Published in final edited form as:

Eur J Pharmacol. 2011 October 15; 668(3): 497–506. doi:10.1016/j.ejphar.2011.07.016.

Effect of treatment of high fat fed/low dose streptozotocin-diabetic rats with Ilepatril on vascular and neural complications

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Abstract

We have previously shown that treating streptozotocin-induced diabetic rats, an animal model of type 1 diabetes, with Ilepatril (an inhibitor of neutral endopeptidase and angiotensin converting enzyme (ACE)) improves vascular and neural function. In this study we sought to determine the effect of Ilepatril treatment of high fat fed/low dose streptozotocin-diabetic rats, a model for type 2 diabetes, on vascular and neural complications. Following 8 weeks on a high fat diet rats were treated with 30 mg/kg streptozotocin (i.p.) and after 4 additional weeks a group of these rats were treated for 12 weeks with Ilepatril followed by analysis of neural and vascular function. Included in these studies were age-matched control rats and rats fed a high fat diet and treated with or without Ilepatril. Diabetic and diet induced obese rats were characteristic for insulin resistance, slowing of nerve conduction velocity, thermal hypoalgesia, reduction in intraepidermal nerve fiber density in the hindpaw and impairment in vascular relaxation to acetylcholine and calcitonin gene-related peptide in epineurial arterioles of the sciatic nerve. Treatment with Ilepatril was efficacious in improving all of these endpoints although improvement of insulin resistance in diabetic rats was minimal. These studies suggest that dual inhibition of angiotensin converting enzyme and neutral endopeptidase activity of type 2 diabetic rats is an effective approach for treatment of diabetic neural and vascular complications.

Keywords

diabetes; diabetic neuropathy; vascular relaxation; neutral endopeptidase; vasoepitidase inhibitor; angiotensin converting enzyme

1. Introduction

My laboratory has focused on the role of microvascular dysfunction in the development and progression of diabetic neuropathy. Studies in both types 1 and 2 diabetic rats have demonstrated that impaired vascular reactivity precedes the development of nerve dysfunction (Coppey, et al. 2000; 2002). Moreover, our studies have indicated that increased oxidative stress is a factor leading to impaired vascular function in diabetes (Coppey, et al. 2001a; 2001b). This has led to the study of the effect of vasoepitidase inhibitors (Davidson,

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The authors have no conflicts of interest to report.

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et al. 2007; 2009; Oltman, et al. 2009). Vasopectidase inhibitors simultaneously inhibit neutral endopeptidase and angiotensin converting enzyme (ACE) activity (Weber, 1999). Inhibition of ACE activity has been shown to be a primary treatment for hypertension (Abassi, et al. 2009; Hanif, et al. 2010). We and others have demonstrated that ACE inhibition is also an effective treatment for diabetic neuropathy (Reja, et al. 1995; Malik, et al. 1998; Coppey, et al. 2006). Neutral endopeptidase is found in many tissues including vascular, renal, adipose and skeletal muscle and its activity is increased by fatty acids and glucose in human microvascular cells (Gonzalez, et al. 1998; Vatter, et al. 1998; Edwards, et al. 1999, Schling and Schafer, 2002; Ebihara, et al. 2003; Muangman, et al. 2003; Broccolini, et al. 2006). Neutral endopeptidase activity has been shown to be activated by protein kinase C, which is increased in vascular tissues by diabetes (Suzki, et al. 2002; Kikkawa, et al. 2004). Neutral endopeptidase degrades vasoactive peptides including natriuretic peptides, adrenomedullin, bradykinin and calcitonin gene-related peptide (Pu and Schiffrin, 2001). Therefore, use of vasopectidase inhibitors would likely promote expression of these peptides by blocking degradation and thus, improving vascular functions. In this regard, vascular conductance in the femoral artery of diabetic rats was improved by a vasopectidase inhibitor (Arbin, et al. 2000). It has also been demonstrated that vasopectidase inhibitors are neuro-protective and prevent nephropathy in ZDF rats (Schafer, et al. 2003; 2004a; 2004b; Cha, et al. 2005).

The goal of this study was to test the efficacy of Ilepatril, a vasopectidase inhibitor, on vascular and neural function in high fat fed/low dose streptozotocin-treated rats. The high fat fed/low dose streptozotocin-treated rats are an animal model for type 2 diabetes (Reed, et al. 2000; Srinivasan, et al. 2005). Rats fed a high fat diet do not become hyperglycemic presumably due to compensatory hyperinsulinemia (Reed, et al. 2000). However, treating high fat fed rats with a low dose of streptozotocin damages insulin producing β -cells so that hyperglycemia develops even though insulin levels are similar or even higher than in chow fed normoglycemia rats (Reed, et al. 2000). The diabetes in these rats is analogous to the development of human type 2 diabetes when the decline in hyperinsulinemia is not able to compensate for insulin resistance and hyperglycemia occurs (Reed, et al. 2000). This diabetic model has been used for pharmacological drug screening; however, little is known about the impact hyperglycemia has on vascular and neural functions in high fat fed animals.

