

α -Lipoic acid corrects neuropeptide deficits in diabetic rats via induction of trophic support

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Received 20 November 1996; revised version received 13 January 1997; accepted 13 January 1997

Abstract

This study compared the effects of treatment of diabetic rats with either α -lipoic acid (100 mg/kg/day i.p. 5 days/week) or with recombinant human nerve growth factor (rhNGF; 0.2 mg/kg s.c. 3 days/week) on NGF-like immunoreactivity (NGFLI) and neuropeptide Y-like immunoreactivity (NPYLI) levels in the sciatic nerve and on the release of substance P-like immunoreactivity (SPLI) from the spinal cord in response to electrical stimulation of the dorsal roots in vitro. Diabetic rats showed depletion of NGFLI and NPYLI, together with reduced release of SPLI. Treatment with NGF increased the sciatic nerve NGFLI (to four times that seen in untreated diabetic rats) and normalised stimulus-evoked release of SPLI, but did not affect the sciatic nerve NPYLI. Treatment with α -lipoic acid caused a small non-significant increase in sciatic nerve NGFLI, but normalised both NPYLI levels and stimulus-evoked release of SPLI. These findings indicate that α -lipoic acid can boost neurotrophic support in diabetic rats, with effects beyond those related to NGF. © 1997 Elsevier Science Ireland Ltd.

Keywords: Diabetes; Lipoic acid; Nerve growth factor; Neuropathy; Neuropeptide Y; Nociception; Substance P

There is overwhelming evidence to support the hypothesis that deficient neurotrophic support contributes to the pathogenesis of diabetic neuropathies. Neurotrophic deficits develop in diabetic rats (see [5] for review) and reduced expression of nerve growth factor (NGF) has been reported in some patients [2]. In addition, there is evidence from both rats [8,21] and patients [4,15] that the neuronal gene targets for NGF are understimulated, resulting in reduced levels of neuropeptides in C-fibres in diabetes. In rats, this deficit is overcome by administration of either murine [7] or human recombinant NGF to diabetic animals and functional effects of such treatment accompany neurochemical changes [3].

The impaired neurotrophic support in diabetic rats has at least two components, reduced expression of NGF by target tissues [11,13] and reduced expression of its receptors, trkA and p75^{NTR}, by responsive neurones [16]. This results in reduced retrograde transport of NGF [9,12], with result-

tant understimulation of its transcription-dependent processes in neurone cell bodies. No mechanisms have been suggested for either of these changes; indeed mechanisms for all of the early neurological deficits in experimental diabetes remain to be proven and the availability of therapy for diabetic neuropathy is similarly restricted. One of the few agents available in sophisticated medical practice is α -lipoic acid (thioctic acid), which is licensed in Germany and has demonstrable efficacy in both experimental [19] and clinical use [22] against diabetes-associated neurological deficits. Indeed, α -lipoic acid has protective properties against other experimental neuropathies [1,14], which may also derive from impaired neurotrophic support. Accordingly we designed the present study to examine effects of α -lipoic acid treatment of diabetic rats, concentrating on aspects of NGF production and action and making a direct comparison with effects of NGF treatment.

Male Wistar rats (Weight range 304–362 g) were randomly assigned to four groups; three of these were made diabetic by a single i.p. injection of streptozotocin (STZ;

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55 mg/kg), freshly dissolved in normal saline and given after an overnight fast. Three days later the glucose concentration in tail vein blood was measured by reflectance photometry ('Reflotest' kit, Boehringer Mannheim, Germany) and animals with glucose <15 mmol/l were rejected from the study. On the same day treatment of one diabetic group with α -lipoic acid was begun and maintained to the end of the protocol, rats were given a single daily dose of 100 mg/kg i.p. in saline from Monday to Friday inclusive. Treatment with recombinant human NGF (rhNGF; Genentech) was begun in another diabetic group after 4 weeks diabetes. These animals were injected with 0.2 mg/kg NGF by s.c. injection at the back of the neck on Monday, Wednesday and Friday for the last 4 weeks of the protocol. The last dose was injected 24 h before killing the rats. The remaining two groups (one control, one diabetic) were left untreated. The total duration of diabetes was 8 weeks. Rats were killed by decapitation and sciatic nerves removed for NGF-like immunoreactivity (NGFLI) and neuropeptide Y-like immunoreactivity (NPYLI) assay and the spinal cords prepared for substance P (SP) release as described below.

