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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Effect of α-lipoic acid on expression of collagen IV of the sciatic nerve of diabetic rats

Mohammad Reza Aldaghi[1], Mahdi Jalali [1*], Mohammad Reza Nikravesh[1], Alireza Fazel[1] and Mojtaba Sankian[2]

[1]Department of Anatomy and Cell Biology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran; [2]Immunology Research Centre, Bu Ali Research Institute, Mashhad University of Medical Sciences, Mashhad, Iran

Abstract

Neuropathy is one of the complications of diabetes which alters structure and function of the basement membranes in nerves. Collagen IV is the most prominent component of basement membranes in sciatic nerve. We examined the effects of α-lipoic acid (ALA), as an antioxidant agent, on blood glucose levels, body weight and alterations in collagen IV expression of sciatic nerve in the diabetic rats. Diabetes was induced in the rats by intraperitoneal injection of streptozotocin (55 mg/kg). After 12 weeks of diabetes, collagen IV expression in sciatic nerves was evaluated by immunohistochemical study and real time polymerase chain reaction (PCR). Our findings revealed that ALA treatment significantly decreased blood glucose levels in the diabetic rats but did not significantly affect the body weight. Collagen IV immunoreactivity was stronger in the perineurium, endoneurium and blood vessels basement membrane of sciatic nerve in untreated diabetic rats compared to control group. ALA treatment significantly decreased collagen IV expression only in the basement membrane of blood vessels. Collagen IV mRNA level of sciatic nerve in untreated diabetic rats significantly increased compared to control group. ALA failed to protect sciatic nerves against collagen IV mRNA up-regulation. We suggest that ALA has the potential in improving hyperglycemia by reducing oxidative stress and may partially prevent collagen IV alteration in sciatic nerves.

Keywords: Diabetic neuropathy; collagen IV; alpha lipoic acid; sciatic nerve

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Introduction

Diabetic neuropathy is the most common of the peripheral neuropathies (Yasuda et al., 2003). The primary cause of diabetic neuropathy is hyperglycemia (Layton et al., 2004). Peripheral nerves sheathed by three layers of connective tissue. The outer layer is the epineurium that covers the nerve and had inter fascicular septum that separates nerve fascicles. Perineurium is the middle layer which covers each nerve fascicle. Perineurium has squamous cell layers; these cells are connected by tight junctions and have basement membranes on both sides. The inner layer connective tissue is the endoneurium that encloses a nerve fibre with its axon and covering Schwann cell (Bahcelioglu et al., 2008). The changes in the extracellular matrix (ECM) of the peripheral nerves have been reported in the diabetic neuropathy (Bradley, 2000; Yasuda, 2003; Layton, 2004). In diabetes, the vascular, perineurial and Schwann cell associated basement membrane increases in thickness and may be associated with alteration in collagen IV metabolism (Bradley, 2000; Yasuda, 2003). Collagen IV is the major component of all basement membranes (Hill et al., 2002). In the peripheral nervous system, collagen IV surrounds Schwann cells and their associated axons and has important properties that promote axonal growth (Chernousov et al., 2008). Collagen IV supports the regeneration of the nerve and found in the blood vessels basement membranes (Bradley, 2000; Hill,

Corresponding author: Jalali Mahdi, Department of Anatomy and Cell biology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran ; E-mail: jalaliM@mums.ac.ir

2002; Yasuda, 2003). Long-lived proteins such as collagen IV are potential targets of glycation (Brownlee, 1995; Duran-Jimenez et al., 2009). Hyperglycemia-induced oxidative stress contributes to the pathology of diabetic neuropathy (Vallianou et al., 2009). Hyperglycemia and increased oxidative stress result in vascular damage and reduced peripheral nerve blood flow (Rajbhandari et al., 2005). Oxygen free radicals activity increased in sciatic nerve in experimental diabetic neuropathy (Ziegler, 2004). In diabetic nerves, ALA decreased oxidative stress and improved blood flow (Nagamatsu, 1995; Vallianou, 2009; Ranieri, 2010). The aim of the present study was to investigate the effect of ALA as an antioxidant agent on blood glucose levels, body weight and collagen IV expression of sciatic nerves basement membrane in the streptozotocin (STZ) induced diabetic rats.