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

Male Sprague-Dawley (Harlan Sprague Dawley, Indianapolis, IN) rats 10–11 weeks of age were housed in a certified animal care facility and food (Harlan Teklad, #7001, Madison, WI) and water were provided ad libitum. All institutional (approval ACURF #0210257) and NIH guidelines for use of animals were followed. At 12 weeks of age the rats were separated into five groups. Four of these groups were placed on a high fat diet (D12451; Research Diets, New Brunswick, NJ). The high fat diet contained 24 gm% fat, 24 gm% protein and 41 gm% carbohydrate. The primary source of the increased fat content in the diet was soybean oil and lard. The remaining group was maintained on the control diet (Harlan Teklad, #7001, Madison, WI), which contained 4.25 gm% fat. Rats were maintained on the high fat diet for 8 weeks. Afterwards, two of the four groups of high fat fed rats were treated with streptozotocin (30 mg/kg in 0.9% NaCl. i.p.). The remaining two groups of high fat fed rats were injected with vehicle alone. Diabetes was verified 96 h later by evaluating blood glucose levels with the use of glucose-oxidase reagent strips (Lifescan Inc., Milpitas, CA). Rats having blood glucose level of 11.1 mM or greater were considered to be diabetic. These

four groups of rats were maintained on the high fat diet for an additional 4 weeks. Afterwards, one of the diabetic groups and non-diabetic groups were placed on the high fat diet containing Ilepatri, (AVE7688 (vasopeptidase inhibitor), Sanofi Aventis 500 mg/kg in the diet). The dose of Ilepatri used in this study was based on previous studies and pharmacokinetic data (Azizi, et al. 2006; Davidson, et al. 2007). Based upon the amount of diet consumed the Ilepatri treated rats received about 20–25 mg/kg rat/day of the drug. The other group of diabetic and non-diabetic rats remained on the high fat diet. These diets were maintained for 12 weeks. In a subset of control rats we tested the effect of streptozotocin (30 mg/kg, i.p.) on the development of hyperglycemia. Six rats were injected with streptozotocin and over the period of 20 weeks none of these rats became hyperglycemic or insulin resistant as determined by a glucose tolerance test (data not shown).

2.2. Glucose tolerance

Glucose tolerance was determined by injecting rats with a saline solution containing 2 g/kg glucose, i.p., after an overnight fast. Rats were briefly anesthetized with isoflurane and the glucose solution was injected. Immediately prior to the glucose injection and at 15, 30, 45, 60, 120, 180 and 240 min blood samples from the tip of the tail that was lanced were taken to measure circulating glucose levels using glucose oxidase reagent strips (Lifescan Inc., Milpitas, CA). Fasting basal levels of insulin and leptin was also determined using Luminex technology (Davidson, et al. 2010). Briefly, 10 μ L of standards or sample was mixed with reagent buffer and placed in the appropriate wells of the 96 well plate. Afterwards, 25 μ L of the mixed beads containing antibody to the protein of interest and the plate was agitated on a plate shaker for 16–18 h at 4° C. After washing, the detection antibody was added and incubated for 1 h with agitation. After washing, Sheath Fluid was added to each well to resuspend the beads and the plate was run on the Luminex system.

2.3. Thermal nociceptive response

Thermal nociceptive response in the hindpaw was measured using the Hargreaves method as previously described (Oltman, et al. 2008). Briefly, the rat was placed in the observation chamber on top of the thermal testing apparatus and allowed 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 heel of the hindpaw and then activated, a process that activates a timer and locally warms the glass surface, when the rat withdrew its paw, the timer, and the heat source was turned off and the time was recorded. The timer was defaulted to go off after 25 sec to avoid injury to the rat. Following an initial recording, which was discarded, four measurements were made for each hindpaw, with a rest period of 5 min between each measurement. The mean of the measurements reported in sec were used as the thermal nociceptive response.

2.4. Motor and sensory nerve conduction velocity and biological and oxidative stress markers

On the day of terminal studies rats were weighed and anesthetized with Nembutal i.p. (50 mg/kg, i.p., Abbott Laboratories, North Chicago, IL). Non-fasting blood glucose was determined. Hemoglobin A₁C levels were determined using a Glyco-tek affinity column kit (Helena Laboratories, Beaumont, TX). Serum samples were collected for determination of free fatty acid, triglyceride and free cholesterol, using commercial kits from Roche Diagnostics, Mannheim, Germany; Sigma Chemical Co., St. Louis, MO; and Bio Vision, Mountain View, CA, respectively. Serum thiobarbituric acid reactive substances levels were determined as a marker of oxidative stress as previously described (Oltman, et al. 2005). Briefly, 200 μ L of serum was boiled in 0.75 ml of phosphoric acid (0.19 M), 0.25 ml thiobarbituric acid (0.42 mM) and 0.3 ml water for 60 min. Afterwards, the samples were precipitated with methanol/NaOH and centrifuged for 5 min. The supernatant was measured

fluorometrically at excitation wavelength of 532 nm and emission wavelength of 553 nm. Standards were prepared by the acid hydrolysis of 1,1,3,3-tetraethoxypropane. The data was reported as $\mu\text{g/ml}$ serum. ACE activity in the serum was quantitated using a colorimetric assay kit from ALPCO diagnostics (Windham, NH) and the data presented as mU/ml serum (Coppey, et al. 2006). One unit of ACE activity is defined as the amount of enzyme required to release one μmol of hippuric acid per minute and per liter of serum at 37°C . Diabetes induced decrease in tissue glutathione levels has been shown to be a marker for an increase in oxidative stress (Coppey, et al. 2002). Lens glutathione levels were determined according to Coppey et al. (2001b). Lenses were weighed and homogenized in 1 ml of cold 10% trichloroacetic acid and centrifuged for 15 min at 1,000g. The supernatant (100 μl) was mixed with 0.89 ml of 1.0 mol/l Tris, pH 8.2, and 0.02 mol/l EDTA. Afterward, 10 ml dithio nitrobenzene was added and change in absorbance measured at 412 nm. A GSH standard curve (100–500 ng) was performed for each assay. The data were recorded as micrograms per milligram of wet weight.

For the determination of sciatic nerve sorbitol, fructose and myo-inositol levels, tissue samples of sciatic nerve were weighed and boiled for 10 min in water containing $\alpha\text{-D-methylmannopyranoside}$ as an internal standard and deproteinized with 0.5 ml each of 0.19 mol/l Ba(OH)_2 and 0.19 mol/l ZnSO_4 . After centrifugation, the supernatant was collected, frozen, and lyophilized. Afterward, the samples were derivatized, and intracellular content of sorbitol, fructose and myo-inositol was determined by gas-liquid chromatography as previously described (Coppey, et al. 2001b).

