facilitation in our study and others (16, 17). Our finding that post-CS facilitation of the Aβ-fiber-mediated withdrawal reflex after the CS was reduced in diabetic mice (Fig. 5B) suggests that the Aδ-fiber-mediated withdrawal reflex is more susceptible to supraspinal descending influences. Enhanced wind-up in diabetic mice may elicit potent supraspinal inhibitory influences on the development of wind-up and post-CS facilitation of the Aδ-fiber-mediated withdrawal reflex. Simultaneously, the magnitude of post-CS facilitation of the C-fiber-mediated response in diabetic mice is probably influenced by this enhanced descending inhibition. However, this inhibitory influence may be counteracted by removal of the presynaptic inhibition on C-fibers from Aδ-fibers (34). Hence, it is likely that we could not detect the differences between post-CS facilitation in control and diabetic mice, although they may differ qualitatively.

The present study has demonstrated that following the development of diabetic allodynia, the excitability of sensitized spinal neurons is not saturated. Rather, sensitized spinal neurons have the capability of being highly excitable, as manifested by the enhanced wind-up of the C-fiber-mediated withdrawal reflex. These changes in the excitability of spinal cord neurons in diabetic mice may represent some aspect of painful diabetic neuropathy.

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