Published in final edited form as:

Eur J Pharmacol. 2011 January 15; 650(2-3): 556–562. doi:10.1016/j.ejphar.2010.10.047.

Role of the effect of inhibition of neutral endopeptidase on vascular and neural complications in streptozotocin-induced diabetic rats

Christine L. Oltman^{a,b}, Eric P. Davidson^b, Lawrence J. Coppey^b, Travis L. Kleinschmidt^a, Brian Dake^b, and Mark A. Yorek^{a,b}

^aDepartment of Veterans Affairs Iowa City Health Care System, University of Iowa, Iowa City, IA, 52246

^bDepartment of Internal Medicine, University of Iowa, Iowa City, IA, 52246

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 individual effect of inhibition of neutral endopeptidase and ACE on diabetes-induced vascular and neural dysfunction. After 4 weeks of untreated diabetes, rats were treated for 12 weeks with Ilepatril, Enalapril (ACE inhibitor) or Candoxatril (neutral endopeptidase inhibitor) followed by analysis of neural and vascular function. Diabetes caused slowing of motor and sensory nerve conduction, 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 and to atrial natriuretic peptide and calcitonin gene-related peptide in renal arteries. Inhibition of neutral endopeptidase or ACE improved neural function; however, dual inhibition of neutral endopeptidase and ACE with Ilepatril tended to have the greatest efficacy. Ilepatril and Candoxatril treatment of diabetic rats was more efficacious in improving vascular responsiveness in epineurial arterioles than treatment with Enalapril. Ilepatril, Enalapril or Candoxatril treatment of diabetic rats were all efficacious in renal arteries. These studies suggest that combination therapy may be the most effective approach for treatment of diabetic neural and vascular complications.

Keywords

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

© Published by Elsevier B.V.

Correspondence should be addressed to: Mark A. Yorek, Room 204, Building 40, Department of Veterans Affairs Iowa City Health Care System, Iowa City, IA 52246, Tel #: 1-319-338-0581 ext. 7696, Fax # 1-319-339-7162, mark-yorek@uiowa.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The authors have no conflicts of interest to report. The authors would like to extend their appreciation to Sanofi Aventis, MERCK, and Pfizer for supplying Ilepatril (AVE7688), Enalapril and Candoxatril, respectively for these studies.

1. Introduction

Evidence suggests that there are at least five major pathways involved in the development of diabetic neuropathy: metabolic, vascular, immunologic, neurohormonal growth factor deficiency, and extracellular matrix remodeling (Brownlee, 2005; Yorek, 2008). In light of the complicated etiologies, an effective treatment for diabetic neuropathy has not yet been identified.

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). Based on these data we have focused our studies on therapies that reduce oxidative stress and improve vascular function. This has led to the study of the effect of vasopeptidase inhibitors (Davidson, et al. 2007; 2009 ; Oltman, et al. 2009). Vasopeptidase 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; Papademetriou, 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 vasopeptidase 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 vasopeptidase inhibitor (Arbin, et al. 2000). It has been demonstrated that vasopeptidase inhibitors are neuro-protective and prevent nephropathy in ZDF rats (Schafer, et al. 2003; 2004a; 2004b; Cha, et al. 2005). Vasopeptidase inhibitors have also been reported to decrease matrix metalloproteinases and advanced glycation end-product formation in type 2 diabetes and improve wound healing (Spenny, et al. 2002; Rizzone, et al. 2004; Wihler, et al. 2005). Lastly, it has been reported that neutral endopeptidase is responsible for up to 50% of the degradation of glucagon-like peptide 1 (Plamboeck, et al. 2005). The objective of this study was to determine the role of neutral endopeptidase inhibition on vascular and neural complications in streptozotocin-induced diabetic rats by examining the individual effect of neutral endopeptidase and ACE inhibition.

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 12 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. Diabetes was induced by intravenously

injecting streptozotocin (55 mg/kg in 0.9% NaCl). Control rats were injected with vehicle alone. Diabetes was verified 48 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 300 mg/dl (16.7 mM) or greater were considered to be diabetic.

Four weeks after the verification of diabetes four groups of rats were established: an untreated group, a group treated with Ilepatril (AVE7688 (vasopeptidase inhibitor), Sanofi Aventis 500 mg/kg in the diet), Enalapril ((ACE inhibitor) MERCK 500 mg/kg in the diet) or Candoxatril ((neutral endopeptidase inhibitor) Pfizer 300 mg/kg in the diet). The supplemental diets were prepared by mixing each compound in the meal form of the diet followed by formation of pellets and drying in a vacuum oven for 16h at 37° C. The treatment phase of the study lasted 12 weeks. The dosage of the compounds used in these studies was based on previous studies and pharmacokinetic data (Coppey, et al. 2001b; 2006; Davidson, et al. 2007; Newaz, et al. 2010). For this study all diabetic rats were treated with 2–3 units of Lantus insulin every other day in order to maintain weight and prevent loss of animals in the study due to extreme affects of diabetes (Calcutt, 2004). In this study only 1 diabetic rat in the Enalapril treated group was lost and this was due to an infection. This dose of insulin did not correct hyperglycemia. Control rats, three in each group, were also treated with each of the compounds described above. Results from these rats demonstrated that these treatments had no effect on neural or vascular function of epineurial arterioles (data not shown).

2.2 Thermal nociceptive response

The day before terminal studies thermal nociceptive response in the hindpaw was measured using the Hargreaves method as previously described (Oltman, et al. 2008).