Motor nerve conduction velocity (MNCV) was determined as previously described using a noninvasive procedure in the sciatic-posterior tibial conducting system (Coppey, et al. 2000). 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). MNCV 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 2 stimulating electrodes measured in millimeters using a Vernier caliper. Sensory nerve conduction velocity (SNCV) was determined using the digital nerve as described by Obrosova et al (Obrosova, et al. 2004). Briefly, hindlimb SNCV 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. The sensory nerve action potential was recorded behind the medial malleolus. Sixteen responses were averaged to obtain the position of the negative peak. The maximal SNCV was calculated by measuring the latency to the onset/peak of the initial negative deflection and the distance between stimulating and recording electrodes. The MNCV and SNCV were reported in meters per second.

2.5. Intraepidermal nerve fiber density in the hindpaw

Immunoreactive intraepidermal nerve fiber profiles, which are primarily sensory nerves, were visualized using confocal microscopy. Samples 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 Serotec, Morpho Sys US Inc., Raleigh, NC) over night followed by treatment with secondary antibody Alexa Fluor 546 goat anti rabbit (Invitrogen, Eugene, OR). Profiles were counted by two individual investigators that were blinded to the sample identity. All immunoreactive profiles within the epidermis were counted and normalized to epidermal length (Beiswenger, et al. 2008; Davidson, et al. 2010).

2.6. Vascular reactivity in epineurial arterioles

Videomicroscopy was used to investigate *in vitro* vasodilatory responsiveness of epineurial arterioles vascularizing the region of the sciatic nerve as previously described (Coppey, et al. 2000; 2001b; 2006). The vessels used for these studies were generally oriented longitudinally in relation to the sciatic nerve; however, radially oriented vessels were also used on occasion. The arterioles used in this study should be regarded as epineurial rather than perineurial vessels. To isolate these vessels, the common iliac was exposed, and the branch points of the internal pudendal and superior gluteal arteries were identified. The vessels were then clamped, and tissue containing these vessels and the branches at the internal pudendal and superior gluteal arteries were dissected en bloc. The block of tissue was immediately submerged in a cooled (4°C), oxygenated (20% O₂, 5% CO₂, and 75% N₂) Krebs-Henseleit physiological saline solution (PSS) of the following composition (in millimoles per liter): NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 20, Na₂EDTA 0.026, and glucose 5.5. Branches of the superior gluteal and internal pudendal arteries (50- to 150-μm internal diameter and 1–2 mm in length) were carefully dissected and trimmed of fat and connective tissue. Both ends of the isolated vessel segment were cannulated with glass micropipettes filled with PSS (4°C) and secured with 10–0 nylon Ethilon monofilament sutures (Ethicon, Cornelia, GA). The pipettes were attached to a single pressure reservoir (initially set at 0 mmHg) under condition of no flow. The organ chamber containing the cannulated vessels was then transferred to the stage of an inverted microscope (CK2; Olympus, Lake Success, NY). Attached to the microscope were a closed-circuit television camera (WV-BL200; Panasonic, Secaucus, NJ), a video monitor (Panasonic), and a video caliper (VIA-100K; Boeckeler Instruments, Tucson, AZ). The organ chamber was connected to a rotary pump (Masterflex; Cole Parmer Instrument, Vernon Hills, IL), which continuously circulated 37°C oxygenated PSS at 30 ml/min. The pressure within the vessel was then slowly increased to 40 mmHg. At this pressure, we found that KCl gave the maximal constrictor response. Therefore, all of the studies were conducted at 40 mmHg. Internal vessel diameter (resolution of 2 μm) was measured by manually adjusting the video micrometer. After a 30-min equilibration, KCl was added to the bath to test vessel viability. Vessels failing to constrict by at least 30% were discarded. After they were washed with PSS, vessels were incubated for 30 min in PSS and then constricted with U46619 (10⁻⁸ to 10⁻⁷ mol/l) (Cayman Chemical, Ann Arbor, MI) to 30–50% of passive diameter. Afterwards, cumulative concentration-response relationships were evaluated for acetylcholine (10⁻⁸–10⁻⁴ M) and calcitonin gene related peptide (10⁻¹¹–10⁻⁸ M) using vessels from each group of rats. At the end of each dose response curve for acetylcholine or calcitonin gene related peptide papaverine (10⁻⁵ M) was added to determine maximal vasodilation.

2.7. Superoxide and nitrotyrosine staining

Hydroethidine (Molecular Probes Inc., Eugene, OR), an oxidative fluorescent dye, was used to evaluate *in situ* levels of superoxide (O₂⁻) in epineurial vessels (Coppey, et al. 2001a; b). Hydroethidine is permeable to cell membranes, and in the presence of O₂⁻ it is oxidized to fluorescent ethidium bromide, where it is trapped by intercalating with DNA. Unfixed frozen vessel segments were cut in 10 μm sections and placed on glass slides. Hydroethidine (2 μM) was topically applied to each tissue section and cover slipped. Slides were incubated in a light-protected humidified chamber at 37° C for 30 min. Images were obtained with a Zeiss LSM710 confocal microscope. Vessels from control and high fat fed rats were processed and imaged in parallel. Laser settings were identical for acquisition of all images from control and high fat/streptozotocin diabetic rats.

