Neuropathy in a rat model of mild diabetes induced by multiple low doses of streptozotocin: effects of the antioxidant stobadine in comparison with a high-dose α-lipoic acid treatment

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Abstract. Hyperglycaemia-induced oxidative stress makes an important contribution to the aetiology of diabetic neuropathy. The aim of our study was to evaluate the effect of the antioxidant stobadine (STB) in comparison with a treatment by a high-dose of α-lipoic acid (ALA), on the neurological consequences of chronic hyperglycaemia in an animal model of diabetes in Wistar rats (16 weeks old), made diabetic by streptozotocin (STZ 3 × 20 mg i.v.).

Neuropathy was evaluated electrophysiologically by measuring motor nerve conduction velocity (NCV) in the 4th and 8th week in vivo and motor NCV and resistance to ischaemic conduction failure (RICF) of the sciatic nerve in the 10th week of the experiment in vitro. The therapy with ALA (100 mg/kg i.p., 5 times a week) and STB (25 mg/kg i.p., 5 times a week) had a significant effect on NCV in vivo in the 8th week of the experiment and no effect in the 10th week in vitro. The RICF elevated in diabetic animals was significantly modified by ALA. The effect of the antioxidant STB on NCV was comparable with that of ALA, while RICF was affected only by ALA.

We conclude that treatment with appropriate antioxidants might partially prevent nerve dysfunction in diabetic rats.

Key words: α-lipoic acid — Diabetes — Neuropathy — Oxidative stress — Stobadine

Abbreviations: ALA, α-lipoic acid; NCV, nerve conduction velocity; MDA, malondialdehyde; MLD, multiple low doses; ROS, reactive oxygen species; RICF, resistance to ischaemic condition failure; STB, stobadine; STZ, streptozotocin.

Introduction

Diabetic neuropathy occurs through diverse pathogenic mechanisms, most of them being initiated by hyperglycaemia and oxidative stress (Baynes 1991; Van Dam et al. 1995; Pop-Busui et al. 2006).

Several factors promote oxidative stress in diabetes, including increased free radical production caused by antioxidation reactions of sugars with proteins and unsaturated lipids (Baynes 1991; Sima and Sugimoto 1999), nerve ischaemia-reperfusion (Schmelzer et al. 1989; Wang et al. 2004) and impairment of tissue anti-oxidant protection systems (Low et al. 1997). Shifts in redox balances due to derangement in energy metabolism of carbohydrates and lipids also contribute to the overt oxidative stress in the diabetic individual. In experimental diabetic neuropathy, oxygen-free-radical activity was increased in the sciatic nerve (Ziegler 2004). Oxidative modification of structural proteins and lipids (Schmelzer et al. 1989; Baynes 1991; Sima et al. 1993) parallels a progressive defect in the paranodal barrier system (Sima et al. 1993) and leads to subsequent impair-
ment in nerve conduction velocity (NCV) and resistance to ischaemic conduction failure (RICF) (Cameron et al. 1993; Biro et al. 1997; Cameron and Cotter 1999).

Recent data stress the importance of mutual interactions between metabolic mechanisms at the endothelial level resulting in focally-increased vascular permeability (Eaton et al. 1996) and perfusion abnormalities (Cameron and Cotter 1994; Cameron et al. 1998; Nangle et al. 2006).

Early impairment of endoneurial nutritive flow leads to insufficient perfusion and nerve ischaemia in diabetic state (Wang et al. 2004). RICF, an adaptive mechanism that increases the ability of the nerve to cope with insufficient perfusion, can to a great extent be explained also by increased glucose, fructose and glycogen stores, and perhaps reduced energy requirements (Low et al. 1985; Sango et al. 1995).

There is a well accepted notion that reactive oxygen species (ROS) affect also neurons and Schwann cells, directly as well indirectly via vascular effects (Cameron et al. 1993; Van Dam et al. 1995; Biro et al. 1997; Cameron and Cotter 1999; Cameron et al. 2008).