2.3. 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). 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 μ mole of hippuric acid per minute and per liter of serum at 37° C. Lens glutathione and sciatic nerve sorbitol, fructose and myoinositol levels were determined 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). Sensory nerve conduction velocity (SNCV) was determined using the digital nerve to the second toe as described by Obrosova et al (Obrosova, et al. 2004; Oltman, et al. 2008). The MNCV and SNCV were reported in meters per second.

2.4. Intraepidermal nerve fiber density in the hindpaw

Immunoreactive intraepidermal nerve fiber profiles 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 serotic, 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).

2.5. 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). 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.

2.6. Vascular reactivity in renal arteries

Vascular ring tension was used to investigate *in vitro* vascular relaxation mediated by atrial natriuretic peptide and calcitonin gene-related peptide in small renal arteries (Oltman, et al. 2000). Vessels were precontracted to 30–50% with phenylephrine prior to the vasodilator curves. Changes in tension measurements were recorded using ADInstruments software during concentration response curves for atrial natriuretic peptide (10^{-11} – 10^{-8} M) and calcitonin gene-related peptide (10^{-11} – 10^{-8} M).

2.7. 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, calcitonin gene-related peptide and atrial natriuretic 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 streptozotocin-diabetic rats on weight and blood glucose

Data in Table 1 demonstrate that untreated or treated diabetic rats failed to gain weight and at the end of the study period weighed significantly less than control rats. All diabetic rats were hyperglycemic at the end of the study period as indicated by significantly elevated blood glucose and hemoglobin A₁C levels (Table 1).

3.2. Effect of treatment of streptozotocin-diabetic rats on serum lipid and thiobarbituric acid reactive substances levels and ACE activity

Data in Table 2 demonstrate that serum thiobarbituric acid reactive substances, a marker for oxidative stress, were significantly increased in diabetic rats. Treating diabetic rats with Ilepatril or Enalapril significantly reduced serum thiobarbituric acid reactive substances. In contrast, treating diabetic rats with Candoxatril had no effect. Diabetes caused a significant increase in serum triglycerides, free fatty acids and cholesterol levels. Treating diabetic rats with Ilepatril significantly improved serum triglyceride levels compared to untreated diabetic rats but levels remained significantly increased compared to control rats. Treatment with Enalapril also lowered triglyceride levels compared to untreated diabetic rats but the

difference was not significant and triglyceride levels in Enalapril treated diabetic rats remained significantly increased compared to control rats. Treating diabetic rats with Candoxatril did not reduce serum triglyceride levels. Treating diabetic rats with Ilepatril or Enalapril significantly reduced serum free cholesterol levels compared to untreated diabetic rats, whereas Candoxatril treatment had no effect on lowering serum free cholesterol levels. None of the treatments of diabetic rats lowered serum free fatty acid levels. As expected treating diabetic rats with Ilepatril or Enalapril lowered serum ACE activity compared to control, untreated diabetic rats or Candoxatril treated diabetic rats.

3.3. Effect of treatment of streptozotocin-diabetic rats on sciatic nerve sorbitol, fructose and myo-inositol levels and lens glutathione

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). None of the treatments used in these studies prevented the increase in sciatic nerve sorbitol and fructose levels and decrease in myo-inositol levels. Diabetes induced decrease in tissue glutathione levels has been shown to be a marker for an increase in oxidative stress (Coppey, et al. 2002). Blocking aldose reductase in diabetic rats has been shown to protect tissue glutathione levels (Coppey, et al. 2002). However, the treatments used in these studies did not prevent the diabetes induced decrease in lens glutathione levels suggesting that these compounds do not block aldose reductase activity (Table 3).

3.4. Effect of treatment of streptozotocin-diabetic rats on nerve conduction velocity, thermal nociception and intraepidermal nerve fiber density

Motor and sensory nerve conduction velocity is significantly decreased in diabetic rats (Fig. 1). Treating diabetic rats with Ilepatril, Enalapril or Candoxatril, were about equally effective in improving motor nerve conduction velocity. Treating diabetic rats with Ilepatril or Candoxatril significantly improved sensory nerve conduction slowing compared to untreated diabetic rats. Treating diabetic rats with Enalapril did not significantly improve sensory nerve conduction velocity. Data in Fig. 2 demonstrate that after 16 weeks of diabetes rats are hypoalgesic. All treatments improved thermal nociception. After 16 weeks of untreated diabetes there is a decrease in intraepidermal nerve fiber profiles in the hindpaw (Fig. 2). This was significantly improved by treating diabetic rats with Ilepatril and to a lesser extent with Candoxatril. Treating diabetic rats with Enalapril did not significantly improve innervation of the hindpaw.

3.5. Effect of treatment of streptozotocin-diabetic rats on vascular relaxation by epineurial arterioles

We have previously demonstrated that 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 compared the effect of these treatments as well as Candoxatril treatment on acetylcholine-mediated vascular relaxation (Fig. 3). Treatment of diabetic rats with Ilepatril or Candoxatril significantly improved acetylcholine-mediated vascular relaxation compared to untreated diabetic rats. Treatment of diabetic rats with Enalapril also improved acetylcholine-mediated vascular relaxation in epineurial arterioles but the effect was not significantly different compared to untreated diabetic rats.