Superoxide anion can interact with nitric oxide to form peroxynitrite (Wattanapitayakul, et al. 2000). This reaction reduces the efficacy of nitric oxide to act as a signal transduction

agent. Peroxynitrite is a highly reactive intermediate known to nitrate protein tyrosine residues and cause cellular oxidative damage (Pryor and Squadrito, 1995; Beckman, 1996). To determine formation of peroxynitrite we measured 3-nitrotyrosine (a stable biomarker of tissue peroxynitrite formation) (Davidson, et al. 2007). Briefly, frozen tissue segments of arterioles were cut into 10 μm sections and then incubated in phosphate buffered saline solution containing 1% Triton X-100 and 0.1% bovine serum albumin for 30 min at room temperature. Afterwards, the samples were incubated in this buffer solution containing mouse anti-nitrotyrosine antibody (Upstate, Lake Placid, NY) overnight at 4°C. After washing, the sections were incubated for 2h with Alexa Fluor 546 goat anti-mouse IgG (Molecular Probes, Eugene, OR). Sections were then rinsed and mounted with VectorShield. The labeled vessels derived from these studies were visualized with a Zeiss LSM710 confocal microscope.

Images for superoxide and nitrotyrosine were quantified using the ZEN image analysis software. The amount of immunostaining was determined by dividing the total intensity of the stained regions by their area. This analysis excludes the area of the unstained lumen.

2.8. Data Analysis

Results are presented as mean $\pm$ S.E.M. Comparisons between the treatment groups and control and non-treated diabetic rats were conducted using one-way ANOVA and Bonferroni post test comparison (Prism software; GraphPad, San Diego, CA). Concentration response curves for acetylcholine and calcitonin gene-related peptide were compared using a two-way repeated measures analysis of variance with autoregressive covariance structure using proc mixed program of SAS (Coppey, 2001b; 2006). A P value of less than 0.05 was considered significant.

3. Results

3.1. Effect of treatment of high fat rats and high fat/streptozotocin-diabetic rats with Ilepatril on weight gain, blood glucose epididymal fat pad and gastrocnemius muscle mass and serum insulin and leptin levels

Data in Table 1 demonstrate that high fat fed rats gained significantly more weight compared to control rats and that treatment with Ilepatril prevented the increased weight gain. Diabetic rats gained about the same amount of weight as control rats and this was not altered by treatment. All diabetic rats were hyperglycemic at the end of the study period as indicated by significantly elevated blood glucose and hemoglobin A_{1c} levels (Table 1). Rats fed a high fat diet only were not hyperglycemic. Epididymal fat pad mass was significantly increased in high fat fed rats and high fat/streptozotocin-diabetic rats. Ilepatril treatment significantly prevented the increase in fat pad mass in high fat fed rats but this was insignificantly prevented in high fat/streptozotocin-diabetic rats. Gastrocnemius muscle mass was not changed in high fat fed rats or high fat/streptozotocin-diabetic rats compared to control rats (Table 1). Serum insulin and leptin levels were significantly increased in high fat fed rats and this was prevented with Ilepatril treatment. Insulin and leptin levels in high fat/streptozotocin-diabetic rats were similar to control rats and this was not changed with treatment.

3.2. Effect of treatment of high fat rats and high fat/streptozotocin-diabetic rats with Ilepatril on thiobarbituric acid reactive substances levels, serum lipids and ACE activity and lens glutathione levels

Data in Table 2 demonstrate that serum thiobarbituric acid reactive substances, a marker for oxidative stress, were significantly increased in high fat fed rats and high fat/streptozotocin-diabetic rats and this was prevented with Ilepatril treatment. Serum triglycerides levels were

significantly increased in high fat/streptozotocin-diabetic rats and this was prevented with Ilepatril treatment (Table 2). Serum triglyceride levels were not increased in high fat fed rats. Serum free fatty acids were significantly increased in high fat fed rats and this was prevented by Ilepatril treatment. Serum free fatty acids were also significantly increased in high fat/streptozotocin-diabetic rats but this was not corrected with treatment. Serum cholesterol levels were significantly increased in high fat fed rats and high fat/streptozotocin-diabetic rats and this was prevented with Ilepatril treatment. Serum angiotensin converting enzyme (ACE) activity was increased in high fat fed rats and high fat/streptozotocin-diabetic rats. As expected treating these rats with Ilepatril lowered serum ACE activity (Table 2). Lens glutathione levels were significantly decreased in high fat/streptozotocin-diabetic rats and this was prevented with Ilepatril treatment (Table 2). Lens glutathione levels were not changed in high fat fed rats.

3.3. Effect of treatment of high fat rats and high fat/streptozotocin-diabetic rats with Ilepatril on sciatic nerve sorbitol, fructose and myo-inositol levels

Diabetes is known to cause an increase in sciatic nerve sorbitol and fructose levels and decrease in myo-inositol levels and this is confirmed in Table 3 (Finegold, et al. 1983). This change has been reported to be due to an increase in activity of aldose reductase and blocking aldose reductase has been shown to improve diabetic neuropathy (Coppey, et al. 2002). Levels of sorbitol, fructose or myo-inositol in sciatic nerve were not changed in rats fed a high fat diet. In contrast, sorbitol and fructose levels were increased and myo-inositol levels decreased in sciatic nerve from high fat/streptozotocin-diabetic rats. Sorbitol in the sciatic nerve was partially (without significant difference) and myo-inositol was significantly corrected in high fat/streptozotocin-diabetic rats treated with Ilepatril, whereas fructose levels remained significantly increased.

3.4. Effect of treatment of high fat rats and high fat/streptozotocin-diabetic rats with Ilepatril on glucose tolerance

Data in Fig. 1 demonstrate that glucose utilization is significantly impaired in high fat fed rats and to a greater extent in high fat/streptozotocin-diabetic rats. Treating high fat fed rats with Ilepatril improved glucose utilization. In contrast, treating high fat/streptozotocin-diabetic rats with Ilepatril did not significantly improve glucose utilization. To examine the effect of treatment of high fat/streptozotocin-diabetic rats on insulin stimulated glucose utilization we examined insulin-stimulated glucose uptake by isolated soleus muscle from control and high fat/streptozotocin-diabetic rats treated without or with Ilepatril as previously described (Bouskila, et al. 2008). Insulin (100 nM) stimulated glucose uptake by soleus muscle by 1.94 ± 0.07 , $1.52 \pm 0.05^*$ and 1.93 ± 0.20 fold for control, high fat/streptozotocin-diabetic and high fat/streptozotocin-diabetic rats treated with Ilepatril, respectively (mean $\pm$ S.E.M. from 8 individual rats for each group, * $P < 0.05$, compared to control).

