Neuropharmacology 116 (2017) 122–131

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Early vs. late intervention of high fat/low dose streptozotocin treated C57Bl/6J mice with enalapril, α-lipoic acid, menhaden oil or their combination: Effect on diabetic neuropathy related endpoints

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ARTICLE INFO

Article history:
Received 24 October 2016
Received in revised form
28 November 2016
Accepted 21 December 2016
Available online 23 December 2016

Chemical compounds:
Enalapril maleate (PubChem CID: 5388961)
α-Lipoic acid (PubChem CID: 864)

Keywords:
Diabetic peripheral neuropathy
Type 2 diabetes
Enalapril
α-Lipoic acid
Menhaden oil
Corneal nerves

ABSTRACT

We have previously demonstrated that enalapril, α-lipoic acid and menhaden (fish) oil has potential as a treatment for diabetic peripheral neuropathy. In this study we sought to determine the efficacy of these treatments individually or in combination on multiple neuropathic endpoints in a high fat fed low dose streptozotocin treated mouse, a model of type 2 diabetes, following early or late intervention. Four or twelve weeks after the onset of hyperglycemia, diabetic mice were treated with enalapril, α-lipoic acid, menhaden oil or their combination for 12 weeks. Afterwards, endpoints including glucose tolerance, motor and sensory nerve conduction velocity, thermal nociception, and intraepidermal and cornea nerve fiber density was determined. Glucose clearance was impaired in diabetic mice and significantly improved only with combination treatment and early intervention. Diabetes caused steatosis, slowing of motor and sensory nerve conduction velocity, thermal hypoalgesia and reduction in intraepidermal and cornea nerve fiber density. Treating diabetic mice with enalapril, α-lipoic acid or menhaden oil partially protected diabetic mice from these deficits, whereas the combination of these three treatments was more efficacious following early or late intervention. These studies suggest that a combination therapy may be more effective for treating neural complications of type 2 diabetes.

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1. Introduction

Peripheral neuropathy is the most common complication of diabetes mellitus and has also been reported to occur in animal models and humans regarded as being pre-diabetic (Cortez et al., 2014; Davidson et al., 2014; Papanas and Ziegler, 2012; Singh et al., 2014; Singleton and Smith, 2007; Vinik et al., 2013; Ziegler et al., 2014a). Studies using rodent models of diabetes have been successful in identifying mechanisms and possible treatments for diabetic peripheral neuropathy, but translation of these findings to humans has failed (Albers and Pop-Busui, 2014; Obrosova, 2009; Singh et al., 2014; Vincent et al., 2011; Yagihashi et al., 2011; Yorek, 2008; Zychowska et al., 2013). Thus far most clinical studies for treatment of diabetic peripheral neuropathy have focused on using mono-therapeutic treatments believing that intervening at a single pathological mechanism will successfully abate this disease. However, diabetic peripheral neuropathy is a complex disease with multiple mechanisms contributing to its etiology and it is unlikely that a mono-therapeutic approach will be maximally successful (Albers and Pop-Busui, 2014; Singh et al., 2014; Vincent et al., 2013; Yagihashi et al., 2011; Yorek, 2008). Furthermore, little is known to what extent an early vs. late intervention can be successful in treatment of diabetic peripheral neuropathy. Studies have been ongoing to identify early marker(s) of peripheral neuropathy (Arimura et al., 2013; Divisova et al., 2016; Malik et al., 2011;

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http://dx.doi.org/10.1016/j.neuropharm.2016.12.022
0028-3908/Published by Elsevier Ltd.

Papanas and Ziegler, 2012, 2013; Pritchard et al., 2011; Ziegler et al., 2014b). As more effective bio-markers for peripheral neuropathy become available, studies need to go forward to identify possible new interventions that can be applied safely in clinical trials. In this regard we have focused on examining the use of a combination of dietary supplements and drugs (enalapril, α-lipoic acid and menhaden [fish] oil) for treatment of diabetic peripheral neuropathy in rodent models (Davidson et al., 2014). The reason for selecting these three compounds is that we have demonstrated that each of these treatments individually had beneficial effects on diabetic peripheral neuropathy and each have also been shown to be safe for use in humans (Coppey et al., 2001, 2006; 2012, 2015; Davidson et al., 2011; Gopinath et al., 2012; Mcllduff and Rutkove, 2011; Papanas and Ziegler, 2014; Rudkowska, 2010; Shevalye et al., 2015; Yee et al., 2010; Ziegler et al., 2011). The primary mechanism of action of these three compounds is oxidative and inflammatory stress. The study design examined the effect of treatments following an early or late intervention on development and progression of diabetic peripheral neuropathy using multiple endpoints in a mouse model of type 2 diabetes, that we had previously characterized (Yorek et al., 2015).