Fig. 4 provides data on the effect of diabetes and treatments 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 diabetes (Yorek, et al. 2004). Calcitonin gene-related peptide is degraded by neutral endopeptidase and we have previously demonstrated that treatment of streptozotocin 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 treatment of diabetic rats with Ilepatril and Candoxatril significantly improved calcitonin gene-related peptide-mediated vascular relaxation in epineurial arterioles. Treatment of diabetic rats with Enalapril also improved vasodilation to calcitonin gene-related peptide but the improvement was not significant compared to untreated diabetic rats.

3.6. Effect of treatment of streptozotocin-diabetic rats on vascular relaxation by renal arteries

We examined the effect of diabetes and treatments on vascular reactivity of renal arteries. It has been demonstrated that vasopeptidase inhibitor treatment of type 2 diabetic rats improves renal function (Schafer, et al. 2003; 2004a; 2004b; Cha, et al. 2005). However, few studies have been performed examining the effect of treating diabetic rats with protease inhibitors on renal artery reactivity. For studies using renal arteries we focused on examining vasodilation mediated by two vasoactive peptides that are degraded by neutral endopeptidase (McDowell, et al. 1997). Atrial natriuretic peptide and calcitonin gene-related peptide are potent dilators in renal arteries. EC_{50} values were -10.33 ± 0.07 M for atrial natriuretic peptide and -9.38 ± 0.03 M for calcitonin gene-related peptide. Data in Fig. 5 demonstrate that vascular relaxation to atrial natriuretic peptide is decreased in renal arteries from diabetic rats. Treating diabetic rats with Ilepatril, Enalapril or Candoxatril significantly improved vascular relaxation to atrial natriuretic peptide in renal arteries with Enalapril treatment having a supra physiological affect.

Data in Fig. 6 demonstrate that vascular relaxation to calcitonin gene-related peptide is decreased in renal arteries from diabetic rats. Similar to results with atrial natriuretic peptide treating diabetic rats with Ilepatril, Enalapril or Candoxatril significantly improved vascular relaxation to calcitonin gene-related peptide in renal arteries.

4. Discussion

We previously reported that the vasopeptidase inhibitor Ilepatril was an efficacious treatment for vascular and neural complications in streptozotocin diabetic rats (Davidson, et al. 2007). We attributed this efficacy to anti-oxidant properties and protection of vasoactive peptides from degradation by neutral endopeptidase. 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 individual effects of inhibition of neutral endopeptidase or ACE on vascular and neural complications in streptozotocin diabetic rats.

Treatment of diabetic rats with Ilepatril, Enalapril or Candoxatril was similarly effective in improving motor nerve conduction velocity and thermal nociception. However, treatment of diabetic rats with Ilepatril or Candoxatril tended to be more effective than treatment with Enalapril in improving sensory nerve conduction velocity. Difference in efficacy of the treatments was also evident in protection of innervation of the hindpaw of diabetic rats. The number of intraepidermal nerve fiber profiles was similar to control values in diabetic rats

treated with Ilepatril a significant improvement compared to untreated diabetic rats. Treatment of diabetic rats with Candoxatril also improved this endpoint but the number of intraepidermal nerve fiber profiles was not significantly improved compared to untreated diabetic rats. In diabetic rats treated with Enalapril the number of intraepidermal nerve fibers were significantly decreased compared to control rats. Therefore, for this endpoint treatment of diabetic rats with Ilepatril was apparently better than treatment with Candoxatril or Enalapril.

All three treatments improved the diabetes-induced impairment in vascular relaxation of epineurial arterioles to acetylcholine and calcitonin gene-related peptide. Vasodilation in response to acetylcholine or calcitonin gene-related peptide in diabetic rats treated with Ilepatril or Candoxatril was significantly improved compared to untreated diabetic rats. In contrast, in diabetic rats treated with Enalapril the improvement in relaxation was not significantly different compared to untreated diabetic rats.

In renal arteries treatment of streptozotocin diabetic rats with Enalapril was supra effective with vasodilation mediated by atrial natriuretic peptide and calcitonin gene-related peptide exceeding reactivity observed in renal arteries from control rats. Treatment of diabetic rats with Ilepatril or Candoxatril also significantly improved vasodilation by atrial natriuretic peptide and calcitonin gene-related peptide in renal arteries compared to untreated diabetic rats but the effect was less than observed with Enalapril treatment of diabetic rats. These results demonstrate that not all vascular beds respond similarly pathologically or to pharmaceutical intervention.

In epineurial arterioles improvement of acetylcholine mediated vascular relaxation in treated diabetic rats was likely due to several mechanisms including decrease in oxidative stress and protection of degradation of vasoactive peptides such as calcitonin gene-related peptide and natriuretic peptides. In epineurial arterioles protection from degradation of C-type natriuretic peptide in diabetic rats treated with Ilepatril or Candoxatril may explain the better vasodilation by acetylcholine observed in these rats compared to diabetic rats treated with Enalapril (Davidson, et al. 2007; Yorek, 2008). The improved vascular function by epineurial arterioles observed in diabetic rats treated with Ilepatril or Candoxatril compared to diabetic rats treated with Enalapril may explain the general better outcome in diabetes impaired sensory nerve function in diabetic rats treated with Ilepatril or Candoxatril. Things seem to be much different in renal arteries. It is likely that impaired vascular relaxation to atrial natriuretic peptide and calcitonin gene-related peptide in renal arteries from streptozotocin diabetic rats in part was due to their increased degradation. Renal tissue express neutral endopeptidase and vasopeptidase inhibition has been shown to improve renal function in pathogenic animal models (Schaffer, et al. 2003; 2004a; 2004b; Skidgel and Erdős, 2004). However, it is unknown why treatment of diabetic rats with Enalapril would be more efficacious compared to diabetic rats treated with Ilepatril or Candoxatril. It is possible that the effective dosages used in our studies may vary depending on the analyses being conducted and the tissues being examined. Even though we used maximal dosing of these compounds based on previous studies and/or pharmacokinetic data we cannot be sure that all tissues were exposed to the same dosage or can we conclude that the effective dose is the same for all tissues (Coppey, et al. 2001b; 2006; Davidson, et al. 2007; Newaz, et al. 2010). This would require an extensive dosing study that is beyond our capability. However, we do know that at the dosage used Ilepatril and to a greater extent Enalapril decreased serum ACE activity and both compounds improved serum lipid abnormalities. In contrast, Candoxatril treatment of diabetic rats had no effect on the serum activity of ACE or diabetes-induced serum lipid levels. Unfortunately, there is no serum marker to determine the efficacy of Candoxatril treatment. Candoxatril like Ilepatril inhibits neutral