3.5. Effect of treatment of high fat rats and high fat/streptozotocin-diabetic rats with Ilepatril on nerve conduction velocity, thermal nociception and intraepidermal nerve fiber density

Motor and sensory nerve conduction velocity was significantly decreased in high fat/streptozotocin-diabetic rats and this was prevented by treatment with Ilepatril (Fig. 2). However, in high fat fed rats only sensory nerve conduction velocity was significantly impaired and this was insignificantly prevented by treatment with Ilepatril. Data in Fig. 3 demonstrate that high fat fed rats and high fat/streptozotocin-diabetic rats are hypoalgesic to thermal stimuli and this was prevented by treatment with Ilepatril. Intraepidermal nerve fiber profiles in the hindpaw of high fat fed rats and high fat/streptozotocin-diabetic rats are significantly decreased and this was significantly prevented by treatment with Ilepatril in

high fat/streptozotocin-diabetic rats but the difference was insignificant in high fat fed rats (Fig. 3).

3.6. Effect of treatment of high fat rats and high fat/streptozotocin-diabetic rats with Ilepatril on vascular relaxation by epineurial arterioles

We have previously demonstrated that type 1 streptozotocin-induced diabetes caused a decrease in acetylcholine-mediated vascular relaxation that was improved with treatment using Ilepatril or Enalapril (Coppey, et al. 2006; Davidson, et al. 2007). In this study we examined the effect of treatment with Ilepatril on acetylcholine-mediated vascular relaxation using high fat fed rats and high fat/streptozotocin-diabetic rats (Fig. 4). Acetylcholine-mediated vascular relaxation was significantly impaired in high fat fed rats and high fat/streptozotocin-diabetic rats. Treatment of these rats with Ilepatril significantly improved acetylcholine-mediated vascular relaxation compared to untreated diabetic rats.

Fig. 5 provides data on the effect of high fat diet and high fat/streptozotocin-induced diabetes and treatment with Ilepatril on vascular relaxation mediated by calcitonin gene-related peptide in epineurial arterioles. Calcitonin gene-related peptide is the most potent vasodilator of epineurial arterioles that we have identified and the potency is decreased by chronic type 1 diabetes (Yorek, et al. 2004). Calcitonin gene-related peptide is degraded by neutral endopeptidase and we have previously demonstrated that treatment of streptozotocin type 1 diabetic rats with Ilepatril improves calcitonin gene-related peptide-mediated vascular relaxation in epineurial arterioles (Davidson, et al. 2007). In this study we demonstrate that vascular relaxation by calcitonin gene-related peptide was significantly impaired in epineurial arterioles from high fat fed rats and high fat/streptozotocin-diabetic rats. Treating high fat fed rats and high fat/streptozotocin-diabetic rats with Ilepatril significantly improved calcitonin gene-related peptide-mediated vascular relaxation in epineurial arterioles.

3.7. Effect of treatment of high fat/streptozotocin-diabetic rats with Ilepatril on superoxide and nitrotyrosine staining by epineurial arterioles

Data in Fig. 6 demonstrate that high fat/streptozotocin-diabetes caused a significant increase in superoxide and nitrotyrosine staining in epineurial arterioles of the sciatic nerve and this was reduced by treatment with Ilepatril. Analysis from three individual groups of rats demonstrated that superoxide and nitrotyrosine staining was increased more than 2 fold in high fat/streptozotocin-diabetic rats and this was completely prevented by treatment with Ilepatril. Superoxide and nitrotyrosine staining is not increased in epineurial arterioles from high fat fed rats (Davidson, et al. 2010).

4. Discussion

We previously reported that Ilepatril was an efficacious treatment for vascular and neural complications in streptozotocin type 1 diabetic rats (Davidson, et al. 2007). We attributed this efficacy to anti-oxidant properties and protection of vasoactive peptides from degradation. Vasopeptidase inhibitors have been previously shown to provide greater renal protection than ACE inhibitors in a rat model subjected to nephrectomy and in lowering blood pressure in hypertensive rats (Taal, et al. 2001). In other rat models of renal vascular disease and hypertension vasopeptidase inhibitor treatment restored renovascular endothelial function, prevented vascular hypertrophy and lowered systolic blood pressure (Quaschnig, et al. 2001; Ferrario, et al. 2002). In Zucker diabetic fatty rats vasopeptidase inhibitor treatment prevented nephropathy (Schafer, et al. 2003; 2004a; 2004b). In this study we sought to determine the effect of Ilepatril treatment on vascular and neural complications in the high fat fed low dose streptozotocin-treated rat (Reed, et al. 2000).

Reed et al (2000) were the first to use the high fat fed streptozotocin treated rat as a model for type 2 diabetes. This group fed Sprague-Dawley rats a high fat diet for 7 weeks and then treated them with streptozotocin (50 mg/kg). These rats were hyperglycemic, insulin resistant and sensitive to the glucose lowering effects of metformin and troglitazone. Others found this model to be suitable for testing anti-diabetic agents for the treatment of type 2 diabetes (Reed, et al. 2000; Zhu, et al. 2010; Wang, et al. 2009; Zhang, et al. 2008; Gaikwad, et al. 2007; Ding, et al. 2005). The literature contains some variability regarding the dose of streptozotocin used to create this model. Generally the high fat diet is about the same ranging from 40–50% calories as fat for a period of 4–8 weeks. However, the dose of streptozotocin used ranges from 20–50 mg/kg (Reed, et al. 2000; Wang, et al. 2011). It has been our experience that after 8 weeks on a high fat diet 20 mg/kg streptozotocin was too low and resulted in a low success rate of hyperglycemia. When we used 40 mg/kg streptozotocin our mortality rate was high with the rats began dying 2–4 weeks following the injection of streptozotocin. With 30 mg/kg streptozotocin our success rate was over 90% and the mortality rate was less than 5%. The rats do not require insulin treatment to maintain weight unlike streptozotocin-induced type 1 diabetic rat models (Davidson, et al. 2007). We have used rats at 12 weeks of age to initialize our studies. In a study by Wang et al (2011) examining parameters for creating the high fat diet/streptozotocin-diabetic rat model they found that duration of high fat diet, dosage of streptozotocin and the age of rats were the most important factors for establishing this model. As created in our laboratory we agree with Reed et al (2000) that the high fat fed/streptozotocin-diabetic rat models late stage type 2 diabetes in patients.