Materials and Methods

This study was performed in the Anatomy and Cell Biology Department and Boali Research Centre of Mashhad University of Medical Sciences (Mums). Thirty two adult male Wistar rats (200–250 g body weight, 6-8 weeks old) were obtained from animal house of Mums. The environmental conditions were set (23-25°C, relative humidity 50-55%, 12 hr light-dark cycle, free access to water and food). The study was approved by the Mums animal ethics committee guidelines. The rats were randomly divided into four groups as follows:

1-Control (C)
2-Diabetic without treatment (D group)
3-Diabetic with insulin treated (D+INS group)
4-Diabetic with ALA treated (D+ALA group)

Diabetes was induced in the rats by a single intraperitoneal injection of streptozotocin (STZ) (Sigma, 55 mg/kg body weight) freshly dissolved in normal saline. The rats were fasting over night prior to STZ injection. After STZ injection, the rats had free access to glucose solution. Diabetes was verified 48 h later by evaluating blood glucose levels using a digital glucometer (Accua Check, Germany). Only the rats with a blood glucose level higher than 300 mg/dl were considered as diabetic. The treatments of diabetic rats were conducted after the verification of diabetes. The D+ALA group was treated with ALA (Sigma). The powder was mixed with saline and 100 mg/kg was injected intraperitoneally five times a week. D+INS group was treated with 4-6 units of insulin (NPH) (EXIR Company, Iran) subcutaneously. The dose of insulin was determined on the basis of a daily blood glucose test. The rats had free access to standard laboratory diet and tap water. The blood glucose levels and body weight were measured once a week. There was no difference in the initial weights in the rats of four groups. Hyperglycemia persisted in diabetic rats during the 12 weeks before they were sacrificed. Finally, the rats were anaesthetized and then killed by cervical dislocation. After shaving, an incision was made in mid thigh and sciatic nerve was carefully removed.

Immunohistochemistry

Sciatic nerves of the rats were fixed in 10% neutral buffered formalin solution for 24 h, embedded in paraffin, sectioned at 5µm thickness and were mounted on poly-L-lysine slides (Sigma). Sections were deparaffinized with the xylene, rehydrated through descending concentrations of ethanol and rinsed in phosphate-buffered saline solution (PBS) (pH 7.4) for 10 min. Enzymatic antigen retrieval were carried out with Trypsin (0.05%) in PBS. The slides were placed in a humidified container and transferred into the 37°C incubator for 20 min and then transferred to a PBS container for 3 min. Sections were pre-incubated in 0.025% Triton X-100 in PBS at room temperature for 10 min. Subsequently, for blocking non specific antibody, followed by 5% goat serum and bovine serum albumin (BSA) 2% in PBS in a humidified container for 1 hr. Then sections were reacted with primary antibody (anti Collagen IV antibody, Abcam 6586) diluted 1:400 in PBS with 1% BSA for over night incubation of primary antibody in a humidified container for 24 hr at 4°C. The next day sections were washed with 0.025% Triton X-100 in PBS at room temperature for two times, each time for 5 min. Endogenous peroxidase activity was blocked with using 0.03% H_2O_2 in methanol for 15 min. Then specimens incubated with goat poly clonal secondary antibody (Abcam 97051) diluted 1:800 in PBS with 1% BSA for 2 hr in room temperature. Then they were washed for 10 min in PBS and finally reacted with 0.03% solution of 3,3-diaminobenzidine tetra hydrochloride (DAB) containing 0.3% H_2O_2 for 20 min. Specimens were washed and then were counter stained with hematoxylin. Finally, the sections were washed, air-dried, dehydrated in increasing graded ethanol, cleared in xylene and mounted in glass slides. Sections stained only with secondary antibody were treated as the negative control. The immunostaining sections were photographed by a light microscope (Olympus DP12, Japan) and collagen IV reaction in the sciatic nerves were evaluated. Assessment of staining for deposition was done semi-quantitatively. The locations which expressed collagen IV were brown, they served as positively stained. The intensity was assessed on a semi quantitative 5 point scale as follows: ++++: very strong expression, +++: strong expression, ++: moderate expression, +: weak expression: no staining, depending on the degree of intensity of staining observed in the sections examined (Jalali et al., 2010).