endopeptidase activity and not necessarily expression. Thus, for these studies we used the maximal dose of Candoxatril as described by Newaz et al. (2010).

In preclinical studies Candoxatril at doses used in our studies has been shown to provide cardioprotection in mice with heart failure, improve hypertension in DOCA-salt sensitive rats by preserving atrial natriuretic peptide levels and reducing bradykinin degradation in rat coronary bed (Hirata, et al., 1994; Dumoulin, et al. 2001; Xu, et al. 2004). In humans Candoxatril has been used to treat chronic heart failure and has been shown to increase circulating levels of vasoactive peptides including calcitonin gene-related peptide and atrial natriuretic peptide (O'Connell, et al. 1992; McDowell, et al. 1997; McDowell and Nicholls, 1999). In our studies Candoxatril treatment of streptozotocin diabetic rats improved neural complications. We attribute this to improved vascular function due to protection of vasoactive peptides. In epineurial arterioles we have shown that acetylcholine induced vasodilation is mediated by the formation of nitric oxide and release of endothelium derived hyperpolarizing factor (Terata, et al. 1999). We have further shown that C-type natriuretic peptide is a candidate for endothelium derived hyperpolarizing factor in epineurial arterioles (Davidson, et al. 2007). Studies using mesenteric arteries have also indicated that C-type natriuretic peptide may be responsible for the endothelium derived hyperpolarizing factor component of acetylcholine-mediated vascular relaxation (Chauhan, et al. 2004). Therefore, preservation of acetylcholine- and calcitonin gene-related peptide-mediated vascular relaxation in epineurial arterioles from streptozotocin diabetic rats treated with Candoxatril is likely due to protection of C-type natriuretic peptide and calcitonin gene-related peptide from degradation by neutral endopeptidase. However, our studies as well as studies of renal and cardiac failure suggest that dual inhibition of neutral endopeptidase and ACE activity provides greater protection than monotherapy (French, et al. 1995; Dumoulin, et al. 2001; Arnal, et al. 2001; Xu, et al. 2004; Grönholm, et al. 2005; Yorek, 2008).

5. Conclusions

Studies were performed on the effect of treating streptozotocin diabetic rats with Ilepatril, Enalapril or Candoxatril on nerve function and vascular reactivity using two different vascular beds. We found that each of these treatments improved neural and/or vascular function with the vasoepitidase inhibitor Ilepatril seemly providing the greatest benefit. Overall, combination therapy may be the most effective approach for treatment of diabetic neural and vascular complications and treatments to consider should include inhibitors of proteolytic degradation and antioxidants.

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 Grants 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.

References

- Abassi Z, Winaver J, Feuerstein GZ. The biochemical pharmacology of renin inhibitors: implications for translational medicine in hypertension, diabetic nephropathy and heart failure: expectations and reality. *Biochem. Pharmacol.* 2009; 78:933–940. [PubMed: 19477166]
- Arbin V, Claperton N, Fournie-Zaluski MC, Roques BP, Peyroux J. Effects of combined neutral endopeptidase 24-11 and angiotensin-converting enzyme inhibition on femoral vascular conductance in streptozotocin-induced diabetic rats. *Brit. J. Pharmacol.* 2000; 130:1297–1304. [PubMed: 10903969]

