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Vasopeptidase inhibitor ilepatril (AVE7688) prevents obesity- and diabetes-induced neuropathy in C57Bl/6J mice

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ABSTRACT

Previously we demonstrated that inhibition of neutral endopeptidase (NEP), a protease that degrades vaso- and neuro-active peptides, and angiotensin converting enzyme (ACE) with a vasopeptidase inhibitor improves vascular and neural function in diabetic rat models. The purpose of this study was to determine whether inhibition of NEP and ACE or deletion of NEP provides protection from nerve impairment caused by diabetes or diet induced obesity (DIO). To determine the role of NEP and ACE inhibition in neuropathy related to insulin-deficient diabetes or DIO we used C57Bl/6J mice treated with AVE7688, a vasopeptidase inhibitor, or NEP deficient (−/−) mice. Mice at 12 weeks of age were fed a high fat diet for 12 weeks or were diabetic for duration of 12 weeks following a single injection of high dose streptozotocin. Both a prevention and intervention protocol was used for AVE7688 treatment. Glucose utilization was impaired in DIO C57Bl/6 J and NEP −/− mice. However, treating DIO C57Bl/6 J or NEP −/− mice with AVE7688 improved glucose tolerance. Thermal hypoalgesia and nerve conduction slowing were present in both streptozotocin-diabetic and DIO C57Bl/6 J mice but not in AVE7688 treated C57Bl/6 J mice or NEP −/− mice exposed to either streptozotocin-induced diabetes or a high fat diet. Intra-epidermal nerve fiber (IENF) profiles were decreased in the hindpaw of C57Bl/6 J diabetic or DIO mice and this improved when the mice were treated with AVE7688. IENF profiles were not decreased in diabetic or DIO NEP (−/−) mice. These studies suggest that NEP plays a role in regulating nerve function in insulin-deficient diabetes and DIO.

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1. Introduction

Diabetes is the most common cause of peripheral nerve damage rendering both diffuse damage referred to as polyneuropathy and focal damage or mononeuropathy (Toth et al., 2004; Zochodne, 2007). It is known that painful sensory neuropathy is also associated with impaired glucose tolerance or metabolic syndrome (Singleton et al., 2001a,b; Sumner et al., 2003; Pittenger et al., 2005). Animal studies of the pathophysiology of diabetic polyneuropathy have provided a long list of mechanisms and possible treatments but these treatments have generally failed in clinical trials (Zochodne, 2007). Thus, at this time there is no effective therapy for diabetic polyneuropathy. Since the etiology of diabetic polyneuropathy is multi factorial it seems unlikely that a single intervention will be beneficial and a more multi targeted approach is necessary.

My laboratory has been examining the role neutral endopeptidase and the efficacy of the vasopeptidase inhibitor AVE7688 on vascular and neural complications associated with obesity and diabetes (Davidson et al., 2007, 2009a; Oltman et al., 2008, 2009). Vasopeptidase inhibitors block angiotensin converting enzyme and neutral endopeptidase activity (Weber, 1999). Neutral endopeptidase degrades a number of vasoactive peptides including natriuretic peptides, adrenomedullin, bradykinin, and calcitonin gene-related peptide (Pu and Schiffrin, 2001). Neutral endopeptidase is found in many tissues including vascular and renal tissue and its activity is increased by fatty acids and glucose in human microvascular cells (Vatter et al., 1998; Gonzalez et al., 1998; Edwards et al., 1999; Ebihara et al., 2003; Muangman et al., 2003). In the peripheral nervous system neutral endopeptidase is located in Schwann cell membranes surrounding dorsal root ganglion cells and nerve fibers (Matsas et al., 1986: Kioussi et al., 1995).

Previously we have demonstrated that treatment of types 1 and 2 diabetic rats and non-diabetic obese Zucker rats with AVE7688 is

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effective in improving microvascular and neural complications (Davidson et al., 2007, 2009a; Oltman et al., 2008, 2009). In order to further investigate the role of neutral endopeptidase in peripheral nerve dysfunction we examined the effect of streptozotocin-induced diabetes and diet induced obesity on nerve conduction velocity and thermal response latency in the hindpaw of C57Bl/6J mice and mice deficient in neutral endopeptidase (Davidson et al., 2009b). In this study we found that neutral endopeptidase deficient mice are protected from the slowing of nerve conduction velocity and thermal hypoalgesia that occur in streptozotocin-induced diabetic- or diet induced obesity-C57Bl/6J mice. For more clinical relevance in these studies we examined the efficacy of AVE7688 treatment on neural complications due to obesity and streptozotocin-induced diabetes in mice. Sciatic nerve conduction velocity slowing and prolonged paw thermal response latency were used as indices of large and small fiber dysfunction respectively in both the streptozotocin-treated and high fat fed mouse models of peripheral neuropathy.

