

## A lipoic acid-gamma linolenic acid conjugate is effective against multiple indices of experimental diabetic neuropathy

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**Summary** Untreated streptozotocin-diabetic (7 weeks duration) rats showed reductions (all  $p < 0.01$ ; percentages in brackets) in motor and sensory nerve conduction velocity (MNCV; 14%, SNCV; 17%) and in sciatic nerve contents of nerve growth factor (NGF; 57%), substance P (SP; 53%) and neuropeptide Y (NPY; 39%). Treatment with a  $\gamma$ -linolenic acid- $\alpha$ -lipoic acid conjugate (GLA-LA; 35 mg  $\cdot$  day<sup>-1</sup>  $\cdot$  rat<sup>-1</sup>) attenuated ( $p < 0.05$ ) these reductions to MNCV (8%), SNCV (5%), NGF (19%), SP (23%), NPY (20%), such that the values in GLA-LA-treated diabetic rats did not differ significantly from those of control non-diabetic animals. Treatment with alpha-

lipoic acid alone at 100 mg/kg i. p. was without effect on these variables except for NGF (33% reduction,  $p < 0.05$ ) and treatment with the antioxidant, butylated hydroxytoluene (1.5% dietary supplement) did not affect any deficits. These data show that GLA-LA is effective in improving both electrophysiological and neurochemical correlates of experimental diabetic neuropathy. [Diabetologia (1998) 41: 839–843]

**Keywords** Alpha-lipoic acid, gamma-linolenic acid, nerve conduction, nerve growth factor, neuropeptide Y, rat, substance P.

Recent attempts to rationalise the aetiology of diabetic neuropathy have focused on two stages of pathogenesis. First, identification of the biochemical deficits that follow directly from hyperglycaemia, with the polyol pathway, protein glycation and free radical attack [1, 2]. Second, attempts to identify the progenitors of dysfunction within the nerve itself. Here there has been a vigorous debate about the exis-

tence and importance of reduced nerve blood flow and consequent ischaemic damage [3–5]. Proponents of this mechanism suggest a chain of consequences comprising hyperglycaemia, exaggerated polyol pathway flux, impaired endoneurial nitric oxide production, reduced nerve blood flow, endoneurial hypoxia and impaired nerve conduction [6–8]. Related to this is the proposition that defective metabolism of essential fatty acids (EFAs) resulting in reduced levels of gamma-linolenic acid and subsequently, arachidonic acid also has a large part to play in the pathogenesis of diabetic neuropathy [9]. Others have argued the importance of impaired neurotrophic support from nerve growth factor (NGF) and neurotrophin 3 (NT-3) [10–13], but there is no rationale to suggest that these different mechanisms need be mutually exclusive. It is clear, however, that although treatment of rats with NGF or NT-3 prevents some of the deficits characteristic of experimental neuropathy [10, 12], these agents do not influence reduced nerve blood flow or motor conduction velocity [14]. Similarly, other agents, such as aldose reductase inhibitors or

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*Abbreviations:* MNCV, Motor nerve conduction velocity; SNCV, sensory nerve conduction velocity; NPY, neuropeptide Y; NPY-LI, neuropeptide Y like-immunoreactivity; SP, substance P; SP-LI, substance P like-immunoreactivity; NGF, nerve growth factor; BHT, butylated hydroxytoluene; GLA-LA, gamma linolenic acid-lipoic acid; EFAs, essential fatty acids; NT-3, neurotrophin-3; STZ, Streptozotocin; BSA, bovine serum albumin.

evening primrose oil offer only partial protection against experimental neuropathy, preventing blood flow and conduction deficits without affecting neurochemical defects derived from impaired neurotrophism [15, 16]. More recently, the antioxidant  $\alpha$ -lipoic acid (thioctic acid) has been found to protect against a broader range of defects in diabetic rats [17–19], including both those related to nerve blood flow and those related to neurotrophins. The present study examined the effects of a novel conjugate of  $\alpha$ -lipoic acid and a triglyceride containing  $\gamma$ -linolenic acid (an active principle of evening primrose oil). This conjugate was compared with native  $\alpha$ -lipoic acid and another antioxidant, butylated hydroxytoluene (BHT). Measurements were designed to reflect both classes of defect referred to above, with motor and sensory conduction velocities indicating the polyol-nerve blood flow axis, and NGF and neuropeptides as markers of neurotrophic support.

## Materials and methods

**Animals and experimental organisation.** Male Wistar rats (range, 300–344 g) were randomly assigned to five groups. Four of these were made diabetic by a single i.p. injection of 50 mg/kg streptozotocin (STZ; Sigma, Poole, Dorset, UK) freshly dissolved in NaCl 154 mmol/l after fasting overnight. Blood glucose was measured 72 h later in tail vein blood by reflectance photometry ('Reflotest', Boehringer Mannheim, Mannheim, Germany) and animals with a resting blood glucose of less than 15 mmol/l were rejected from the study. Treatment was begun on the same day as hyperglycaemia (a resting blood glucose of > 15 mmol/l) was confirmed with diabetic animals receiving 100 mg/kg  $\alpha$ -lipoic acid (Asta Medica, Frankfurt am Main, Germany) i.p. five times a week, or butylated hydroxytoluene 1.5% dietary supplementation or  $\gamma$ -linolenic acid- $\alpha$ -lipoic acid conjugate (GLA-LA; Scotia Pharmaceuticals, Carlisle, UK) via dietary supplementation at a dose of 50 mg per 100 g of food, giving a daily consumption of approximately 35 mg. The last dose of either treatment was given 24 h before killing the rats. The remaining two groups, were one control (age matched) and one diabetic (untreated). The total duration of diabetes was 7 weeks.