- Arnal JF, Castano C, Maupas E, Mugniot A, Darblade B, Gourdy P, Michel JB, Bayard F. Omapatrilat, dual angiotensin-converting enzyme and neutral endopeptidase inhibitor, prevents fatty streak deposit in apolipoprotein E-deficient mice. *Atherosclerosis*. 2001; 155:291–295. [PubMed: 11254898]
- Beiswenger KK, Calcutt NA, Mizisin AP. Epidermal nerve fiber quantification in the assessment of diabetic neuropathy. *Acta Histochem*. 2008; 110:351–362. [PubMed: 18384843]
- Broccolini A, Gidaro T, Morosetti R, Gliubizzi C, Servidei T, Pescatori M, Tonali PA, Ricci E, Mirabella M. Neprilysin participates in skeletal muscle regeneration and is accumulated in abnormal muscle fibres of inclusion body myositis. *J. Neurochem*. 2006; 96:777–789. [PubMed: 16405511]
- Brownlee M. The pathobiology of diabetic complications, a unifying mechanism. *Diabetes*. 2005; 54:1615–1625. [PubMed: 15919781]
- Calcutt NA. Modeling diabetic sensory neuropathy in rats. *Methods Mol. Med*. 2004; 99:55–65. [PubMed: 15131329]
- Cha YM, Redfield MM, Shah S, Shen WK, Fishbein MC, Chen PS. Effects of omapatrilat on cardiac nerve sprouting and structural remodeling in experimental congestive heart failure. *Heart Rhythm*. 2005; 2:984–990. [PubMed: 16171755]
- Chauhan SD, Hobbs AJ, Ahluwalia A. C-type natriuretic peptide: new candidate for endothelium-derived hyperpolarising factor. *Int J Biochem Cell Biol*. 2004; 36:1878–1881. [PubMed: 15203101]
- Coppey LJ, Davidson EP, Dunlap J, Lund DD, Yorek MA. Slowing of motor nerve conduction velocity in streptozotocin-induced diabetic rats is preceded by impaired vasodilation in arterioles that provide circulation to the sciatic nerve. *Int. J. Exp. Diabetes Res*. 2000; 1:131–143. [PubMed: 11469397]
- Coppey LJ, Davidson EP, Rinehart TW, Gellert JS, Oltman CL, Lund DD, Yorek MA. ACE inhibitor or angiotensin II receptor antagonist attenuates diabetic neuropathy in streptozotocin-induced diabetic rats. *Diabetes*. 2006; 55:341–348. [PubMed: 16443766]
- Coppey LJ, Gellert JS, Davidson EP, Dunlap J, Lund DD, Salvemini D, Yorek MA. Effect of M40403 Treatment of diabetic rats on endoneurial blood flow, motor nerve conduction velocity, and vascular function of epineurial arterioles of the sciatic nerve. *Brit. J. Pharmacol*. 2001a; 134:121–129.
- Coppey LJ, Gellert JS, Davidson EP, Dunlap J, Lund DD, Yorek MA. Effect of antioxidant treatment of streptozotocin-induced diabetic rats on endoneurial blood flow, motor nerve conduction velocity, and vascular reactivity of epineurial arterioles of the sciatic nerve. *Diabetes*. 2001b; 50:1927–1937. [PubMed: 11473057]
- Coppey LJ, Gellert JS, Davidson EP, Dunlap JA, Yorek MA. Effect of treating streptozotocin-induced diabetic rats with sorbinil, myo-inositol or aminoguanidine on endoneurial blood flow, motor nerve conduction velocity and vascular function of epineurial arterioles of the sciatic nerve. *Int. J. Experimental Diab. Res*. 2002; 3:21–36.
- Davidson EP, Coppey LJ, Kleinschmidt TL, Oltman CL, Yorek MA. Vascular and Neural Dysfunction in obese Zucker rats: Effect of AVE7688. *Experimental Diabetes Res* 2009. 2009; 2009:912327.
- Davidson EP, Coppey LJ, Calcutt NA, Oltman CL, Yorek MA. Diet Induced Obesity in Sprague Dawley Rats: Effect on Microvascular and Neural Function. *Diabetes. Metabolism Res. Rev*. 2010; 26:306–318.
- Davidson EP, Kleinschmidt TL, Oltman CL, Lund DD, Yorek MA. Treatment of Streptozotocin-induced Diabetic Rats with AVE7688, a Vasoepitidase Inhibitor, on Vascular and Neural Disease. *Diabetes*. 2007; 56:355–362. [PubMed: 17259379]
- Dumoulin MJ, Adam A, Rouleau JL, Lamontagne D. Comparison of a vasoepitidase inhibitor with neutral endopeptidase and angiotensin-converting enzyme inhibitors on bradykinin metabolism in the rat coronary bed. *J. Cardiovasc. Pharmacol*. 2001; 37:359–366. [PubMed: 11300648]
- Ebihara F, Di Marco GS, Juliano MA, Casarini DE. Neutral endopeptidase expression in mesangial cells. *J. Renin-Angiotensin-Aldosterone System*. 2003; 4:228–233. 2003. [PubMed: 14689370]
- Edwards RM, Pullen M, Nambi P. Distribution of neutral endopeptidase activity along the rat and rabbit nephron. *Pharmacology*. 1999; 59:45–50. [PubMed: 10352425]