Real Time Study

RNA extraction and cDNA synthesis

Total RNA was isolated by the RNeasy Mini Kit (Qiagen, 74104) according to the manufacturer's instructions. Briefly, 25 to 30 mg of sciatic nerves were lysed with buffer containing 1% mercaptoethanol and homogenized by using polytron homogenizer (PT 1200E, Switzerland) and subsequent ultrasonication. The homogenate was centrifuged at 13200 g for 3 min to remove insoluble debris, and the supernatant was transferred to a fresh micro tube. An equal volume of 70% ethanol was added and samples were transferred to Rneasy Mini Kit columns (Qiagen, Germany) and RNA extraction proceeded according to the manufacturer's instructions. The RNA integrity was checked by visualization of 18S and 28S ribosomal bands on 1% agarose gel. First strand cDNA was made by using a cDNA synthesis kit (Fermentas, Lithuania) according to the manufacturer instructions. 7µl of total RNA with 1µl of random primer and 3µl of water were mixed and incubated at 65°C for 5 min and then added the filling component: 4µl of 5x reaction buffer, 1µl of ribolock RNase, 2µl of 10mm dNTP mix, 1µl of reverse transcriptase. The mixture was incubated for 15 min at 25°C followed by 60 min at 42°C and terminated reaction by heating at 70°C for 5 min cDNA. Samples were stored at -20°C.

Real-time polymerase chain reaction (PCR)

Real-time PCR was performed by using the Stratagene Max3000p (USA). The reaction mixture (total volume of 20 µl per well) consisted of 10 µl of SYBR Green PCR Master Mix (Pars Tous, Iran), 1µl of each forward and reverse primer, 0.25µl of Taq DNA polymerase, 6.75 µl of H_2O and 1µl of cDNA template. The following primers were used:
Collagen IV (α1), 5-ATTCCTTTGTGATGCACACC AG-3' (forward) 5'-AAGCTGTAAGCATTCGCGTAG TA-3' (reverse) and 5'-AACTCCCATTCTTCCACCT TTG-3' (forward) 5'-CTGTAGCCATATTCATTGTCA TACCAG-3' (reverse) for GAPDH. The cDNA were denatured for 10 min at 94°C and then 40 cycles of 94°C for 30s, 58°C for 30s and 72°C for 45s. At the end of the runs, the temperature was 95°C to construct a melting curve. The cDNA content in each specimen was determined by using a comparative cycle threshold (Ct) method. The results showed a specific gene normalized to the GAPDH gene. The averages of the relative amount of each mRNA in control group were considered as 1.0.

Statistical analysis

All values are expressed as mean ± SEM. Statistical evaluation was done by using one way ANOVA followed by Tukey test. The data were analyzed by using SPSS software. P values less than 0.05 was considered significant.

Results

Data in Table 1 showed information on the final body weight and blood glucose levels for all groups. Blood glucose level was significantly increased in the D group compared to the control group ($P<0.001$). The ALA treatment significantly decreased blood glucose levels in the D+ALA group compared to untreated diabetic group ($P<0.05$). Also in D+INS group, blood glucose levels decreased significantly compared to D group ($P<0.001$). The diabetic rats without treatment failed to gain weight compared to the control rats ($P<0.001$). ALA treatment did not significantly affect the final body weight of the diabetic rats. Collagen IV expression in fascicles with similar size of sciatic nerves were determined according to the intensity of colour darkness. In normal sciatic nerves, collagen IV was present in the perineurim, endoneurium around the Schwann cells and in the basement membranes of epineurial and endoneurial blood vessels. In diabetic sciatic nerves, collagen IV was prominent especially in the perineurium and thickened layers of basement membranes around the epineurial and endoneurial blood vessels (Fig. 1, 2 and Table 2). In the D+INS group collagen IV intensity of immunoreactivity significantly decreased compared to the D group ($P<0.05$). Immunoreactivity pattern of collagen IV was similar in the D+INS and C groups. In the D+ALA group, collagen IV reactivity significantly decreased only in the epineurial and endoneurial blood vessels compared to D group ($P<0.05$) (Fig. 2 and Table 2). Collagen IV (α1) mRNA expressions in sciatic nerves were detected by real-time PCR. Data analysis revealed that collagen IV (α1) mRNA levels significantly increased (0.7 fold) in the D group compared to the control group ($P<0.05$) (Fig. 3). Insulin treatment reduced collagen IV (α1) mRNA expression to levels not different from those in the control rats. ALA failed to protect the sciatic nerves against up-regulation of collagen IV (α1) mRNA (Fig. 3).