A potent lipophilic antioxidant, α-lipoic acid (ALA) prevented or improved hyperglycaemia-induced neurovascular and metabolic abnormalities in various organ systems (Low et al. 1997). The results of numerous pre-clinical and clinical studies have confirmed its efficacy in the treatment of diabetic neuropathy (Ford et al. 2001; Van Dam et al. 2001; Gouty et al. 2003; Tankova et al. 2004; Ziegler 2004; Varkonyi and Kempler 2008; Ziegler 2008).

The pyridinolone antioxidant stobadine (STB) has antioxidant properties and the ability to scavenge ROS, such as hydroxyl, peroxyl, and alkoxyl radicals (Horakova and Stolc 1998). Moreover, it is an effective quencher of singlet oxygen. Under conditions of the experimental glycation model in vitro, STB was found to protect bovine serum albumin against glycoxidative damage (Stefek et al. 1996). STB ameliorated the NCV impairment of the motor nerves in young streptozotocin (STZ)-diabetic rats (Skalska et al. 2008) and improved sympathetic neurotransmission and abnormal function of the diabetic vas deferens (Gunes et al. 2005). These findings, along with the high oral bioavailability of STB, its toxic safety, as well as efficient detoxification pathways, render this drug a prospective agent in the prevention of late diabetic complications (Stefek et al. 2000; Sotnikova et al. 2001; Stelek et al. 2002 a,b; Pekiner et al. 2002; Ulusu et al. 2003; Kyselova et al. 2005; Sotnikova et al. 2006; Vlkovicova et al. 2006; Vrbjar et al. 2007; Yulek et al. 2007).

The aim of the present study was to compare the effect of the antioxidant STB and ALA, a naturally occurring free radical scavenger (Packer and Tritschler 1996; Cameron et al. 1998) commonly used in therapy of diabetic polyneuropathy (Nagamatsu et al. 1995; Low et al. 1996; Ziegler and Griess 1997; Cameron et al. 1998; Ziegler et al. 1999), on neurological consequences of diabetes induced in adult Wistar rats by multiple low doses (MLD) of STZ. The consequences were studied as changes in NCV and RICF.

Materials and Methods

Experimental diabetes

The study was approved by the Ethics Committee of the Institute and performed in accordance with the Principles of Laboratory Animal Care (NIH publication 83–25, revised 1985) and the Slovak law regulating animal experiments (Decree 289, Part 139, July 9th 2003). Male Wistar rats, 16 weeks old, weighing 250–300 g were used. The laboratory animals were of monitored conventional quality and were supplied by the Breeding Facility of the Institute of Experimental Pharmacology at Dobra Voda, Slovak Republic.

Experimental diabetes was induced by MLD of STZ (20 mg/kg b.w. for 3 consecutive days i.v.). STZ was dissolved in saline. Induction of diabetes with STZ was done according to Larsen et al. (2002) modified and applied to rats. The laboratory animals were fasting overnight prior to every STZ administration. After STZ injection the rats had free access to glucose solution (5 mmol/l) to avoid and/or attenuate subsequent inevitable hyperinsulinaemia and hypoglycaemic shock. Water and food were available immediately after the dosing. No animals perished along this procedure. Control animals received saline intravenously. Eleven days after STZ administration, animals with postprandial plasma glucose level >20 mmol/l were considered to be diabetic and were included in the study.

Diabetic animals were randomly assigned to groups: STZ, diabetic rats; STZ+ALA, diabetic rats treated with ALA (100 mg/kg b.w.) and STZ+STB, diabetic rats treated with STB (25 mg/kg b.w.). The therapy was administered 5 times a week i.p., over a period of 10 weeks.

The animals were housed in groups of two in T4 Velaz cages (Prague, Czech Republic) with wood shaving bedding exchanged daily. Tap water and pelleted standard diet KKZ-P-M (Dobra Voda, Slovak Republic) were available ad libitum. The animal room was kept at 23 ± 1°C, relative humidity 40–70%, 10 air changes per hour. The animals were kept under a stable regimen of 12 h light/12 h darkness.