- Ferrario CM, Smith RD, Brosnihan B, Chappell MC, Campese VM, Vesterqvist O, Liao W, Ruddy MC, Grim CE. Effects of Omapatrilat on the renin-angiotensin system in salt-sensitive hypertension. *Am. J. Hypertension*. 2002; 15:557–564.
- Finegold D, Lattimer SA, Nolle S, Bernstein M, Greene DA. Polyol pathway activity and myo-inositol metabolism. A suggested relationship in the pathogenesis of diabetic neuropathy. *Diabetes*. 1983; 32:988–992. [PubMed: 6416910]
- French JF, Anderson BA, Downs TR, Dage RC. Dual inhibition of angiotensin-converting enzyme and neutral endopeptidase in rats with hypertension. *J. Cardiovasc. Pharmacol*. 1995; 26:107–113. [PubMed: 7564349]
- Gonzalez W, Soleilhac JM, Fournie-Zaluski MC, Roques, Michel BP. Characterization of neutral endopeptidase in vascular cells, modulation of vasoactive peptide levels. *European J. Pharmacol*. 1998; 345:323–331. [PubMed: 9592033]
- Grönholm T, Cheng ZJ, Palojoki E, Eriksson A, Bäcklund T, Vuolteenaho O, Finckenberg P, Laine M, Mervaala E, Tikkanen I. Vasoepitidase inhibition has beneficial cardiac effects in spontaneously diabetic Goto-Kakizaki rats. *Eur. J. Pharmacol*. 2005; 519:267–276. [PubMed: 16137672]
- Hanif K, Bid HK, Konwar R. Reinventing the ACE inhibitors: some old and new implications of ACE inhibition. *Hypertens. Res*. 2010; 33:11–21.
- Hirata Y, Suzuki E, Hayakawa H, Matsuoka H, Sugimoto T, Kangawa K, Matsuo H. Mechanisms of the natriuretic effects of neutral endopeptidase inhibition in Dahl salt-sensitive and salt-resistant rats. *J. Cardiovasc. Pharmacol*. 1994; 23:283–290. [PubMed: 7511759]
- Kikkawa F, Shibata K, Suzuki T, Kajiyama H, Ino K, Nomura S, Mizutani S. Signal pathway involved in increased expression of neutral endopeptidase by gonadotropin releasing hormone in choriocarcinoma cells. *Placenta*. 2004; 25:176–183. [PubMed: 14972450]
- Malik RA, Williamson S, Abbott C, Carrington AL, Iqbal J, Schady W, Boulton AJ. Effect of angiotensin-converting-enzyme (ACE) inhibitor trandolapril on human diabetic neuropathy: randomized double-blind controlled trial. *Lancet*. 1998; 352:1978–1981. [PubMed: 9872248]
- McDowell G, Coutie W, Shaw C, Buchanan KD, Struthers AD, Nicholls DP. The effect of the neutral endopeptidase inhibitor drug, candoxatril, on circulating levels of two of the most potent vasoactive peptides. *Brit. J. Clin. Pharmacol*. 1997; 43:329–332. [PubMed: 9088591]
- McDowell G, Nicholls DP. The endopeptidase inhibitor, candoxatril, and its therapeutic potential in the treatment of chronic cardiac failure in man. *Expert Opin. Investig. Drugs*. 1999; 8:79–84.
- Muangman P, Spenny ML, Tamura RN, Gibran NS. Fatty acids and glucose increase neutral endopeptidase activity in human microvascular endothelial cells. *Shock*. 2003; 19:508–512. [PubMed: 12785004]
- Newaz M, Yousefipour Z, Oyekan A. Natriuretic and renoprotective effect of chronic oral neutral endopeptidase inhibition in acute renal failure. *Ren. Fail*. 2010; 32:384–390. [PubMed: 20370457]
- Obrosova IG, Li F, Abatan OI, Forsell MA, Komjati K, Pacher P, Szabo C, Stevens MJ. Role of poly(ADP-ribose) polymerase activation in diabetic neuropathy. *Diabetes*. 2004; 53:711–720. [PubMed: 14988256]
- O'Connell JE, Jardine AG, Davidson G, Connell JM. Candoxatril, an orally active neutral endopeptidase inhibitor raises plasma atrial natriuretic factor and is natriuretic in essential hypertension. *J. Hypertens*. 1992; 10:271–277. [PubMed: 1315825]
- Oltman CL, Coppey LJ, Gellett JS, Davidson EP, Lund DD, Yorek MA. Progression of Vascular and Neural Dysfunction in Sciatic Nerves of Zucker Diabetic Fatty (ZDF) and Zucker Rats. *Am. J. Physiol*. 2005; 289:E113–E122.
- Oltman CL, Davidson EP, Coppey LJ, Kleinschmidt TL, Lund DD, Yorek MA. Attenuation of Vascular/Neural Dysfunction in Zucker Rats Treated with Enalapril or Rosuvastatin. *Obesity*. 2008; 16:82–89. [PubMed: 18223617]
- Oltman CL, Davidson EP, Coppey LJ, Kleinschmidt TL, Lund DD, Yorek MA. Treatment of Zucker Diabetic Fatty Rats with AVE7688 Improves Vascular and Neural Dysfunction. *Diabetes, Obesity and Metabolism*. 2009; 11:223–233.
- Oltman CL, Kane NL, Gutterman DD, Bar RS, Dellsperger KC. Mechanisms of coronary vasodilation to insulin and insulin-like growth factor 1 is dependent on vessel size. *Am. J. Physiol*. 2000; 279:E176–E181.