At the end of the study period the diabetic rats weighed about the same as control rats, insulin and leptin levels were near control values and they were hyperlipidemic. Interestingly, there is little information available about the impact of hyperglycemia on the background of a high fat diet on neural endpoints or neurovascular function. Compared to high fat fed rats we found that following induction of hyperglycemia glucose utilization was further impaired. Furthermore, we found that at the end of the study period weight of the epididymal fat pad was significantly less in the high fat/streptozotocin-diabetic rats compared to high fat fed rats. However, epididymal fat pad weight in the high fat/streptozotocin-diabetic rats was still greater than control rats. Serum insulin and leptin levels are significantly decreased in high fat/streptozotocin-diabetic rats compared to high fat fed rats but similar to control rats. Two interesting differences between high fat fed rats and high fat/streptozotocin-diabetic rats is that in diabetic rats motor nerve conduction velocity was significantly decreased and superoxide levels and nitrotyrosine staining was increased in epineurial arterioles (Davidson, et al. 2007). This would indicate that in the microvasculature oxidative stress is increased with hyperglycemia. In epineurial arterioles from high fat fed rats superoxide levels and nitrotyrosine staining were not increased (Davidson, et al. 2010). Sensory nerve conduction velocity, thermal nociception and intraepidermal nerve fiber profiles are impaired in both high fat fed rats and high fat/streptozotocin-diabetic rats. Interestingly, acetylcholine- and CGRP-mediated vascular relaxations of epineurial arterioles are impaired to a similar degree in high fat fed rats and high fat/streptozotocin-diabetic rats. It was expected that because of the increase in superoxide and nitrotyrosine staining in epineurial arterioles from high fat/streptozotocin-diabetic rats compared to high fat fed rats that vascular relaxation to acetylcholine would be impaired to a greater degree in high fat/streptozotocin-diabetic rats. This suggests that mechanisms in addition to oxidative stress contribute significantly to impairment of vascular function in epineurial arterioles from high fat fed rats and high fat/streptozotocin-diabetic rats. This could include increased expression of neutral endopeptidase. We have shown that diet induced obesity and diabetes causes an increase in the expression of neutral endopeptidase in epineurial arterioles (Davidson, et al. 2007; 2010). In addition, it has been shown that hyperlipidemia and hyperglycemia increases neutral endopeptidase activity in

human microvascular endothelial cells (Muangman, et al. 2003). We propose that Ilepatril in part may be protecting vascular reactivity and neural function by inhibiting neutral endopeptidase and the degradation of C-type natriuretic peptide and calcitonin gene-related peptide (Davidson, et al. 2007; 2009; Oltman, et al. 2009; Yorek, 2008).

In this study treatment of high fat fed rats and high fat/streptozotocin-diabetic rats with Ilepatril improved both vascular and neural dysfunction. With only one exception Ilepatril treatment improved markers of oxidative stress and hyperlipidemia in both high fat fed rats and high fat/streptozotocin-diabetic rats. The only exception was that Ilepatril treatment did not improve the increase in serum free fatty acid levels observed in high fat/streptozotocin-diabetic rats. In addition, Ilepatril treatment did not significantly improve glucose utilization in high fat/streptozotocin-diabetic rats. However, in insulin-stimulated glucose uptake studies by isolated soleus muscle Ilepatril treatment did significantly improve glucose utilization by soleus muscle from high fat/streptozotocin-diabetic rats in vitro. Although not studied it is possible that treatment with Ilepatril for 12 weeks improved insulin signaling by skeletal muscle but when insulin resistance was tested in the whole animal by examining glucose utilization the insulin response in the Ilepatril treated diabetic rat in vivo after 24 weeks on the high fat diet and 16 weeks of hyperglycemia was not sufficient to improve glucose uptake.