Glycaemia measurement

Preprandial and postprandial glycaemia was measured in the 4th and 8th week after STZ administration. Preprandial glycaemia was measured after overnight fasting period (approximately 14 h, at 8 a.m.). Then water and food were im-
mediately available to all experimental animals ad libitum. Postprandial glycaemia was measured two hours after daily main meal course (at 3 p.m.). Plasma glucose levels were determined by the commercial Bio-La-Test kit (Lachema, Brno, Czech Republic). Body weight was followed during the experiment.

**In vivo recordings of nerve conductivity**

For NCV measurement in vivo we used surface contact clip electrodes placed on the gastrocnemius muscle. The sciatic nerve was stimulated by external bipolar electrode. Stimuli of supramaximal intensity were elicited by a constant current stimulator. Impulse duration was 0.1 s; two points of stimulation were chosen – sciatic notch and popliteal fossa; timebase 1 ms/div, filter band 2 Hz–10 kHz, sensitivity 10 mV. Five measurements of compound muscle action potential were then recorded from both lower extremities by electromyography, device Nicolet Viking IV P. NCV was calculated as the quotient of distance between the two stimulation points along the sciatic nerve, measured with a calliper, and the average value of all compound muscle action potential latencies measured was established. Measurements were performed in the 4th and 8th week of the study. The rats were anaesthetised with Narcotan (2.5 %) during recording.

**Ex vivo recordings of nerve conductivity**

After termination of the study (10 weeks after MLD-STZ administration), NCV and RICF were measured in the sciatic nerves ex vivo, by methods essentially the same as those described by Cameron et al. (1991). The rats fasting overnight with free access to water were anaesthetised with thiopental (65 mg/kg b.w. i.p.). Both sciatic nerves were removed with branches supplying the gastrocnemius muscle. The nerves were stored in oxygenated (O₂ 100%) Krebs solution (pH = 7.3) consisting of (in mmol/l) NaCl 136, KCl 5.6, CaCl₂·2H₂O 2.2, MgCl₂·6H₂O 1.2, HEPES 5, glucose 4.9 until transferred to an air-tight experimental chamber. The chamber was filled with mineral oil pre-gassed with O₂ (100%). It comprised a system of platinum wire electrodes for stimulation, grounding and registration. Action potential was evoked by supramaximal (max. +50%) square wave pulses lasting 0.07 ms at 0.3 Hz. Compound action potential amplitude was monitored during adaptation period lasting typically 15 min. The NCV was calculated in axons dominating in the preparation from the delay between stimulation artefact and the peak of the action potential amplitude, and the distance between the stimulation and recording electrodes. The NCV was typically ~30 m/s, hence the axons belonged apparently to the Aα group. After assessment of NCV, the O₂-equilibrated mineral oil was replaced by oil equilibrated with N₂ to asses RICF. The declining of the action potential was measured at 5-min intervals for the next 30 min.

**Drugs and reagents used**

ALA (Thioctacid Asta Medica, Germany), stobadine dihydrochloride (IEPT SAS Bratislava, Slovakia), STZ (Sigma Aldrich, Germany), HEPES (Calbiochem, USA), thiopental (VÚAB Prague, Czech Republic), Narcotan (Zentiva Prague, Czech Republic), mineral oil (paraffinum liquidum, ČsL.IV, Slavus Bratislava, Slovakia). All other chemicals used were of analytical purity.

**Statistical analysis**

One-way analysis of variance was performed and any significant (p < 0.05) differences were assigned to individual between-group comparisons using Student’s t-test (INSTAT GraphPad, San Diego, CA, USA). All data are expressed as means ± SEM.

**Results**

The first measurement of blood glucose levels was made 11 days after the induction of diabetes. The rats, with postprandial hyperglycaemia ≥20 mmol/l, were chosen for further study. Preprandial and postprandial blood glucose levels, measured in the 4th and 8th week after STZ administration, were found significantly elevated in diabetic groups compared to control rats (Fig. 1A,B). The blood glucose levels in the control rats were in the physiological range.

Besides hyperglycaemia, diabetic animals revealed further typical signs of diabetes, such as polyphagia, polydipsia and polyuria.

