Effects of U83836E on nerve functions, hyperalgesia and oxidative stress in experimental diabetic neuropathy

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Abstract

Oxidative stress has been implicated to play an important role in the pathogenesis of diabetic neuropathy, which is the most common complication of diabetes mellitus affecting more than 50% of diabetic patients. In the present study, we have investigated the effect of U83836E [(-)-2-((4-(2,6-Di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl)methyl)-3,4-dihydro-2,3,7,8-tetramethyl-2H-1-benzopyran-6-ol, 2HCl], a potent free radical scavenger in streptozotocin (STZ)-induced diabetic neuropathy in rats. STZ-induced diabetic rats showed significant deficit in motor nerve conduction velocity (MNCV), nerve blood flow (NBF) and thermal hyperalgesia after 8 weeks of diabetes induction, indicating development of diabetic neuropathy. Antioxidant enzyme (superoxide dismutase and catalase) levels were reduced and malondialdehyde (MDA) levels were significantly increased in diabetic rats as compared to the age-matched control rats, this indicates the involvement of oxidative stress in diabetic neuropathy. The 2-week treatment with U83836E (3 and 9 mg/kg, i.p.) started 6 weeks after diabetes induction significantly ameliorated the alterations in MNCV, NBF, hyperalgesia, MDA levels and antioxidant enzymes in diabetic rats. Results of the present study suggest the potential of U83836E in treatment of diabetic neuropathy.

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Keywords: Diabetic neuropathy; Oxidative stress; U83836E; Nerve function; Hyperalgesia; Lipid peroxidation; Antioxidant enzyme activity

Introduction

Diabetes mellitus is one of the serious problems in developing as well as developed countries. The number of people affected with diabetes worldwide is projected to be 366 million by year 2030 (Wild et al., 2004). Uncontrolled chronic hyperglycemia in diabetic patients leads to several complications including retinopathy, nephropathy, autonomic dysfunctions and neuropathy. Diabetic neuropathy is the most common complication affecting more than 50% of diabetic patients. Diabetes-induced deficits in motor and sensory nerve conduction velocities and other manifestations of peripheral diabetic neuropathy (PDN) have been well correlated with chronic hyperglycemia. Hyperglycemia has been reported to result in increased polyol pathway activity, oxidative stress, advanced glycation end product formation (AGE), nerve hypoxia/ischemia, increased activation of protein kinase C and impaired nerve growth factor support (Sima and Sugimoto, 1999; van Dam, 2002; Feldman, 2003; Obrosova, 2003; Vincent et al., 2004). All these pathways contribute to the development of diabetic neuropathy.

Oxidative stress resulting from enhanced free radical formation and/or a defect in antioxidant defenses has been implicated in the pathogenesis of experimental diabetic neuropathy. Reactive oxygen species (superoxide radical, hydrogen peroxide and hydroxyl radical) and reactive nitrogen species (peroxynitrite) contribute to pathophysiological changes in diabetic neuropathy (Vincent et al., 2004). Antioxidant enzyme defense system (Superoxide dismutase, catalase and glutathione peroxidase) is also attenuated in peripheral nerves of diabetic animals indicating the vital role of oxidative stress in diabetic neuropathy (Low et al., 1997). Several mechanisms including auto oxidative glycosylation, formation of AGE and increased polyol pathway activity contribute to increased oxidative stress (Van Dam, 2002; Vincent et al., 2004). Oxidative stress also activates down stream pathways such as poly(ADP-ribose) polymerase (PARP) and mitogen-activated...
protein kinases (MAPK) (Evans et al., 2002; Obrosova et al., 2004). Oxidative stress causes vascular impairment leading to endoneurial hypoxia resulting in impaired neural function (Wang et al., 2002; Yorek et al., 2004), reduced nerve conduction velocity and loss of neurotrophic support. Long-term oxidative stress can also mediate apoptosis of neurons and Schwann cells leading to nerve damage (Sekido et al., 2004). Above-mentioned studies suggest that oxidative stress may be one of the major pathway in the development of diabetic neuropathy and that an antioxidant can prevent or reverse hyperglycemia-induced nerve dysfunctions.

Several antioxidants such as α-lipoic acid, taurine, acetyl-L-carnitine, M40403 and β-carotene have demonstrated to ameliorate nerve function deficit in experimental diabetic neuropathy (Cameron and Cotter, 1999; Coppey et al., 2001a, b; Heller et al., 2004; Karasu et al., 1995; Love et al., 1996; Nickander et al., 1996; Rosen et al., 1995). These reports suggest that reducing oxidative stress may inhibit the development of diabetic neuropathy. Therefore, in the present study, we have investigated the effect of U83836E, a potent free radical scavenger on nerve functions, nociception and oxidative stress in streptozotocin (STZ)-induced diabetic neuropathy model in rats.