Discussion

In the present study, we investigated the effect of α-lipoic acid administration on the blood glucose levels, body weight and collagen IV immunoreactivity of sciatic nerves in the STZ-induced diabetic rats. Our finding revealed that ALA with 100 mg/kg, intraperitoneally injection has shown hypoglycemic effects in diabetic rats. Previous showed that ALA decreased the blood glucose levels in diabetes (Nagamatsu, 1995; Obrosova, 1998; Baydas, 2004 and

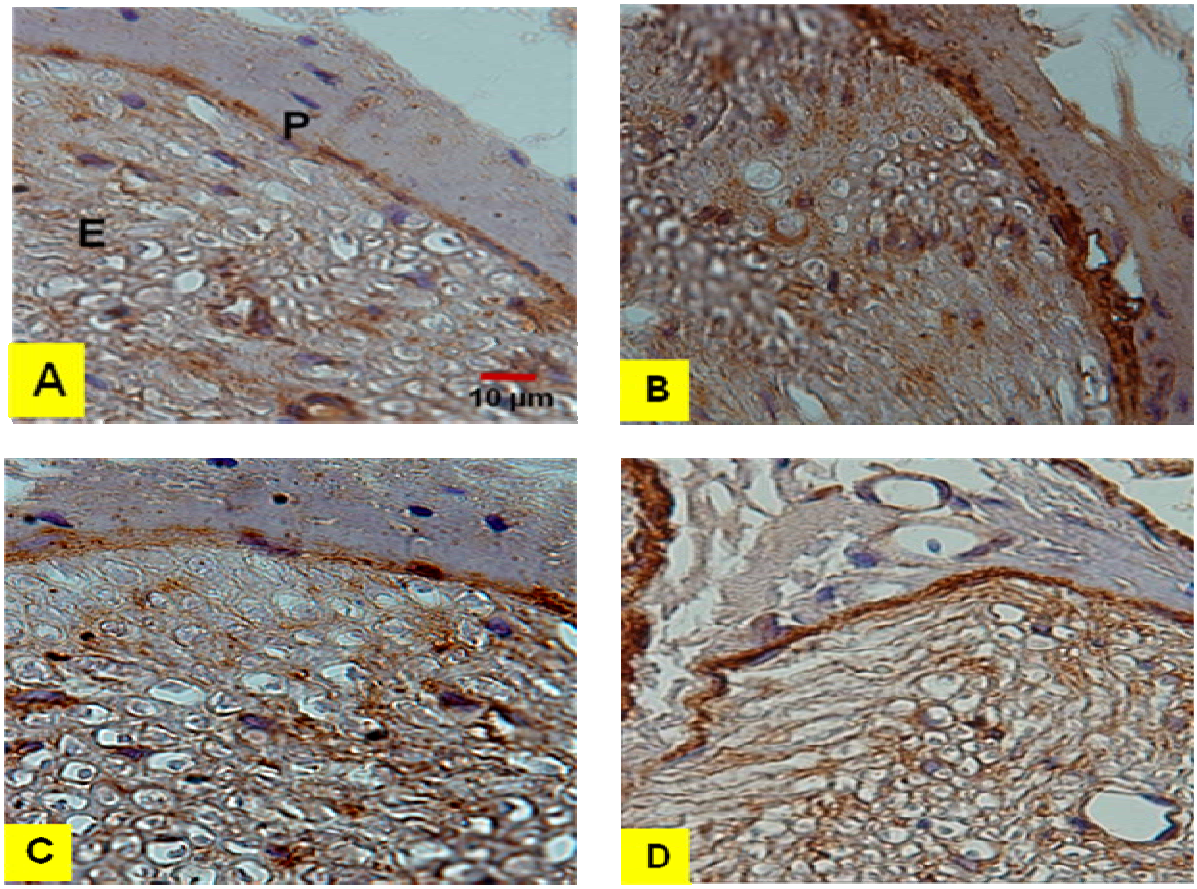

Fig. 1: Immunohistochemical staining in control and diabetic nerve samples for collagen IV reaction in both the perineurium (P) and endoneurium (E). The locations which expressed collagen IV were brown.