Horizontal spinal cord slices were obtained from rats as previously described [17]. Briefly, hemisected dorsal lumbosacral slices with attached dorsal roots were isolated and mounted in a three-compartment bath. The tissue was positioned in the central compartment where it was continuously perfused with Krebs's solution at 1 ml/min and the dorsal roots were laid across two pairs of bipolar electrodes and immersed in mineral oil in the lateral compartments. Leak-proof partition barriers of perspex and paraffin grease ensured electrical isolation. After 1 h equilibration, normal Krebs's solution (in mmol/l: NaCl, 118; KCl, 4; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25; CaCl₂, 2.5 and glucose, 5.5) was substituted with Krebs's solution containing 0.1% bovine serum albumin (BSA), 100 μ M captopril, 1 μ M phosphoramidon, 20 μ g/ml bacitracin and dithiothreitol (6 μ M) to prevent metabolic breakdown and oxidation of SP. Eight minute fractions were collected in the following order: three fractions to measure basal out-

flow, one fraction to measure release during electrical stimulation at 20 V, 0.5 ms, 1 Hz (11 ± 2.1 mA) and three fractions to evaluate recovery to basal levels. Samples (8 ml volume) were desalted by using 100 mg SEP-PAK C₁₈ reverse phase silica gel cartridges (Millipore) as already described [17]. The eluates were dried by evaporation at 55°C under nitrogen and stored at -70°C until they could be assayed for SP-like immunoreactivity (SPLI) content by radioimmunoassay (RIA) using the scintillation proximity assay bead technique already described [17].

Sciatic nerve samples were boiled for 15 min in 0.5 ml of 2 M acetic acid containing 10 mM hydrochloric acid, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol. After boiling, tissues were homogenised (Polytron, Kinematica), the remaining solution was centrifuged (9000 \times g for 5 min) and the supernatant was freeze-dried overnight. Samples were stored at -70°C until assay. NPYLI was determined by scintillation proximity assay using commercially available antisera (Peninsula Labs., UK).

A 1 cm portion of sciatic nerve was homogenised in a solution containing 50 mM Tris-HCl, 0.2 M NaCl, 1% BSA, 0.05% sodium azide, 0.1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride (PMSF), 40 U ml⁻¹ aprotinin and 4 mM EDTA. Samples were then centrifuged at 100 000 \times g for 20 min and the resulting supernatant was collected and freeze dried overnight. The assay was performed in 96-well Nunc immunosorb plates. Wells were coated with solution containing 50 mM Na₂CO₃, 50 mM NaHCO₃, 0.1% sodium azide and monoclonal mouse NGF antibody, specific for the β -subunit of mouse NGF (Boehringer Mannheim, UK), at a concentration of 1.0 μ g ml⁻¹. After a 2 h incubation period at 37°C, the wells were washed (as in subsequent washes) with a solution containing 50 mM Tris-HCl, 0.2 M NaCl, 10 mM CaCl₂, 0.1% Triton X-100 and 0.05% sodium azide and then incubated with blocking solution (coating solution plus 0.5% BSA) at 37°C for 30 min to inhibit non-specific binding. After washing, mouse β -NGF standard (rat NGF is not available) and samples (100 μ l of each) were added, in triplicate

Table 1

Effects of diabetes and treatments on body weight, glycemia and neuropeptides

	Final body weight (g)	Final blood glucose (mmol/l)	Sciatic nerve content		SPLI release from spinal cord	
			NGFLI (pg/cm)	NPYLI (pmol/cm)	Basal (fmol/min)	Evoked (% basal)
Control (8)	511 \pm 56	4.6 \pm 0.4	79.8 \pm 27.1*	3.02 \pm 1.02*	8.3 \pm 1.4	193 \pm 27*
Diabetic, untreated (7)	288 \pm 41	19.8 \pm 2.5	36.4 \pm 28.4	2.27 \pm 0.73	9.3 \pm 2.1	118 \pm 17
Diabetic + NGF (8)	292 \pm 41	20.2 \pm 1.8	159.6 \pm 66.9*	1.92 \pm 0.93	8.6 \pm 0.7	167 \pm 21
Diabetic + α -lipoic acid (7)	302 \pm 31	20.0 \pm 2.7	50.0 \pm 40.6	2.79 \pm 1.19*	9.8 \pm 0.2	204 \pm 62*