- Papademetriou V. Inhibition of the renin-angiotensin-aldosterone system to prevent ischemic and atherothrombotic events. *Am. Heart J.* 2009; 157:S24–S30. [PubMed: 19450721]
- Plamboeck A, Holst JJ, Carr RD, Deacon CF. Neutral endopeptidase 24.11 and dipeptidyl peptidase IV are both involved in regulating the metabolic stability of glucagon-like peptide-1 in vivo. *Diabetologia.* 2005; 48:1882–1890. [PubMed: 16025254]
- Pu Q, Schiffrin EL. Effect of ACE/NEP inhibition on cardiac and vascular collagen in stroke-prone spontaneously hypertensive rats. *Am. J. Hypertension.* 2001; 14:1067–1072.
- Quaschnig T, D'uscio LV, Shaw S, Grone H, Ruschitzka F, Luscher TF. Vasopeptidase inhibition restores renovascular endothelial dysfunction in salt-induced hypertension. *J. Am. Soc. Nephrol.* 2001; 12:2280–2287. [PubMed: 11675404]
- Reja A, Tesfaye S, Harris ND, Ward UK. Is ACE inhibition with lisinopril helpful in diabetic neuropathy? *Diabetic Med.* 1995; 12:307–309. [PubMed: 7600744]
- Rizzone D, Rossi GP, Porteri E, Sticchi D, Rodella L, Rezzani R, Sleiman I, De Ciuceis C, Paiardi S, Bianchi R, Nussdorfer GG, Agabiti-Rosei E. Bradykinin and matrix metalloproteinases are involved in the structural alterations of rat small resistance arteries with inhibition of ACE and NEP. *J. Hypertension.* 2004; 22:759–766.
- Schafer S, Linz W, Bube A, Gerl M, Huber J, Kurznel GU, Bleich M, Schmidts H-L, Busch AE, Rutten H. Vasopeptidase inhibition prevents nephropathy in Zucker diabetic fatty rats. *Cardiovascular Res.* 2003; 60:447–454.
- Schafer S, Linz W, Vollert H, Biemer-Daub G, Rutten H, Bleich M, Busch AE. The vasopeptidase inhibitor AVE7688 ameliorates Type 2 diabetic nephropathy. *Diabetologia.* 2004a; 47:98–103. [PubMed: 14618238]
- Schafer S, Schmidts H-L, Bleich M, Busch AE, Linz W. Nephroprotection in Zucker diabetic fatty rats by vasopeptidase inhibition is partly bradykinin B2 receptor dependent. *Brit. J. Pharmacol.* 2004b; 143:27–32. [PubMed: 15289289]
- Schling P, Schafer T. Human adipose tissue cells keep tight control on the angiotensin II levels in their vicinity. *J. Biol. Chem.* 2002; 277:48066–48075. [PubMed: 12196514]
- Skidgel RA, Erdös EG. Angiotensin converting enzyme (ACE) and neprilysin hydrolyze neuropeptides: a brief history, the beginning and follow-ups to early studies. *Peptides.* 2004; 25:521–525. [PubMed: 15134871]
- Spenny ML, Muangman P, Sullivan SR, Bunnett NW, Ansel JC, Olerud JE, Gibran NS. Neutral endopeptidase inhibition in diabetic wound repair. *Wound Repair Regeneration.* 2002; 10:295–301.
- Suzuki T, Ino K, Kikkawa F, Uehara C, Kajiyama H, Shibata K, Mizutani S. Neutral endopeptidase/CD10 expression during phorbol ester-induced differentiation of choriocarcinoma cells through the protein kinase C- and extracellular signal-regulated kinase-dependent signaling pathway. *Placenta.* 2002; 23:475–482. [PubMed: 12137745]
- Taal MW, Nenov VD, Wong W, Satyal SR, Sakharova O, Choi JH, Troy JL, Brenner BM. Vasopeptidase inhibition affords greater renoprotection than angiotensin-converting enzyme inhibition alone. *J. Am. Soc. Nephrol.* 2001; 12:2051–2059. [PubMed: 11562403]
- Terata K, Coppey LJ, Davidson EP, Dunlap JA, Gutterman D, Yorek MA. Acetylcholine-induced arteriolar dilation is reduced in streptozotocin-induced diabetic rats with motor nerve dysfunction. *Brit. J. Pharmacol.* 1999; 128:837–843. [PubMed: 10516670]
- Vatter H, Schilling L, Schmiedek P, Ehrenreich H. Evidence for functional endothelin-converting enzyme activity in isolated rat basilar artery: effect of inhibitors. *J. Cardiovascular Pharma.* 1998; 31:S64–S67.
- Weber M. Emerging treatments for hypertension: potential role for vasopeptidase inhibition. *Am. J. Hypertension.* 1999; 12:139S–147S.
- Wihler C, Schafer S, Schmid K, Deemer EK, Munch G, Bleich M, Busch AE, Dingermann T, Somoza V, Baynes JW, Huber J. Renal accumulation and clearance of advanced glycation end-products in type 2 diabetic nephropathy: effect of angiotensin-converting enzyme and vasopeptidase inhibition. *Diabetologia.* 2005; 48:1645–1653. [PubMed: 16010524]

- Xu J, Carretero OA, Liu YH, Yang F, Shesely EG, Oja-Tebbe N, Yang XP. Dual inhibition of ACE and NEP provides greater cardioprotection in mice with heart failure. *J. Card. Fail.* 2004; 10:83–89. [PubMed: 14966779]
- Yorek MA, Coppey LJ, Gellett JS, Davidson EP. Sensory nerve innervation of epineurial arterioles of the sciatic nerve containing calcitonin gene-related peptide: effect of streptozotocin-induced diabetes. *Exp. Diabetes Res.* 2004; 5:197–193.
- Yorek MA. The potential role of angiotensin converting enzyme and vasopeptidase inhibitors in the treatment of diabetic neuropathy. *Current Drug Targets.* 2008; 9:77–84. [PubMed: 18220715]

Fig. 1. Effect of treatment of streptozotocin-diabetic rats with Ilepatril, Enalapril or Candoxatril on motor and sensory nerve conduction velocity

At 12 weeks of age rats were made diabetic using streptozotocin and 4 weeks later were divided into 4 groups. One group was fed a normal diet and the other three groups were fed diets containing Ilepatril, Enalapril or Candoxatril, respectively for 12 weeks. A non-diabetic control group was also included. Afterwards, motor and sensory nerve conduction velocity was examined as described. 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 rats fed the standard diet; + $P < 0.05$, compared to untreated diabetic rats.