Ilepatril, a vasopeptidase inhibitor, blocks both neutral endopeptidase and angiotensin converting enzyme activities. The mechanisms proposed for the improvement in glucose tolerance with treatment of rodent models of obesity or type 2 diabetes with angiotensin converting enzyme inhibitors are increased energy expenditure, liver and adipose tissue metabolic modulation, lower concentration of leptin, improved insulin signaling and increased glucose and fatty acid utilization by muscle (Santos, et al. 2008; 2009; Weisinger, et al. 2009; Araki, et al. 2006; Jayasooriya, et al. 2008; Kouyma, et al. 2005; Nawano, et al. 1999; Henriksen, et al. 1995; 1996; Sugimoto, et al. 2008). In a study comparing the effects of Ramipril, an angiotensin converting enzyme inhibitor, to Ilepatril in JCR:LA-cp rats, an obese, insulin resistant, hyperinsulinemic, normoglycemic model, it was found that both compounds reduced the surge of plasma insulin in a meal tolerance test by about 50% but Ilepatril was more beneficial in improving vascular reactivity (Russell and Kelly, 2004). In another study using obese Zucker rats it was found that dual inhibition of angiotensin converting enzyme and neutral endopeptidase improved insulin mediated glucose disposal more effectively than monotherapy and this effect was linked to increased activation of the kinin-nitric oxide pathway (Arbin, 2001). In a similar independent study it was found that Omapatrilat, a vasopeptidase inhibitor, induced insulin sensitization and increased myocardial glucose uptake in obese Zucker rats and that the effect of Omapatrilat was greater than Ramipril in part due to stimulation of the B₂ receptor (Wang, 2003). Later this group reported that treatment of obese Zucker rats with a vasopeptidase inhibitor increased muscle glucose uptake independent of insulin signaling (Wong, 2006). In two of these studies protection of bradykinin from degradation by neutral endopeptidase was found to improve insulin action (Arbin, 2001; Wang, 2003). Interestingly, it has been shown that natriuretic peptides promote muscle mitochondrial biogenesis and fat oxidation as to prevent obesity and glucose intolerance (Miyashita, et al. 2009). The natriuretic peptides are also degraded by neutral endopeptidase (Yorek, 2008). Because neutral endopeptidase is expressed in skeletal muscle in relatively large amounts and being located on the cell surface, neutral endopeptidase is able to hydrolyze peptides in the vicinity of their receptors thereby neutralizing their bioactivity (Yorek, 2008; Broccolini, et al. 2006). Since bradykinin and natriuretic peptides may have a role in regulating glucose and fatty acid metabolism by muscle protecting their bioactive function by preventing degradation may be a therapeutic approach for treatment of obesity and type 2 diabetes (Yorek, 2008; Broccolini, et al. 2006).

5. Conclusions

Studies were performed on the effect of treating high fat fed rats and high fat/streptozotocin-diabetic rats, a model for type 2 diabetes, with Iliopril on nerve function and neurovascular reactivity. We found that treatment with Iliopril improved neural and vascular function in rat models for diet induced obesity and type 2 diabetes. In summary, vasopeptidase inhibitors may be an effective treatment for neural and vascular complications associated with metabolic syndrome and type 2 diabetes.

Acknowledgments

This material is based upon work supported in part by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development, by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK073990 from NIH and by a research grant from the Juvenile Diabetes Research Foundation. The content of this manuscript are new and solely the responsibility of the authors and do not necessarily represent the official views of the granting agencies. The authors would like to extend their appreciation to Sanofi Aventis for supplying Iliopril (AVE7688) for these studies.

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Fig. 1. Effect of a high fat diet, high fat diet and streptozotocin-induced diabetes and treatment with Ilepatril on glucose tolerance

Glucose tolerance was determined as described in the Methods section. Data are presented as the mean $\pm$ S.E.M. in mg/dl. The area under the curve (AUC) was significantly different $p < 0.01$ for high fat fed rats and high fat/streptozotocin-diabetic rats vs. control and high fat fed rats vs. high fat/streptozotocin-diabetic rats ($p < 0.05$). There was a significant improvement in AUC for high fat fed rats treated with Ilepatril compared to high fat fed rats ($p < 0.05$). There was no significant improvement for AUC between high fat/streptozotocin-diabetic rats treated with Ilepatril compared to high fat/streptozotocin-diabetic untreated rats. The number of rats in each group was the same as shown in Table 1.

Fig. 2. Effect of a high fat diet, high fat diet and streptozotocin-induced diabetes and treatment with Ilepatril on motor and sensory nerve conduction velocity

Motor and sensory nerve conduction velocity was examined as described in the Methods section. Data are presented as the mean $\pm$ S.E.M. in m/sec. The number of rats in each group was the same as shown in Table 1. * $P < 0.05$ compared to control; + $P < 0.05$ compared to high fat; # $P < 0.05$ compared to high fat + Ilepatril; \$ $P < 0.05$ compared to diabetic.

Fig. 3. Effect of a high fat diet, high fat diet and streptozotocin-induced diabetes and treatment with Ilepatril on thermal nociception and intraepidermal nerve fiber density

Thermal nociception and intraepidermal nerve fiber density was examined as described in the Methods section. Data are presented as the mean $\pm$ S.E.M. for thermal nociception in sec and intra epidermal nerve fiber profiles per mm. The number of rats in each group was the same as shown in Table 1. * $P < 0.05$ compared to control; + $P < 0.05$ compared to high fat; # $P < 0.05$ compared to high fat + Ilepatril; \$ $P < 0.05$ compared to diabetic.

Fig. 4. Effect of a high fat diet, high fat diet and streptozotocin-induced diabetes and treatment with Ilepatril on vascular relaxation by acetylcholine in epineurial arterioles

Pressurized arterioles (40 mm Hg and ranging from 60–100 μ m luminal diameter) were constricted with U46619 (30–50%) and incremental doses of acetylcholine were added to the bathing solution while recording steady state vessel diameter. Data are presented as the mean of % relaxation $\pm$ S.E.M. For these studies two vessels were collected from each rat, studied and the data combined. The number of rats in each group was the same as shown in Table 1. * $P < 0.05$, compared to control rats fed the standard diet; + $P < 0.05$, compared to the respective untreated high fat fed rats or high fat/streptozotocin-diabetic rats.

Fig. 5. Effect of a high fat diet, high fat diet and streptozotocin-induced diabetes and treatment with Ilepatril on vascular relaxation by calcitonin gene-related peptide in epineurial arterioles Arterioles were treated as described in Fig. 4. Incremental doses of calcitonin gene-related peptide (CGRP) were added to the bathing solution while recording steady state vessel diameter. Data are presented as the mean of % relaxation $\pm$ S.E.M. The number of rats in each group was the same as shown in Table 1. * P 0.05, compared to control rats fed the standard diet; + P < 0.05, compared to the respective untreated high fat fed rats or high fat/streptozotocin-diabetic rats.