2. Materials and methods

Unless stated otherwise all chemicals used in these studies were obtained from Sigma Chemical Co. (St. Louis, MO).

2.1. Animals

C57Bl/6J wild type mice were purchased from Jackson Laboratories. Breeding pairs of neutral endopeptidase deficient (NEP −/−) mice were provided by Drs. Lu and Gerard and are on the C57Bl/6J background (Lu et al., 1995). These mice have been bred and a colony created at the Veterans Affairs Medical Center, Iowa City, Iowa. The C57Bl/6J and NEP −/− mice were age matched for these studies. Deficiency of neutral endopeptidase activity was confirmed in the mice by measuring the specific activity of neutral endopeptidase in kidney homogenates using the method described by Ayoub and Melzig (2005) with modification. Activity of neutral endopeptidase in kidney from C57Bl/6J and NEP −/− mice was 3.04 ± 0.39 and $0.14 \pm 0.03^{\#}$ mM 7-amido-3-methylcoumarin (AMC)/min/mg protein, respectively ($\#p < 0.001$ versus C57Bl/6J by unpaired t-test). This test was performed on all mice used in these studies in order to confirm that NEP was functionally "knocked out" in the NEP −/− mice.

Mice were housed in a certified animal care facility and food (Harlan Teklad, #7001, Madison, WI) and water were provided ad libitum. Adequate measures were taken to minimize pain or discomfort and all of the experiments were conducted in accordance with international standards on animal welfare and were compliant with all institutional and National Institutes of Health guidelines for use of animals (ACURF protocol 0802033).

C57Bl/6J and NEP −/− mice at 12 weeks of age were divided into three groups. One group was fed a standard chow diet with or without AVE7688 (500 mg/kg in the diet). We have found that this dose provides maximal inhibition of neutral endopeptidase and angiotensin converting enzyme activity in vivo. A second group was fed a high fat diet containing 24 g% fat, 24 g% protein and 41 g% carbohydrate (D12451; Research Diets, New Brunswick, NJ) with or without AVE7688. The primary source of the increased fat content in the diet was soybean oil and lard. The average fat content of the control diet was 4.25 g% (Harlan Teklad, #7001, Madison, WI). The third group was treated with streptozotocin (150 mg/kg i.p in saline) to induce diabetes. Mice having blood glucose level of 300 mg/dl (16.7 mM) or greater were considered to be diabetic. This group was fed a standard chow diet with or without AVE7688. Treatment with AVE7688 was started at two different time points at the onset of the high fat diet or after verification of hyperglycemia (prevention group) or after 6 weeks duration of the high fat diet or diabetes (intervention group). The experimental period lasted for 12 weeks thus the intervention group was treated with AVE7688 for the final 6 weeks of the experimental period.

2.2. Glucose tolerance

Prior to behavioral and nerve conduction studies control and high fat fed mice were fasted overnight for study of glucose utilization. Mice were injected with a saline solution containing 2 g/kg glucose, i.p. Immediately prior to the glucose injection and for 120 min afterwards blood samples were taken to measure circulating glucose levels.

2.3. Thermal nociceptive response

The day before the terminal studies thermal nociceptive response in the hindpaw was measured using the Hargreaves method with instrumentation provided by IITC Life Science; Woodland Hills, CA (model 390G) or UARD (San Diego). The test was performed when possible in a blind manner. Thermal nociceptive responses were measured by placing the mouse in the observation chamber on top of the thermal testing apparatus and allowing it to acclimate to the warmed glass surface (30 °C) and surroundings for a period of 15 min. The mobile heat source was maneuvered so that it was under the heal of the hindpaw and then activated, a process that starts a timer and locally warms the glass surface, when the mouse withdrew its paw, the timer, and the heat source was turned off (Calcutt et al., 1996). Following an initial recording, which was discarded, four measurements were made for each hindpaw, with a rest period of 5 min between each set of measurements. The mean of the measurements, reported in seconds, were used as a measure of the thermal nociceptive response latency.