Body weights in the all experimental groups are shown in Table 1. Despite polyphagia, none of diabetic groups did

| Table 1. Body weight of rats within the experiment (in g) |
|-------------------|--------|--------|
| Treatment        | Day 10 | Week 10 |
| C                | 302 ± 3.5 | 423 ± 9.3 |
| STZ              | 251 ± 5.1 | 250 ± 12.1 |
| STZ+ALA           | 261 ± 3.8 | 237 ± 6.7 |
| STZ+STB           | 265 ± 3.6 | 256 ± 5.6 |

Body weights in all experimental groups at the beginning of the experiment (10 days after STZ administration) and 10 weeks after STZ administration. C, control group; STZ, diabetic group; STZ+ALA, diabetic animals treated with ALA; STZ+STB, diabetic animals treated with STB. Means with SEM are shown (n = 4 for control group, n = 8 for diabetic groups).
gain weight throughout the experiment, besides the differences among diabetic groups were not significant. On the contrary, control rats were continuously gaining weight. Administration of the antioxidants did not significantly affect blood glucose and body weights either in control or diabetic animals.

NCV measured in vivo in the 4th and 8th week after administration of MLD-STZ was significantly decreased in the diabetic group ($p < 0.01$ and $p < 0.001$, respectively). Both drugs significantly diminished ($p < 0.05$) this decrease, compared to the untreated nerves in the 8th week of the experiment (Fig. 2).

In vitro measurement of the NCV in sciatic nerves from diabetic rats 10 weeks after diabetes induction confirmed a significant decrease ($p < 0.01$) compared to that in nerves from the control rats (Fig. 3). The effect of drug treatment in this case was not significant, as shown by comparison of diabetic-treated vs. untreated experimental groups.

Nerve exposure to hypoxia in vitro induced a steady decline of the compound action potential amplitude with increasing hypoxia duration (Fig. 4). However, the nerves from diabetic animals were less sensitive to hypoxia than the non-diabetic ones with significance starting at minute 5 of hypoxia. The therapy with ALA significantly modified...
Impairment of the endoneurial nutritive flow leads to insufficient perfusion and nerve ischaemia in diabetic state (Wang et al. 2004), hence the physiological condition of the endothelium has considerable importance in optimal nerve function (Cameron and Cotter 1999; Camera et al. 2008). Consequently, oxidative stress may diminish endothelium-dependent vasodilatation. This was observed in chronic diabetic animals (Archibald et al. 1996) and even in resistance of isolated mesenteric artery in acute exposure to hyperglycaemia (Taylor and Poston 1994). Dietary supplementation of the antioxidant STB was shown to reduce vascular impairment in STZ-diabetic rats (Sotnikova et al. 2001; Sotnikova et al. 2006). Superoxide radicals react with NO giving rise to peroxynitrite, an important source of hydroxyl radicals inducing endothelial damage (Beckman et al. 1990; Pieper et al. 1993). STB could prevent endothelial damage by scavenging hydroxyl radicals, although its effect against superoxide radical was not so evident (Horakova and Stolc 1998).

Dyslipidaemia, including hypertriglyceridaemia, is considered a significant and independent risk factor for diabetic complications (Januszewski et al. 2003; Metz et al. 2003). STB was found to cause a significant correction of hypercholesterolaemia and hypertriglyceridaemia in diabetic rats (Stefek et al. 2000; Pekiner et al. 2002), consistent with beneficial effects of STB on dyslipidaemia in a short-term clinical trial in humans (Horakova and Stolc 1998).

Decomposition of lipid peroxides, increasingly generated in diabetes mellitus, may initiate chain reactions that produce reactive carbonyl compounds (Habib et al. 1994; Baynes et al. 2000; Januszewski et al. 2003). The ability of STB to attenuate lipoxidation reactions in diabetes and thus the production of toxic aldehydes may account, at least partly, also for its observed neuroprotective action. According to previous findings (Pekiner et al. 2002; Kyselova et al. 2005), STB supplementation of diabetic animals significantly attenuated plasma levels of malondialdehyde (MDA), an index of systemic oxidative damage. In our previous papers we reported on the ability of STB to reduce oxidative damage of the diabetic heart (Stefek et al. 2000, Ulusu et al. 2003) and kidney tissue (Stefek et al. 2002a), as measured by conjugated dienes or MDA.