Materials and methods

Chemicals and drug solution preparation

U83836E [((-)-2-((4-(2,6-Di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl)methyl)-3,4-dihydro-2,3,7,8-tetramethyl-2H-1-benzopyran-6-ol, 2HCl] was purchased from Calbiochem, Germany. Atropine sulphate and STZ were procured from Sigma, U.S.A. Thiopentone sodium was obtained from Neon, India. Glucose oxidase–peroxidase (GOD/POD) glucose kit was purchased from Accurex, India. All other chemicals of analytical grade were purchased locally. STZ was dissolved in citrate buffer (pH 4.4) and U83836E was dissolved in distilled water.

Animals

Healthy male Sprague–Dawley rats (250–270 g, 8–10 weeks old) were obtained from the Central Animal Facility (CAF), National Institute of Pharmaceutical Education and Research (NIPER). Animals were provided with standard diet and water ad libitum. They were housed in plastic cages (three in each) at a controlled temperature of 24±1°C and humidity of 55±5%, with 12-h light and dark cycle. All animals were acclimatized for minimum period of 1 week prior to the beginning of study. The experimental protocols were approved by the Institutional Animal Ethics Committee of NIPER.

Induction of diabetes

A single dose of STZ (55 mg/kg, i.p.) was used for induction of type I diabetes in rats. Age-matched control rats received the equal volume of vehicle (citrate buffer). Diabetes was confirmed after 48 h of STZ injection by estimating plasma glucose levels using GOD/POD kit. Rats with plasma glucose levels of >250 mg/dl were included for further studies.

Experimental design and drug treatment

We have reported earlier that diabetic neuropathy develop within 6 weeks after the induction of diabetes (Kumar et al., 2005). In this study, development of diabetic neuropathy was ensured before starting the treatment by measuring motor nerve conduction velocity (MNCV) and hyperalgesia after 6 weeks of diabetes induction. Six weeks after the diabetic induction therapeutic treatment with U83836E (3 and 9 mg/kg, i.p., daily) was given for 2 weeks (7th and 8th weeks). All parameters (MNCV, nerve blood flow (NBF), hyperalgesia, lipid peroxidation and antioxidant enzyme activity level) were evaluated on completion of treatment.

MNCV measurement

Power Lab 8sp system (ADInstruments, Australia) was used for the measurement of MNCV. MNCV was measured in anesthetized rats by stimulating the sciatic (proximal to sciatic notch) and tibial (proximally to ankle) nerve using bipolar needle (26.5-gauge) electrodes with 3 V, single stimulus exactly as described previously (Kumar et al., 2005; Saini et al., 2004). During the study, body temperature of rats was maintained at 37°C using homeothermic blanket system (Harvard, UK). MNCV was calculated by following formula:

\[ \text{MNCV} = \frac{\text{distance between sciatic and tibial nerve stimulation point}}{\text{(sciatic M wave latency–tibial M wave latency)}}. \]

Composite NBF measurement

Nerve blood flow was measured using laser Doppler flowmeter (Perimed, Sweden). Animals were anaesthetized by administering 1.5% thiopentone sodium (30 mg/kg, i.p.) and placed on stereotaxic apparatus to achieve uniform position of laser Doppler probe. Sciatic nerve of left flank was exposed and laser probe placed just above the nerve. Animals were stabilized for 15 min prior to 10-min continuous NBF recording, as per previously described procedure (Kumar et al., 2005; Saini et al., 2004). Average values of 10-min recording were represented as perfusion unit. During the study, body temperature of rats was maintained at 37°C using homeothermic blanket system (Harvard, UK) and sciatic nerve temperature was monitored using digital thermometer (Century, India). Laser Doppler technique is considered to be an appropriate method for the measurement of composite sciatic NBF (Stevens et al., 1994), even though it cannot discriminate between endoneurial and epineurial blood flow. Data obtained from laser Doppler flowmeter have been reported to have a significant correlation between conventional techniques such as the hydrogen clearance method (Takeuchi and Low, 1987) and the $^{14}$C iodoantipyrine method (Rundquist et al., 1985).
Thermal hyperalgesia: cold and hot immersion test

Hyperalgesia was assessed by tail immersion test (Courteix et al., 1993). Rat tail was immersed in cold water (10°C—for cold immersion test) or warm water (45°C—for hot immersion test) and the tail flick response latency (withdrawal response of tail) was observed as the end point response.