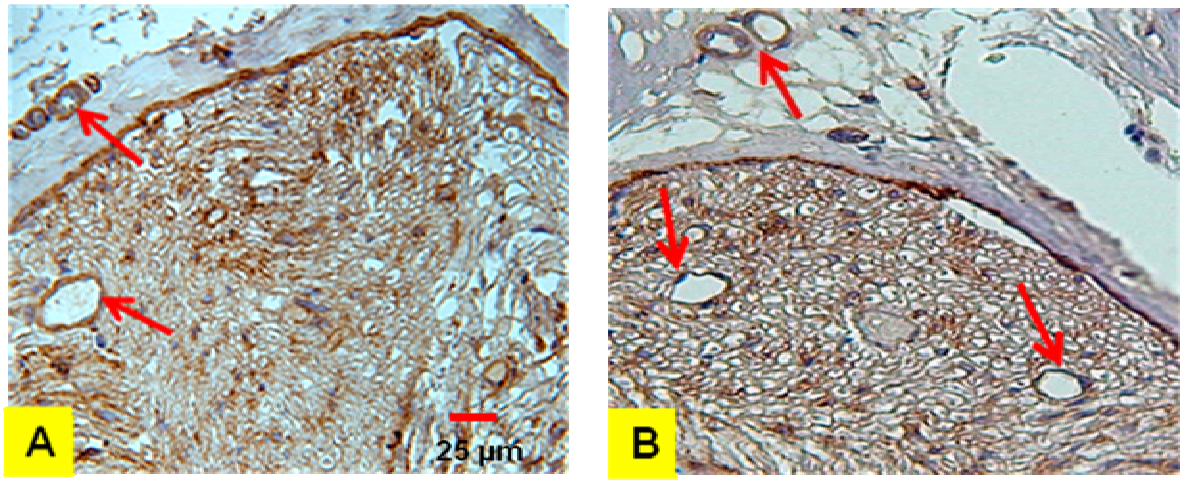

Fig. 2: The arrows in A indicated epineurial and endoneurial blood vessels have strongly reactivity for collagen IV in untreated diabetic group. The arrows in B indicated decreased immunoreactivity in diabetic with ALA treatment group.

Siti Balkis, 2008). This may be due to increase glucose transport by ALA (Stevens et al., 2000). Unsuccessful nerve regeneration in diabetic neuropathy suggested that may be in part due to changes in extracellular matrix (ECM) composition (Bradley et al., 2000). ECM proteins of peripheral nerve may alterations in the

Table 1: Mean body weight and blood glucose level of the rats in different groups.

Group	Body Weight (g)		Blood glucose level (mg/dl)
	Initial	Final	End of study
C	235.1±7.7	304.2±6.5	111.1±8.9
D	226.1±35.3	201.8±10.6*	480.5±32.0*
D+INS	218.0±31.6	253.6±20.8	128.0±10.0***
D+ALA	218.8±32.6	214.8±11.7*	423.8±13.0**

C: control, D: diabetic, D+INS:diabetic treated with Insulin, D+ALA:diabetic treated with ALA; Data are expressed as means ± SEM. n=8 for each group. *P<0.001**:** compare to control group; **P<0.05 and ***P<0.001**:** compare to untreated diabetic group.

Table 2: Comparison of collagen IV immunoreactivity in the sciatic nerves of different groups

Group	Perineurium	Endoneurium	Blood vessels
C	++	++	+++
D	++++*	+++*	++++*
D+INS	++**	++**	+++**
D+ALA	++++*	+++*	++**

C: control, D: diabetic, D+INS:diabetic treated with Insulin, D+ALA:diabetic treated with ALA; ++++**:** very strong expression, +++**:** strong expression, ++**:** moderate expression, n=8 for each group; *P<0.05**:** compare to control group, **P<0.05**:** compare to diabetic group.

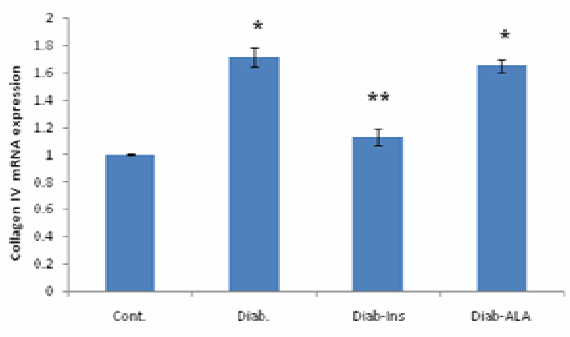

Fig. 3: Comparative analysis of collagen IV (α1) mRNA expression in sciatic nerves of the rats by using real-time PCR. Significant up regulation in diabetic animals was prevented with insulin treatment, but ALA failed to prevent against mRNA up regulation. Data are mean ± standard error of the mean. n=5. Cont:control; Diab: Diabetic; Diab-Ins: Diabetic with insulin treatment; Diab-ALA:Diabetic with ALA treatment. Significance at: *P<0.05: compare to control group, **P<0.05: compare to diabetic group.

