ONLINE APPENDIX

SUPPLEMENTARY METHODS

Skin Biopsy, Immunohistochemical Staining, and Quantification of Epidermal Innervation

The skin biopsy was performed immediately or within one week after the contact heat evoked potential (CHEP) test. A 3-mm-diameter skin punch was taken from the right distal leg 10 cm proximal to the lateral malleolus under local anesthesia with 2% lidocaine. The sampled skin tissue was fixed overnight in PLP. Sections 50 μm perpendicular to the dermis were immunostained with rabbit antiserum to protein gene product 9.5 (PGP 9.5, 1: 1000; UltraClone, Isle of Wight, UK). Sections were then incubated with biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA) for 1 h and the avidin-biotin complex for another hour. The reaction product was demonstrated using chromogen SG (Vector Laboratories).

Epidermal innervation was quantified according to established criteria in a coded fashion. Observers were blinded to the clinical information. PGP 9.5 (+) nerves in the epidermis of each skin section were counted at a magnification of 40x with a BX40 microscope (Olympus, Tokyo, Japan). The length of the epidermis along the upper margin of the stratum corneum in each skin section was was measured with ImageJ vers. 1.43 (Image Processing and Analysis in Java, National Institutes of Health, Bethesda, MD: http://rsbweb.nih.gov/ij/download.html). The intraepidermal nerve fiber (IENF) density was expressed as the number of fibers/mm of epidermal length. In the distal leg, normative values from our laboratory (mean ± SD, 5th percentile) of IENF were 11..2 ± 3.7, 5.9 fibers/mm for subjects aged < 60 years and 7.6 ± 3.1, 2.5 fibers/mm for subjects aged ≥ 60 years (1)

Records of Contact Heat Evoked Potential

A contact heat evoked potential stimulator (CHEP stimulator, Medoc, Ramat Yishai, Israel)

was used to deliver the heat stimuli (2). The diameter of the circular thermode is 30 mm; the heating rate is 70 °C/s; and the cooling rate is 40 °C/s. Cooling begins immediately after the thermode reaches its target stimulus temperature based on the default algorithms. To avoid the interference of medications on CHEP, fresh cases were examined before the prescription of anti-neuropathic-pain agents and patients who were taking anti-neuropathic-pain agents were asked to withhold the drugs for at least 3 days before the study.

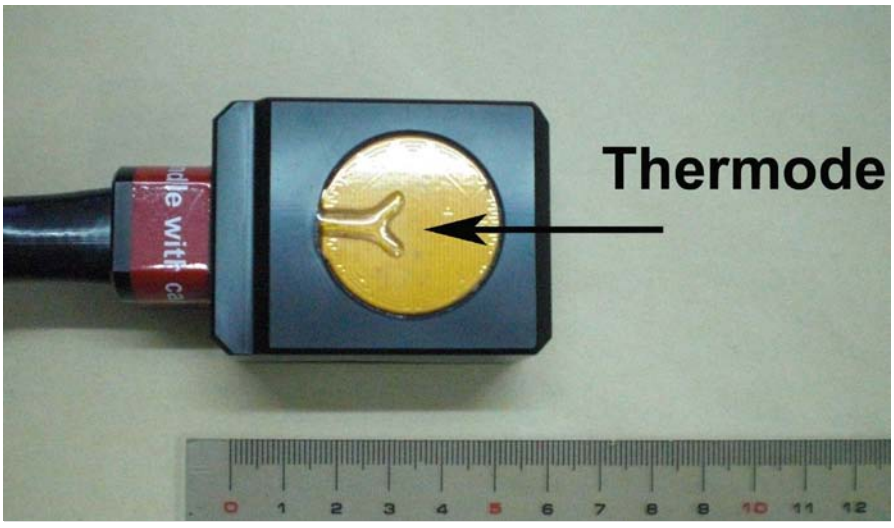
CHEP was evoked by methods described previously (3). In brief, subjects lay on a bed, with their eyes closed, in a semi-dark room with the room temperature controlled to 25 °C. The heat stimulus was applied to the hairy skin area of the right lateral leg around 10 cm proximal to the lateral malleolus. The adaptive temperature (baseline) of the thermode was set to 35 °C , and the heat pulse was delivered from the baseline to 51 °C. The inter-stimulus interval was randomly set to around 20~22 s. Subjects were asked to pay consistent attention to the stimulation during the study. CHEP was recorded using a Nicolet Bravo evoked potential system (Nicolet Biomedical, Madison, WI). The recording electrode was placed at the Cz positions according to the standard international 10-20 system. The reference was set at the left ear lobe, and the ground was placed at the Fpz. To control artifacts, we monitored the electrooculogram from supra- and infraorbital electrodes. The impendence of all electrodes was kept below 3 kΩ. The evoked potentials were filtered with a bandpass filter at 0.1~30 Hz. The recording was triggered by the onset of a stimulus, and the sweep time was 1500 ms.