Lipid peroxidation

Lipid peroxidation was estimated by measuring the formed malondialdehyde (MDA) colorimetrically using method described by Zhang et al. (2004). Sciatic nerve was isolated bilaterally from the inguinal ligament to its trifurcation. Isolated nerve was homogenized in 1 ml of 100 mM phosphate buffer solution (PBS) having pH 7.4 using polytron homogenizer. An aliquot (100 μl) of homogenate was added to a reaction mixture containing 50 μl of 8.1% sodium dodecyl sulphate, 300 μl of 20% acetic acid, 300 μl of 0.8% thiobarbituric acid and 60 μl distilled water in a tightly closed capped tubes. Samples were then incubated at 95°C for 1 h, after incubation samples were cooled to room temperature and then centrifuged at 4000 × g for 10 min. Supernatant was removed and absorbance was taken at 532 nm; quantification was done using the standard curve generated with authentic MDA following similar conditions. The MDA content was expressed as μmol mg−1 of tissue protein.

Antioxidant enzyme activity

Catalase activity

Sciatic nerve was isolated bilaterally from the inguinal ligament to its trifurcation. The isolated nerve was then homogenized in 1 ml of 100 mM PBS pH 7.4 using polytron homogenizer. Homogenate was then centrifuged at 4°C, 15,000 rpm for 10 min. Then, supernatant was used for the measurement of catalase activity by H2O2 degradation method according to Stevens et al. (2000).

SOD activity

SOD activity was assessed using NADH oxidation method described by Paoletti and Mocali (1990). Briefly, the following reagents were added in reaction cuvette: 800 μl of triethanolamine–diethanolamine buffer (pH 7.4), 40 μl of 7.5 mM NADH, 25 μl of EDTA/MnCl2 (100:50 mM) (pH 7), 100 μl of homogenate (sample) or phosphate buffer (blank). After thorough mixing, the reaction was initiated by adding 100 μl of 10 mM mercaptoethanol and reaction was followed at 340 nm using Perkin Elmer spectrophotometer. The rate of nucleotide oxidation (in blank) and inhibition of the same by homogenate was calculated.

Statistical analysis of the results

All the results are expressed as mean ± standard error of mean (S.E.M.). Significance of difference between the two groups was evaluated using Student’s t-test. For multiple comparisons, one-way analysis of variance (ANOVA) was used. When ANOVA showed significant difference, post hoc analysis was performed with Tukey’s test. P < 0.05 was considered statistically significant. Statistical analysis was carried out using Jandel Sigma Stat Version 2, software.

Results

Plasma glucose levels and body weight

STZ-induced diabetic rats showed approximately five-fold increase in the blood glucose levels after STZ administration, which was consistent throughout the study period. Diabetic rats showed significant decrease in body weight as compared to age-matched control rats. U83836E treatment did not produce any change in body weight and plasma glucose levels (Table 1).

Motor nerve conduction velocity

A significant decrease (12% and 14%) in MNCV was observed after sixth and eighth weeks of diabetes induction. MNCV in diabetic rats was 44.22 ± 1.55 m/s (sixth week) and 42.76 ± 1.38 m/s (eighth week) as compared to their age-matched control group (50.39 ± 2.17 m/s and 49.90 ± 1.94 m/s, respectively). Two-week U83836E (3 and 6 mg/kg, i.p.) treatment produced significant reversal (78% and 100%, respectively) of MNCV deficits in diabetic rats (Fig. 1).

Composite nerve blood flow

Eight-week diabetic rats showed 64% reduction in NBF; this deficit was reversed up to 13% upon treatment with U83836E 3 mg/kg, which was not statistically significant, but treatment with U83836E 9 mg/kg significantly (p < 0.01) reversed the deficit up to 20% (Fig. 2).

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Normal control</th>
<th>Diabetic control</th>
<th>Diabetic + U83836E 3 mg/kg</th>
<th>Diabetic + U83836E 9 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>393.33±4.45</td>
<td>253.83±19.19***</td>
<td>249.67±5.63</td>
<td>264.2±11.76</td>
</tr>
<tr>
<td>Plasma glucose (mg/dl)</td>
<td>106.96±3.15</td>
<td>581.51±17.48***</td>
<td>567.34±11.15</td>
<td>560.07±34.06</td>
</tr>
</tbody>
</table>

Values are represented as mean±S.E.M.