2.4. Motor and sensory nerve conduction velocity

Mice were anesthetized with Nembutal (75 mg/kg, i.p., Abbott Laboratories, North Chicago, IL) and non-fasting blood glucose levels determined with the use of glucose oxidase reagent strips (Lifescan Inc., Milpitas, CA). Afterwards, motor and sensory nerve conduction velocities were determined as previously described (Davidson et al., 2007, 2009b; Oltman et al., 2008, 2009). Briefly, motor nerve conduction velocity was determined using a noninvasive procedure in the sciatic-posterior tibial conducting system in a temperature controlled environment. The left sciatic nerve was stimulated first at the sciatic notch and then at the Achilles tendon. Stimulation consisted of single 0.2-ms supramaximal (8 V) pulses through a bipolar electrode (Grass S44 Stimulator; Grass Medical Instruments, Quincy, MA). The evoked potentials were recorded from the interosseous muscle with a unipolar platinum electrode and displayed on a digital storage oscilloscope (model 54600A; Hewlett-Packard, Rolling Meadows, IL). Motor nerve conduction velocity was calculated by subtracting the distal from the proximal latency (measured in milliseconds) from the stimulus artifact of the take-off of the evoked potential, and the difference was divided into the distance between the two stimulating electrodes (measured in millimeters using a Vernier caliper). Sensory nerve conduction velocity was recorded in the digital nerve to the second toe by stimulating with a square-wave pulse of 0.05-ms duration using the smallest intensity current that resulted in a maximal amplitude response (Grass S44 Stimulator; Grass Medical Instruments, Quincy, MA). The sensory nerve action potential was recorded behind the medial malleolus. The maximal sensory nerve conduction velocity was calculated by measuring the latency to the onset/peak of the initial negative deflection and the distance between stimulating and recording electrodes. The motor and sensory nerve conduction velocity were reported in meters per second.

2.5. Intraepidermal nerve fiber density

Immunoreactive intraepidermal nerve fiber profiles were visualized using confocal microscopy. Biopsies of skin of the right hindpaw were fixed, dehydrated and embedded in paraffin. Sections (7 μm) were collected and immuno stained with anti-PGP9.5 antibody (rabbit anti human, AbD Serotic, Morpho Sys US Inc., Raleigh,

Table 1
Weight change and blood glucose values for C57Bl/6J and NEP −/− mice.

C57Bl/6J	Control (20)	Control + AVE7688 (14)	Diabetic (26)	Diabetic + AVE7688 (25)	High Fat (27)	High Fat + AVE7688 (28)
Start weight (g)	27.2 ± 0.4	26.7 ± 0.6	27.8 ± 0.4	27.2 ± 0.4	28.0 ± 0.4	27.6 ± 0.3
End weight (g)	31.4 ± 0.4	30.2 ± 0.5	$25.2 \pm 0.5^{\#}$	$24.9 \pm 0.5^{\#}$	$42.2 \pm 0.9^{\#}$	$30.0 \pm 0.4^{+}$
Blood glucose (mg/dl)	221 ± 9	176 ± 11	$572 \pm 9^{\#}$	$499 \pm 28^{\#,+}$	181 ± 9	183 ± 8
NEP −/−	Control (24)	Control + AVE7688 (10)	Diabetic (18)	Diabetic + AVE7688	High Fat (34)	High Fat + AVE7688 (22)
Start weight (g)	27.3 ± 0.4	26.1 ± 0.4	26.1 ± 0.3	ND	25.1 ± 0.3	26.4 ± 0.3
End weight (g)	31.4 ± 0.4	28.6 ± 0.5	$23.5 \pm 0.8^{\#}$	ND	$42.2 \pm 0.9^{\#}$	$29.6 \pm 0.6^{+}$
Blood glucose (mg/dl)	150 ± 4	147 ± 9	$592 \pm 7^{\#}$	ND	161 ± 7	152 ± 6

Data are presented as the mean ± SEM. # $p < 0.05$ compared to control, + $p < 0.05$ compared to the respective group. Parentheses indicate the number of experimental animals. ND not determined.

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