Fig. 2. Effect of treatment of streptozotocin-diabetic rats with Ilepatril, Enalapril or Candoxatril on thermal nociception and intraepidermal nerve fiber density

Rats were treated as describe in Fig. 1 and the group identification is the same. Thermal nociception and intraepidermal nerve fiber density was examined as described. 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 rats fed the standard diet ; + $P < 0.05$, compared to untreated diabetic rats.

Fig. 3. Effect of treatment of streptozotocin-diabetic rats with Ilepatril, Enalapril or Candoxatril on vascular relaxation by acetylcholine in epineurial arterioles

Rats were treated as describe in Fig. 1. 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 untreated diabetic rats.

Fig. 4. Effect of treatment of streptozotocin-diabetic rats with Ilepatril, Enalapril or Candoxatril on vascular relaxation by calcitonin gene-related peptide in epineurial arterioles
Arterioles were derived from rats as described in Fig. 3. 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 untreated diabetic rats.

Fig. 5. Effect of treatment of streptozotocin-diabetic rats with Ilepatril, Enalapril or Candoxatril on vascular relaxation by atrial natriuretic peptide in renal arteries

Rats were treated as described in Fig. 1. Renal arteries were harvested as described and relaxation to incremental doses of atrial natriuretic peptide (ANP) was determined. 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 untreated diabetic rats.

Fig. 6. Effect of treatment of streptozotocin-diabetic rats with Ilepatril, Enalapril or Candoxatril on vascular relaxation by calcitonin gene-related peptide in renal arteries

Rats were treated as described in Fig. 1. Renal arteries were harvested as described and relaxation to incremental doses of calcitonin gene-related peptide (CGRP) was determined. 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 untreated diabetic rats.

Effect of Treatment of Streptozotocin-Diabetic Rats with Ilepatriil, Enalapril or Candoxatril on Change in Body Weight, Blood Glucose and Hemoglobin A₁C

Table 1

Determination	Control (11)	Diabetic (12)	Diabetic + Ilepatriil (8)	Diabetic + Enalapril (7)	Diabetic + Candoxatril (8)
Start weight (g)	359 ± 4	362 ± 4	360 ± 3	348 ± 6	354 ± 6
End weight (g)	524 ± 9	334 ± 14 ^a	382 ± 18 ^a	322 ± 17 ^a	348 ± 20 ^a
Blood glucose (mg/dl)	110 ± 5	578 ± 13 ^a	575 ± 25 ^a	570 ± 22 ^a	546 ± 14 ^a
Hb A ₁ C (%)	5.3 ± 0.3	13.9 ± 0.4 ^a	13.8 ± 1.8 ^a	16.1 ± 0.9 ^a	15.5 ± 1.1 ^a

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

^ap < 0.05 compared to control.

Parentheses indicate the number of experimental animals.

Table 2

Effect of Treatment of Streptozotocin-Diabetic Rats with Ilepatri, Enalapril or Candoxatril on Serum Thio Barbituric Acid Reactive Substances, Triglycerides, Free Fatty Acids, Cholesterol and ACE activity

Determination	Control (11)	Diabetic (12)	Diabetic + Ilepatri (8)	Diabetic + Enalapril (7)	Diabetic + Candoxatril (8)
Thiobarbituric acid reactive substances ($\mu\text{g/ml}$)	0.78 ± 0.07	1.07 ± 0.06^a	0.81 ± 0.06^b	0.60 ± 0.06^b	1.04 ± 0.06^a
Triglycerides (mg/dl)	25 ± 3	206 ± 27^a	$81 \pm 14^{a,b}$	118 ± 24^a	223 ± 51^a
Free fatty acids (mmol/l)	0.11 ± 0.03	0.56 ± 0.07^a	0.42 ± 0.10^a	0.76 ± 0.17^a	0.78 ± 0.18^a
Cholesterol (mg/dl)	82 ± 13	223 ± 38^a	46 ± 10^b	58 ± 15^b	166 ± 31^a
ACE (mU/ml/min)	51 ± 3	68 ± 3^a	$28 \pm 5^{a,b}$	$3 \pm 1^{a,b}$	59 ± 6

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

^a $p < 0.05$ compared to control;

^b $p < 0.05$ compared to diabetic.

Parentheses indicate the number of experimental animals.

Table 3

Effect of Treatment of Streptozotocin-Diabetic Rats with Ilepatriil, Enalapril or Candoxatril on Sciatic Nerve Sorbitol, Fructose and myo-Inositol content and Lens Glutathione levels

Determination	Control (11)	Diabetic (12)	Diabetic + Ilepatriil (8)	Diabetic + Enalapril (7)	Diabetic + Candoxatril (8)
Sorbitol (nmol/mg prot)	0.8 ± 0.1	9.2 ± 1.1 ^a	7.2 ± 1.2 ^a	8.6 ± 0.7 ^a	8.4 ± 0.6 ^a
Fructose (nmol/mg prot)	0.52 ± 0.03	3.44 ± 0.23 ^a	2.83 ± 0.38 ^a	3.44 ± 0.11 ^a	3.36 ± 0.12 ^a
Myo-Inositol (nmol/mg prot)	7.4 ± 0.5	4.9 ± 0.4 ^a	4.8 ± 0.4 ^a	5.3 ± 0.9 ^a	6.3 ± 0.8
Lens glutathione (μg/mg)	3.0 ± 0.1	0.5 ± 0.1 ^a	0.8 ± 0.3 ^a	0.7 ± 0.2 ^a	0.6 ± 0.1 ^a

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

^aP < 0.05 compared to control.

Parentheses indicate the number of experimental animals.