*** p<0.001 vs. normal control.
Thermal hyperalgesia

A significant decrease in tail flick latency was observed after sixth and eighth weeks of diabetes induction in cold and hot immersion test (Fig. 3A and B). This deficit in tail flick response latency was significantly ($p<0.05$) reversed on 2-week treatment with U83836E 3 and 9mg/kg (Fig. 3A and B).

Lipid peroxidation

MDA level in eighth week diabetic rats was significantly ($p<0.05$) increased as compared to the age-matched control rats (15.06±0.70 vs. 12.86±0.56 μM/mg of protein). Two-week treatment with U83836E inhibited this increase in MDA levels in treated diabetic rats (Fig. 4).

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**Fig. 1.** Effect of 2-week treatment with U83836E on motor nerve conduction velocity in diabetic rats. *$p<0.05$ vs. age-matched control rats, †$p<0.05$ vs. diabetic rats without treatment, ‡$p<0.05$ vs. their respective pretreatment group.

**Fig. 2.** Effect of 2-week treatment with U83836E on composite nerve blood flow. Composite nerve blood flow was measured in eighth week diabetic rats with or without treatment. ***$p<0.001$ vs. age-matched control rats, ‡‡$p<0.01$ vs. diabetic rats without treatment.

**Fig. 3.** Effect of 2-week treatment with U83836E on hyperalgesia (A: cold immersion test and B: hot immersion test). *$p<0.05$ vs. age-matched control rats, †$p<0.05$ vs. diabetic rats without treatment, ‡$p<0.05$ vs. their respective pretreatment group.

**Fig. 4.** Effect of 2-week treatment with U83836E on MDA levels. MDA levels were measured in eighth week diabetic rats with or without treatment. *$p<0.05$ vs. age-matched control rats, ‡$p<0.05$ vs. diabetic rats without treatment.
Catalase activity

In the eighth week diabetic rats, catalase activity was significantly decreased (0.063±0.01 U/mg of protein) as compared to the age-matched control rats (0.12 ±0.01 U/mg of protein). Dose dependent improvement was observed up on 2-week treatment with U83836E at 3 and 9mg/kg (Fig. 5).

SOD activity

In the eighth week diabetic rats, SOD activity was significantly decreased (0.62±0.19 U/mg of protein) as compared to the age-matched control rats (1.72 ±0.18 U/mg of protein). Two-week treatment with U83836E 3mg/kg and 9mg/kg significantly ($p<0.05$) inhibited the decrease in SOD activity in treated diabetic rats as compared to vehicle-treated rats (Fig. 6).

Discussion

In our study, development of diabetic neuropathy in STZ-induced diabetic rats was evident from reduction in MNCV, NBF and decrease in tail flick latency. We observed 14% and 64% deficit in MNCV and NBF respectively after 8 weeks of diabetes induction. These results are consistent with the previous reports, wherein similar reductions of MNCV and NBF in STZ-induced diabetic rats were reported (Cameron and Cotter, 2002; Cotter et al., 2002).

Generation of ROS and reduced generation and/or bioactivity of nitric oxide by the endothelial nitric oxide synthase in diabetic condition are well established. It has been demonstrated that oxidative stress produce nerve conduction velocity deficits in diabetic rats (Cameron and Cotter, 1995; Cameron et al., 1994; Cameron et al.1998; Cotter et al., 1995; Karasu et al., 1995; Love, et al., 1996). Reduction in nerve conduction velocity in our study was prevented up to 78% at U83836E (3mg/kg) and complete reversal was observed with U83836E (9mg/kg).

A number of reports indicate that peripheral diabetic neuropathy (PDN) is a hypoxic neuropathy. Decrease in NBF and resulting endoneurial hypoxia may cause MNCV slowing (Cameron and Cotter, 1997; Coppey et al., 2001a, b; Obrosova et al., 2002). Impaired endothelium dependent vasodilatation has been demonstrated in various vascular beds of animal models of diabetes and humans with type 1 and type 2 diabetes (De Vriese et al., 2000; Prieper and Siebeneich, 1997). Free radicals such as $O_2^-$ and $OH^-$ cause vascular endothelial damage and reduced NO$^-$ mediated vasodilatation (Cameron and Cotter, 1997). Studies have provided evidence that $O_2^-$ and peroxynitrite impairs endothelium dependent vascular relaxation of epineural arterioles of the sciatic nerve from diabetic rats (Coppey et al., 2002; Nishikawa et al., 2000). We observed 64% decrease in sciatic NBF in diabetic rats. This deficit in composite NBF was improved significantly but not completely reversed upon U83836E treatment. It has been reported that complete normalization of NBF is not required for correction of MNCV in diabetic rats (Obrosova, 2003).