structure and function that induced by hyperglycemia or advanced glycation end products (Chiu et al., 2008; Duran-Jimenez et al., 2009). Based on our immunohistochemial results diabetes in rats resulted in significantly increased expression of collagen IV in the perineurium, endoneurium and epineurial and endoneurial blood vessels, but it was not detected in the epineurium in general. These results are consistent with Muona et al. (1993) who found collagen IV to be up regulated in Schwann cells and perineurial cells in the presence of excess glucose. Also Bradley et al. (2000) in a human study found that collagen IV was up-regulated in the perineurium, endoneurium and in the endoneurial microvessels. Our finding was consistent with Layton et al. (2004) who reported larger amount of collagen IV in diabetic nerves. They also showed significantly higher abundance of collagen IV in the endoneurium and perineurium in nerves than the control group. Previous studies have shown collagen IV is glycated in diabetic condition and increased synthesis of type IV collagen might make a massive deposit of microfibrils between perineurial cell layers (Muona et al., 1993; Bradley et al., 2000; Yasuda et al. 2003). Hill et al. (2009) indicated that in human diabetic neuropathy although the mean value of perineurial and endoneurial collagen IV content in diabetic group was greater than the control group, but the difference was not significant. Serafin et al. (2010) assessed the changes of collagen IV expression in peripheral nerves in a low-dose STZ-induced diabetic mouse. In contrast to our results, they reported did not differ in collagen IV expression in the diabetic nerves. This difference may be due to difference in methodology. In Serafin et al. (2010) study, mice were induced diabetic with a low dose of streptozotocin and in short time period studied (four weeks). Diabetes duration and long-term hyperglycemia are the most important reasons for polyneuropathy (Bureković et al., 2008). Impairment of the nutritive flow leads to nerve ischemia in diabetes (Wang et al., 2004). The antioxidant reduces vascular impairment in diabetic rats (Sotnikova et.al., 2006). Beneficial effect of ALA on the neuropathic symptoms due to diabetic neuropathy has been reported (Ametov, 2003; Ziegler, 2004; Yorek, 2004). ALA prevents neural injury by inhibiting oxidative stress in STZ diabetic rats (Baydas et al., 2004) and reduced symptom of diabetic polyneuropathy (Zeigler, 2006; Ranieri 2010). Hyperglycemia leads to the formation of advanced glycation end products (AGEs). AGEs stimulates production of oxygen free radicals that leading to oxidative stress (Bhatti et al., 2005). Oxidative stress has been suggested to contribute to defective nerve blood supply and ALA has been shown to protect peripheral nerves from ischemia in experimental diabetic neuropathy (Vallianou et al., 2009). Our findings showed that ALA treatment decreased collagen IV expression in the basement membrane of epineurial and endoneurial blood vessels in the rat's diabetic nerves. Coppey (2001) and Okudan (2011) suggested that ALA prevents the vascular complications in the STZ induced diabetic rats. Chernousov et al. (2008) reported that collagen IV (α1)

subunit is present in all basement membranes and may be essential for Type-IV collagen assembly. Our results revealed that collagen IV (α1) mRNA expression increased in untreated diabetic rats compare to control rats. Muona et al. (1991) reported that incubation of cell cultures consisting of Schwann cells and perineurial cells in high glucose concentrations resulted in elevation of collagen IV (α1) mRNA expression. Stitt et al. (2002) reported that experimental diabetes in rats is associated with over expression of collagen IV mRNA in retina. Chiu (2008) and Yuan (2011) demonstrated collagen IV (α1) mRNA expression increased in kidney or heart of diabetic rats. In our study, ALA treatment in contrast to insulin treatment did not affect collagen IV (α1) mRNA expression in sciatic nerves. Diabetic neuropathy is a multi factorial disorder and it seems that ALA alone had no affect in improving collagen IV (α1) mRNA up regulation in diabetic nerves.

Conclusions

Based on our data, we suggest that ALA has the potential in improving hyperglycemia by reducing oxidative stress and may partially prevent alteration collagen IV in diabetic nerves.

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