Fig. 6. Effect of a high fat diet, high fat diet and streptozotocin-induced diabetes and treatment with Ilepatril on superoxide and nitrotyrosine staining in epineurial arterioles

Presented are representative fluorescent photomicrographs of confocal microscopic sections of epineurial arterioles of the sciatic nerve. Each of the 3 vessels was examined on the same day. Arterioles were labeled with the oxidative dye hydroethidine for superoxide determination (top) or anti-nitrotyrosine for peroxynitrite determination (bottom) as described in the Materials and Methods section. Recording of fluorescence was taken at identical laser and photomultiplier settings for each vessel cross section.

Effect of Treatment of High Fat or High Fat/Streptozotocin-Diabetic Rats with Ilepatriil on Change in Body Weight, Blood Glucose, Hemoglobin A₁C, Epididymal fat and Gastrocnemius muscle mass and Serum Insulin and Leptin levels

Table 1

Determination	Control (17)	High Fat (17)	High Fat + Ilepatriil (11)	Diabetic (15)	Diabetic + Ilepatriil (11)
Start weight (g)	329 ± 5	338 ± 5	324 ± 3	332 ± 5	329 ± 3
End weight (g)	481 ± 8	575 ± 17 ^a	464 ± 7 ^b	472 ± 12 ^b	452 ± 11 ^b
Blood glucose (mM)	6.0 ± 0.2	6.6 ± 0.3	6.3 ± 0.2	20.9 ± 1.2 ^{a,b,c}	14.8 ± 2.2 ^{a,b,c,d}
Hb A ₁ C (%)	6.6 ± 0.6	7.1 ± 0.6	5.6 ± 0.3	14.2 ± 1.7 ^{a,b,c}	9.4 ± 0.5 ^{a,b,c,d}
Epididymal fat pad (g)	6.2 ± 0.4	14.1 ± 0.9 ^a	6.4 ± 0.4 ^b	9.1 ± 0.4 ^{a,b,c}	8.1 ± 0.8 ^b
Gastrocnemius muscle (g)	6.4 ± 0.1	6.7 ± 0.2	5.6 ± 0.3	5.8 ± 0.1	5.9 ± 0.2
Insulin (ng/ml)	1.6 ± 0.3	4.6 ± 0.5 ^a	1.7 ± 0.3 ^b	2.2 ± 0.5 ^b	2.9 ± 0.7
Leptin (pM)	319 ± 49	2208 ± 289 ^a	527 ± 54 ^b	325 ± 84 ^b	330 ± 101 ^b

Data are presented as the mean ± S.E.M.

^ap < 0.05 compared to control;

^bp < 0.05 compared to high fat;

^cp < 0.05 compared to high fat + Ilepatriil;

^dp < 0.05 compared to diabetic.

Parentheses indicate the number of experimental animals.

Table 2

Effect of Treatment of High Fat or High Fat/Streptozotocin-Diabetic Rats with Ilepatriil on Serum Thio Barbituric Acid Reactive Substances, Triglycerides, Free Fatty Acids, Cholesterol and ACE activity, and Lens Glutathione levels

Determination	Control (17)	High Fat (17)	High Fat + Ilepatriil (11)	Diabetic (15)	Diabetic + Ilepatriil (11)
Thiobarbituric acid reactive substances ($\mu\text{g/ml}$)	0.70 ± 0.03	0.94 ± 0.03^a	0.62 ± 0.04^b	$1.03 \pm 0.05^{a,c}$	$0.68 \pm 0.04^{a,b,d}$
Triglycerides (mg/dl)	23 ± 2	23 ± 2	23 ± 2	$61 \pm 13^{a,b,c}$	31 ± 8^d
Free fatty acids (mmol/l)	0.11 ± 0.02	0.26 ± 0.02^a	0.14 ± 0.02^b	$0.33 \pm 0.04^{a,c}$	$0.35 \pm 0.06^{a,c}$
Cholesterol (mg/dl)	130 ± 11	227 ± 34^a	165 ± 18	238 ± 37^a	$103 \pm 9^{b,d}$
ACE (mU/ml/min)	39 ± 5	56 ± 4^a	22 ± 3^b	$66 \pm 10^{a,c}$	$28 \pm 1^{b,d}$
Lens glutathione ($\mu\text{g/mg}$ tissue)	2.5 ± 0.1	2.7 ± 0.1	2.4 ± 0.1	$1.1 \pm 0.1^{a,b,c}$	2.2 ± 0.2^d

Data are presented as the mean $\pm$ S.E.M.

^a $p < 0.05$ compared to control;

^b $p < 0.05$ compared to high fat;

^c $p < 0.05$ compared to high fat + Ilepatriil;

^d $p < 0.05$ compared to diabetic.

Parentheses indicate the number of experimental animals.

Effect of Treatment of High Fat or High Fat/Streptozotocin-Diabetic Rats with Ilepatriil on Sciatic Nerve Sorbitol, Fructose and myo-Inositol content

Table 3

Determination	Control (17)	High Fat (17)	High Fat + Ilepatriil (11)	Diabetic (15)	Diabetic + Ilepatriil (11)
Sorbitol (nmol/mg prot)	0.7 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	3.4 ± 0.6 ^{a,b,c}	2.0 ± 0.5
Fructose (nmol/mg prot)	0.45 ± 0.05	0.48 ± 0.04	0.42 ± 0.03	1.79 ± 0.23 ^{a,b,c}	1.56 ± 0.25 ^{a,b,c}
Myo-Inositol (nmol/mg prot)	7.7 ± 1.1	9.2 ± 1.2	7.7 ± 1.5	4.5 ± 0.6 ^{a,b,c}	7.3 ± 1.3 ^d

Data are presented as the mean ± S.E.M.

^a $P < 0.05$ compared to control;

^b $P < 0.05$ compared to high fat;

^c $P < 0.05$ compared to high fat + Ilepatriil;

^d $P < 0.05$ compared to diabetic. Parentheses indicate the number of experimental animals.

Parentheses indicate the number of experimental animals.