Before the formal recording of CHEP, we delivered 2~3 heat stimuli to patients at the skin area other than the formal stimulation site for subjects to get used to the intensity of the heat. During the formal records, the recording began from the second stimulus to prevent the startle effects by the first stimulus. We recorded the consecutive tracings of CHEP by series heat stimuli. To prevent interference by repeated heat stimuli, the total number of heat stimuli

never exceeded 30. During the study, subjects were asked to verbally rate the intensity of pain perception 3 s after each stimulus using a verbal rating scale (VRS; 0~10), on which ‘0’ means no sensation, ‘4’ represents the threshold of pain, and ‘10’ corresponds to intolerable pain.

Consecutive tracings of CHEP in response to heat stimuli were recorded. Recordings with artifacts from motion or eye movement were discarded. The first 16 artifact-free evoked potential tracings were averaged for analysis. This is justified by preliminary studies, in which we compared the CHEP averaged by current protocol and that averaged from all tracings in six control subjects. The CHEP amplitude by averaging the first 16 artifact-free tracings was similar to that by averaging all tracings (41.9 ± 10.2 vs. 38.1 ± 8.5 μV, $p = 0.50$). To prevent potential habituation due to repeated heat stimuli, we thus followed established protocols to analyze the first 16 artifact-free tracings (3). In most of experimental subjects, no more than 20 tracings were required to get the first 16 artifacts-free tracings, i.e. only less than 5 tracings had artifacts.

To determine the background activities as a comparison for CHEP, we performed a control test before the formal recordings of CHEP. A CHEP stimulator with the thermode side facing upwards is shown in the following diagram.



During the formal CHEP recordings, the CHEP stimulator contacted the skin with the

thermode side delivering heat stimulus to the skin. In the control test, the CHEP stimulator contacted the skin with the non-thermode side and brain activities were recorded with the same settings as in the formal CHEP recordings (3).

To examine whether CHEP was mediated by Aδ fibers, we recorded CHEP from a proximal site (the skin at the S1 paraspinal area) in addition to the regular site (the distal site) in 5 healthy subjects and 4 of our 32 patients. The peripheral conduction velocity of CHEP in the lower limb was calculated by the equation: (the distance between distal and proximal stimulation sites / the difference of N-wave latencies between the distal and proximal CHEP) (3). In these healthy subjects and patients, the peripheral conduction velocities of CHEP were 2.07~12.42 m/s, in the range of Aδ fibers.

Quantitative Sensory Testing for Thermal Thresholds

We performed quantitative sensory testing with a Thermal Sensory Analyzer (Medoc Advanced Medical System, Minneapolis, MN) to measure sensory thresholds of warm and cold sensations as reported before (4). The stimulator was applied to the skin of the big toe. The examiner explained the procedures to the subjects, and the subjects made several trials to become familiar with the test. For the measurement of thermal threshold temperatures, reference temperatures were set to 32 °C. We used two testing strategies: the method of limits and the method of level. The results of these two algorithms were correlated and the thresholds for the method of level were presented here. The method of level was independent of reaction time, and the results of this algorithm are presented in this report. Briefly, the machine delivered a stimulus of constant intensity which had been determined by the algorithm. The intensity of the next stimulus was either increased or decreased by a fixed ratio according to the response of the subject i.e., whether or not the subject perceived the stimulus. Such procedures were repeated until a pre-determined difference of intensity was reached.

The mean intensity of the last two stimuli was the threshold for the level method. Thermal thresholds were expressed as warm threshold temperature and cold threshold temperature. These temperatures were compared with normative values for age.

Nerve Conduction Studies

Nerve conduction studies were performed with a Viking IV Electromyographer (Nicolet, Madison, WI) in all patients following established methods. The sural and peroneal nerves were studied. The results of nerve conduction studies were compared to normative data in our laboratory (5). Abnormal results in nerve conduction studies were defined as having reduced amplitude of compound motor action potential or sensory action potential, prolonged distal latencies, or slowing of the nerve conduction velocities.

References

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nerve disease in SARS: report of a case. Neurology 2003;61:1820-1821.