Protection of nerve mitochondria may participate in the neuroprotective effect of STB. Mitochondrial dysfunction was recently proposed as an aetiologic factor in diabetic neuropathy (Fernyhough et al. 2003). Other authors reported its ability to preserve fine mitochondrial structure in cerebral capillaries and neurons under conditions of global cerebral ischaemia reperfusion injury in dogs (Franko et al. 1999). STB was found to ameliorate the mitochondrial energy production reduced in the diabetic kidney (Stefek et al. 2002b). STB was also reported to prevent calcium accumulation in diabetic tissues (Pekiner et al. 2002).
The measurements carried out in vitro in the 10th week of the experiment are in accordance with those observed in vivo in the 4th and 8th week. However, the in vitro beneficiary effect of the drugs used was not significant when compared to the untreated diabetic group; the in vivo measurement technique seems to be more sensitive in demonstrating changes in NCV.

Contrary to its effect on NCV, STB was ineffective on diabetes-induced RICF changes. The mechanisms underlying RICF are controversial and have not been fully explained yet. RICF can be produced acutely by an oral glucose load and corrected by insulin treatment. RICF therefore may be related to the metabolic availability of high glucose (Baba et al. 2006). Another hypothesis suggests that it is a polyol-pathway-related consequence of reduced Na,K-ATPase activity; hence the diminished demand for ATP and oxygen (Lattimer et al. 1989). Nerve function could also be adversely affected by hyperglycaemic pseudohypoxia caused by excessive flux through the second half of the polyol pathway, which may promote an elevation in cell NADH/NAD ratio with wide-spread effects on intermediate metabolism (Williamson et al. 1993). The vascular hypothesis attributes hypoxic resistance to adaptation to the hypoxic endoneurium, involving increased reliance on anaerobic energy metabolism (Low et al. 1989; Cameron et al. 1994). Results from vasodilator treatment experiments showed that RICF could be prevented without any effect on nerve polyol levels (Cameron et al. 1992).

Processes of adaptation may be partially responsible for increased RICF in diabetic state. Rat hearts with chronic diabetes were less sensitive to ischaemic injury and exhibited lower susceptibility to ventricular arrhythmias (Ravingerova et al. 2001). It was also shown that besides their negative role in the pathogenesis of diabetes, ROS, and particularly the products of non-enzymatic glycation of proteins, may participate in the mechanism of development of calcium resistance of the heart (Ziegelhofer et al. 1999).

RICF is an apparently more sensitive indicator of diabetic nerve dysfunction than NCV and correspondingly more difficult to correct (Cameron et al. 1992). In addition, hyperglycaemic exposure can independently stimulate nerve anaerobic metabolism (Strupp et al. 1991) and our interven-tion had no effect on the severity of diabetes.

The high-dose therapy with ALA improved RICF and the nerves were significantly more sensitive to hypoxia than the diabetic ones with no ALA treatment. Radical scavenging, metal chelation, amphiphilic character, interaction with other antioxidants (e.g. GSH, vitamin E, ubiquinol, vitamin C) and metabolic regeneration are important properties of ALA (Packer et al. 2001). In addition to its powerful antioxidant properties, ALA increases glucose uptake through recruitment of the glucose transporter-4 to plasma membranes, a mechanism that is shared with insulin-stimulated glucose uptake. Further, recent trials showed that ALA improved glucose disposal in patients with type II diabetes (Packer et al. 2001; Jacob et al. 1995; Evans et al. 2000). ALA is also a co-factor of α-ketoglutarat dehydrogenase and pyruvate dehydrogenase, which can be useful in supporting aerobic metabolism (Packer et al. 1995; Packer et al. 2001). These mechanisms could be considered to underlie its efficient effect on RICF.

In conclusion, the observations of the current study suggest that STB has a potential in the management of diabetic neuropathy. Even though STB did not improve RICF its effect on NCV was convincing and comparable with ALA. We conclude that treatment with appropriate antioxidants might partially prevent nerve dysfunction in STZ diabetic rats, possibly on the basis of attenuating the enhanced oxidative stress that corresponds with chronic hyperglycaemia in diabetic conditions.

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