Several groups have suggested that vascular mechanism play an important role in MNCV deficits in PDN (Cameron et al., 2001; Coppey et al., 2001a,b; Low et al., 1997). In addition to vascular mechanisms, nonvascular mechanisms have also been reported to cause conduction deficits (Li et al., 2004; Obrosova, 2003). Enhancement of neurotrophic factors by prosaposin-derived peptide has shown to preserve nerve conduction, but not NBF by reversible modification of the metabolic and neurotrophic state of the neuron and Schwann cells in the peripheral nervous system (Mizisin et al., 2001).

![Fig. 5](image1.png)

**Fig. 5.** Effect of 2-week treatment with U83836E on catalase activity. Catalase activity was measured in eighth week diabetic rats with or without treatment. **$p<0.01$ vs. age-matched control rats, $p<0.05$ vs. diabetic rats without treatment.**

![Fig. 6](image2.png)

**Fig. 6.** Effect of 2-week treatment with U83836E on SOD activity. SOD activity was measured in eighth week diabetic rats with or without treatment. **$p<0.01$ vs. age-matched control rats, $p<0.05$ vs. diabetic rats without treatment.**
Also the PARP inhibitors known to inhibit energy depletion due to oxidative stress restore normal nerve energy state and prevent reduction in conduction deficits (Obrosova et al., 2004). P334, a PARP inhibitor normalized nerve energy level to state that completely reversed diabetes induced MNCV deficits (Obrosova, 2003).

U83836E might increase energy metabolism, reduce AGE formation and inflammation as reported with other antioxidants (α-lipoic acid, vitamin E, vitamin C and N-acetyl-l-cysteine). α-Lipoic acid has shown to increase energy metabolism and myo-inositol level in diabetic nerves (Kishi et al., 1999; van Dam, 2002). Vitamin C and vitamin E have been reported to decrease formation of AGE products (Singh et al., 2001). N-Acetyl-l-cysteine and α-tocopherol have shown to reduce pro-inflammatory cytokines (IL, TNF-α), chemokines and C-reactive proteins in diabetic rats (Jialal et al., 2002; Sagara et al., 1996).

U83836E treatments have shown reduction of apoptotic cell death, which is usually a consequence of oxidative stress (Patel and Gores, 1997).

In diabetic rats, we observed a significant increase in MDA levels and reduction in antioxidant enzyme activity. U83836E treatment significantly reduced MDA levels and increased antioxidant enzyme (SOD and catalase) activity in diabetic rats. U83836E have already been reported to increase SOD levels and decrease MDA levels in streptozotocin low dose-treated mice (Papaccio et al., 1995). α-Lipoic acid, an antioxidant, has also been reported to improve levels of these antioxidant enzymes and restore deficits in diabetic neuropathy (Stevens et al., 2000). Vitamin C and E normalize antioxidant enzyme expression in diabetic rats (Sindhu et al., 2004). Melatonin, a potent antioxidant, has shown to produce an increase in antioxidant enzyme expression (Esparza et al., 2005). There might be possibility that U83836E enhance antioxidant enzyme expression, thereby increasing antioxidant enzyme levels and decrease the formation of free radicals leading to inhibition of lipid peroxidation.

Neuropathic pain is commonly associated with diabetic neuropathy. We observed reduction in tail flick latencies in hot and cold immersion test in diabetic rats, which indicate thermal hyperalgesia. Several researchers have reported similar hyperalgesia in diabetic rats (Anjaneyulu and Chopra, 2004; Ciruela et al., 2003; Wuarin-Bierman et al., 1987). Mechanisms, such as tissue injury due to ischemia, sensitization of peripheral receptors and ectopic activity in sprouting fibers and alterations in dorsal root ganglia cells, are reported to contribute to change in nociception (Jensen and Baron, 2003). In the present study, we observed a significant improvement in tail flick response latency for hot as well as cold immersion performance upon U83836E treatment, thus suggesting the role of oxidative stress in nociceptive changes in diabetic rats.

In conclusion, U83836E showed significant protection in diabetic neuropathy as evident from improvement in MNCV, NBF, antioxidant enzyme levels, reduction in thermal hyperalgesia and lipid peroxidation in treated diabetic rats. This study suggests the role of oxidative stress in diabetic neuropathy and beneficial effects of U83836E in diabetic neuropathy.

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