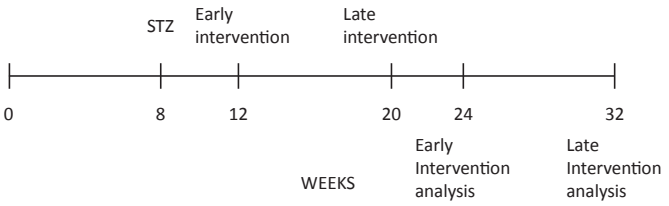
2. Materials and methods

2.1. Materials

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

2.2. Animals

C57Bl6/J male mice were purchased from Jackson Laboratories. Mice were housed in a certified animal care facility with standard diet (Harlan Teklad, #7001, Madison, WI) and water provided ad libitum. All experiments were conducted in accordance with international standards on animal welfare and were compliant with institutional and National Institutes of Health guidelines for use of animals (ACORP protocol 1390201). Two separate experiments (early and late intervention design) were conducted (see graphical timeline below). For the early intervention study mice at 12 weeks of age were divided into six groups. After 1 week on a standard diet (Harlan Teklad, #7001; Madison, WI) five of the groups were fed a 60 kcal% high fat diet (D12492; Research Diets, New Brunswick, NJ). The other group (control) remained on the standard diet for the duration of the study. For the late intervention study mice were divided into eight groups and six groups were fed the 60 kcal% high fat diet while the other two groups remained on the standard diet. To create the type 2 diabetic model the high fat fed mice, after 8 weeks on the high fat diet, were treated with 75 mg/kg streptozotocin (EMD Chemicals, San Diego, CA) followed three days later with a second dose of streptozotocin (50 mg/kg) as needed. Mice with blood glucose ≥13.8 mM (250 mg/dl) were considered diabetic. These groups remained on the high fat diet for an additional four weeks for the early intervention study and twelve weeks for the late intervention study. Afterwards, one group designated as the untreated diabetic group in each of the early and late intervention studies remained on the 60 kcal% high fat diet for the entire period of the study. The other four groups received the 60 kcal% high fat diet containing: menhaden oil (½ of the fat [derived primarily from lard] in the 60 kcal% high fat diet was substituted with menhaden oil), or enalapril (500 mg/kg in the 60 kcal% high fat diet; BOC Sciences, Shirley, NY), or α-lipoic acid (2.5 g/kg in the 60 kcal% high fat diet) or the combination of all three. The modified diets were prepared by Research Diets. The doses of the agents used in the modified diets were based on our previous studies (Coppey et al., 2001, 2006; 2012; Davidson et al., 2015). The base diet was the same for all the diabetic mice in these studies and we found that each group ate about the same amount of chow throughout the study (~75 g/kg mouse). These diets (treatment phase) were maintained for 12 weeks for both the early and late intervention. For the late intervention study, a group of control and untreated diabetic mice were studied at the time treatment began to establish a baseline for the pathology present after 12 weeks of untreated hyperglycemia. All studies were performed in a masked fashion by providing a coding for each animal that was unknown by the examiner.



2.3. Intraperitoneal glucose tolerance test

After 12 weeks of diet intervention intraperitoneal glucose tolerance test was performed after an overnight fast as previously described (Coppey et al., 2011).

2.4. Behavioral test

Immediately prior to the terminal studies thermal nociceptive response in the hindpaw was measured using the Hargreaves method as previously described (Coppey et al., 2011).

2.5. Motor and sensory nerve conduction velocity

On the day of terminal studies mice were weighed and anesthetized with sodium pentobarbital (75 mg/kg, i.p., Diamondback Drugs, Scottsdale, AZ). Non-fasting blood glucose was determined. Motor and sensory nerve conduction velocities were determined as previously described (Coppey et al., 2011).

2.6. Corneal innervation in vivo

Sub-epithelial corneal nerves were imaged *in vivo* non-invasively using the Rostock cornea module of the Heidelberg Retina Tomograph (Heidelberg Engineering, Vista, CA) confocal microscope as previously described (Yorek et al., 2014, 2015).

2.7. Immunohistochemistry analysis of nerves in the skin and cornea in vitro

After completion of all *in vivo* analyses skin from the footpad and corneas were collected. These tissues were fixed and analyzed for presence of intraepidermal nerve fibers and corneal nerves (sub-epithelial and nerves penetrating the epithelium), respectively, as previously described (Yorek et al., 2014, 2015).

2.8. Analyses in liver and serum

To examine steatosis, liver samples were frozen in OCT compound (Sakura FineTek USA, Torrance, CA) in liquid nitrogen. Liver sections, 5 μm, were incubated with BODIPY (Molecular Probes, Carlsbad, CA), at a 1:5000 dilution in 1% bovine serum albumin for 1 h at room temperature. After washing liver sections were