Data are mean \pm 1 SD. Numbers of rats are in brackets. NGFLI, nerve growth factor-like immunoreactivity; NPYLI, neuropeptide Y-like immunoreactivity; SPLI, substance P-like immunoreactivity. * P < 0.05 in comparison to diabetic untreated by one-way ANOVA with Duncan's multiple range tests.

for the standards and in duplicate for the samples, to the plate and incubated overnight at 4°C. After washing, 100 μ l of mouse anti-NGF antibody, conjugated to the enzyme β -galactosidase (again specific for the β -subunit of mouse NGF), were added at a concentration of 400 mU ml⁻¹ and the plate incubated for 4 h at 37°C. Colour was developed by the addition of substrate solution containing 100 mM HEPES, 150 mM NaCl, 2 mM MgCl₂, 0.1% sodium azide, 1% BSA and 2 mg ml⁻¹ CPRG (chlorophenolred-B-D-galactopyranoside). The optical density was measured at 574 nm (Microplate reader, BIORAD Labs., USA).

All data are presented in Table 1, which gives a reasonably clear picture of the changes seen. As usual diabetic rats were hyperglycaemic and showed weight loss; these changes were unaffected by either treatment. As previously reported by ourselves and others, diabetic rats showed reduced sciatic nerve content of NGFLI [9,11]. However, we have now demonstrated that this is associated with reduced sciatic nerve content of NPYLI and reduced release of SPLI from the lumbar spinal cord on stimulation of dorsal roots. There was no change in basal release of SPLI in preparations made from diabetic rats.

Treatment of diabetic rats with rhNGF increased stimulus-evoked release of SPLI to a value that was not different from that seen in control preparations. However, rhNGF treatment was without effect on the sciatic nerve content of NPYLI.

Treatment of diabetic rats with α -lipoic acid tended to increase sciatic nerve NGFLI levels, but the effect did not attain significance, giving a value that differed significantly neither from controls nor from untreated diabetic rats. There was, however, a marked effect of α -lipoic acid treatment on stimulus-evoked release of SPLI, which was completely normalised. In contrast to NGF treatment, α -lipoic acid also normalised the sciatic nerve content of NPYLI.

It is clear that there is a link between reduced NGF neurotrophic support in diabetic rats and the levels of SP expressed in lumbar afferents. This is reported elsewhere [3,6,7,9] and substantiated by the present study. Treatment of diabetic rats with rhNGF restores levels of sciatic nerve SP to normal, or even exceeding those of controls at higher NGF doses [9] and, as seen here, can increase the pool of SP available for release from C-fibre central projections. These observations can be explained by the established influence of NGF on transcription of preprotachykinin A (PPT-A), leading to increased synthesis of SP [10].

It is possible that α -lipoic acid protects diabetic rats against NGF depletion [18]. Although a stimulatory effect of α -lipoic acid on sciatic nerve NGF was not marked here, a full examination of retrograde transport of NGF would be required to determine the extent of stimulation of NGF delivery to the cell body. Thus, even the modest effect seen here might result in a marked increase in tonic stimulation of NGF-responsive genes. Certainly the effect of α -lipoic acid on SP release was as marked as that of a dose of NGF

that quadrupled sciatic nerve NGFLI levels in diabetic rats. This implies an effect on SP over and above a stimulation of NGF undergoing retrograde transport. For example, it would be interesting to know whether α -lipoic acid had a stimulatory effect on NGF receptors or their second messengers.

The positive effect of α -lipoic acid on sciatic nerve NPYLI levels was clearly unrelated to any action on NGF, because NGF supplementation did not increase NPYLI levels. If much of the NPY was present in sympathetic postganglionic fibres, then this observation indicates that, although NGF can reinforce expression of the noradrenergic phenotype, via induction of tyrosine hydroxylase [20], this effect does not extend to the peptide co-transmitter. The effect of α -lipoic acid on NPYLI indicates that it might stimulate other neurotrophic factors.

Clearly, the mechanisms involved here demand much more detailed study, but the observations here offer an interesting insight into the potential efficacy of α -lipoic acid as an agent directed at diabetic neuropathy. Stimulation of a range of neurotrophic factors would seem to offer a distinct advantage over any therapy currently available or currently envisaged.

We are grateful to Genentech for a gift of rhNGF. This study was supported by Asta Medica; we are indebted to Dr. Hans Tritschler for his support and advice.

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