SUPPLEMENTARY RESULTS

SUPPLEMENTARY Table A1. Clinical and sensory profiles of the diabetic neuropathy

Clinical profiles	n = 32	Neuropathic pain symptoms	n (%)
Age*	51.63 ± 10.93	Evoked pain	10 (31.3)
Gender (Male : Female)	20 : 12	Tingle or shooting	25 (78.1)
Duration of diabetes (years)*	8.25 ± 6.60	Lacerating	2 (6.3)
Duration of neuropathic pain (years)*	1.79 ± 1.96	Hot or burning	13 (40.6)
Fasting plasma glucose (mg/dL)*	196.5 ± 72.7	Cold or freezing	4 (12.5)
Post-prandial plasma glucose (mg/dL)*	276.9 ± 82.1	Electric shock	14 (43.8)
HbA1C (%)*	9.2 ± 2.3	Numbness	25 (78.1)
Distribution of neuropathic pain	n (%)	Neurological signs	n (%)
Limited to feet	5 (15.6)	Small fibers	29 (90.6)
Feet and legs	13 (40.6)	Impaired pinprick sensation	28 (87.5)
Both lower and upper limbs	14 (43.8)	Impaired heat sensation	19 (59.4)
		Impaired cold sensation	16 (50)
		Large fibers	21 (65.6)
		Impaired vibratory sensation	21 (65.6)

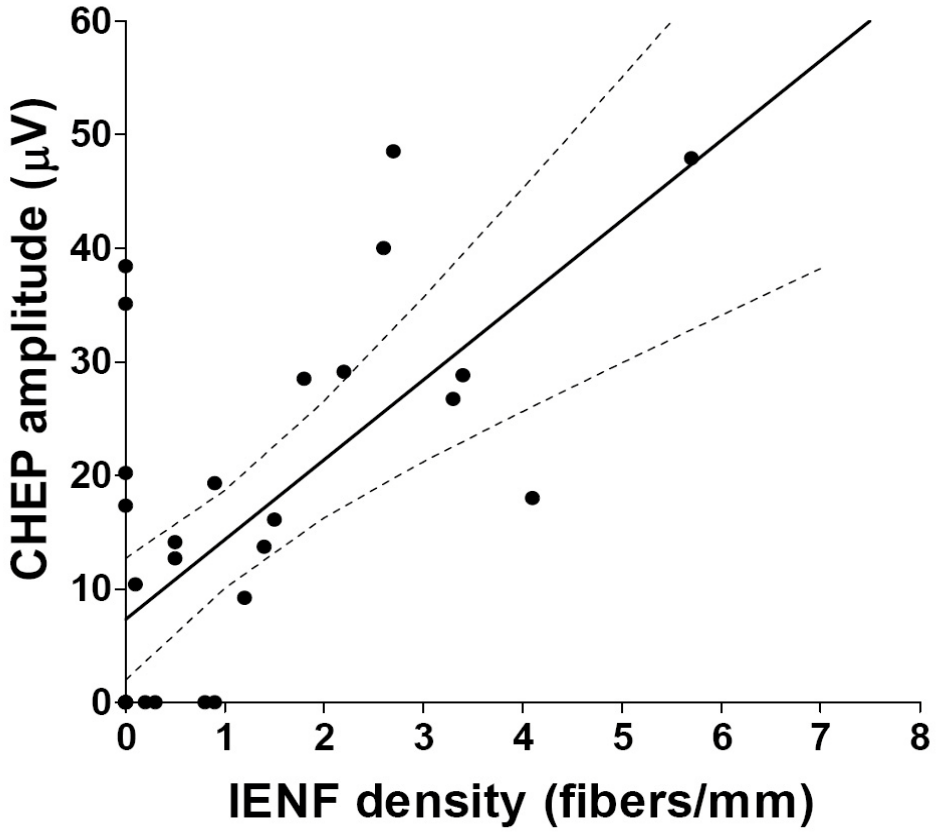
*** mean ± SD**

Effects of diabetes duration on examination results

In these 32 diabetic patients, the duration of diabetes was 8.25 ± 6.60 years and only 3

patients (9.4%) had diabetes duration less than 6 months (3 months in one patient and 4 months in two patients). There was no difference between the established diabetic patients (29 patients) and newly diagnosed diabetic patients (3 patients) in the following examination results: amplitude and latency on CHEP, conduction velocities and amplitudes on nerve conduction studies, thermal and vibratory thresholds on quantitative sensory testing, clinical signs of small-fibers (pinprick and thermal sense) and large-fibers (vibratory sense) on neurological examinations. This probably was due to the relatively small proportion of patients with early diabetes in this study group.

SUPPLEMENTARY Figure A1. Relationship between contact heat evoked potential (CHEP) and skin innervation.



The CHEP amplitude is positively correlated with the intraepidermal nerve fiber (IENF) IENF density (slope = 7.02 ± 1.47, $p < 0.001$).