**Measurements of nerve conduction velocity.** The procedure is described in full elsewhere [20]. Briefly, rats were anaesthetized with hypnorm (0.6 ml/kg i.m.) and diazepam (2.5 mg/kg i.p.). Rectal temperature was monitored throughout the procedure and rats placed on a heating blanket to counteract the hyperthermic effects of hypnorm anaesthesia. Nerve temperature was monitored using a thermocouple probe and maintained in the range of 36.5 to 37.5°C by radiant heat. The right sciatic nerve was stimulated first at the sciatic notch and then at the Achilles tendon. Stimulation comprised of 0.1 ms pulses of varying amplitude (1–4 V) were delivered via fine percutaneous needle electrodes using a Neurolog stimulus isolator (Digitimer Research Instrumentation, Welwyn Garden City, UK). Consequent to each stimulation M-waves and h-reflexes were recorded using fine needle electrodes from the second interosseus muscle via a MacLab bridge amp and a digital storage type oscilloscope (Gould, Cambridge, UK) before being transferred to a MacLab/4e (AD Instruments, Castle Hill,

New South Wales, Australia). Initially the h-reflex was recorded before subsequently increasing stimulation amplitude to generate M-waves. The reliability of the h-reflex was validated by its disappearance on generation of a maximal M-wave. The temporal separation of the peaks of M-waves and h-reflexes were calculated using Chart v3.5 on an Apple Macintosh computer and the means of at least six measurements each of M-waves and h-reflexes were taken. Nerve length from sciatic notch to Achilles tendon was measured at death and used to calculate motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV) in m/s.

**Peptide and NGF measurements.** Rats were exsanguinated after MNCV/SNCV measurements and sciatic nerves were removed for substance P (SP), NGF and neuropeptide Y (NPY) like immunoreactivity (LI). Sciatic nerve (1 cm) samples were boiled for 15 min in 0.8 ml of 2 mol/l acetic acid containing 10 mmol/l hydrochloric acid, 1 mmol/l EDTA and 1 mmol/l dithiothreitol. After boiling, samples were homogenised (Polytron, Kinematica, Lucerne, Switzerland) and centrifuged. Nerve samples were centrifuged at 11000  $\times$  g for 5 min. Supernatants were freeze dried overnight and lyophilates stored at -70°C until assay. SP-LI in the sciatic nerve was determined by RIA using commercially available rabbit antisera (Amersham, Little Chalfont, UK) as published previously [21]. Neuropeptide Y like-immunoreactivity (NPY-LI) was determined by RIA from the same aliquot as that used for substance P like-immunoreactivity (SP-LI) measurements, using a secondary antibody precipitation method. Tissue lyophilates were reconstituted in 50 mmol/l phosphate buffer containing 0.5% bovine serum albumin (BSA) and 0.01% Triton X-100. We preincubated 100  $\mu$ l of samples (in duplicate) and standards (3.9–1000 fmol) overnight at 4°C with 100  $\mu$ l 4RG2 (polyclonal rabbit anti-porcine NPY antibody; [22]; 1:480 000 final concentration) and added 100  $\mu$ l of <sup>125</sup>I-NPY labelled with Bolton Hunter reagent (Amersham, UK), total counts of approximately 10000 cpm, the next day. Samples were left to equilibrate at 4°C for another 24 h. Separation was achieved by the addition of 100  $\mu$ l of normal rabbit serum (1:80), 100  $\mu$ l sheep anti-rabbit secondary antibody (raised against the Fc fragment; IGi Ltd, Gateshead, UK; 1:16). Following a 3 h equilibration at 4°C samples were centrifuged at 3000 rpm for 30 min after the addition of 500  $\mu$ l buffer. Supernatants were discarded and pellets counted in a gamma counter. NGF levels were determined by ELISA using a kit (Boehringer Mannheim, Germany) as previously described [23].

**Statistical analysis.** Data are expressed as the arithmetic mean  $\pm$  1 SD. One way analysis of variance (ANOVA) was performed, followed by Duncan's multiple range tests to correct for multiple comparisons. *P* less than 0.05 was considered to be significant.

## Results

The weight loss and glycaemia present in the diabetic rats are shown in Table 1. None of the treatments affected the severity of diabetes as judged by these indices. Neurological data are also presented in Table 1. Untreated diabetic animals showed both conduction velocity deficits and reduced levels of NGF, neuropeptide Y (NPY) and SP in their sciatic nerves. Treatment with BHT was without effect on any of these variables. Treatment with  $\alpha$ -lipoic acid signifi-

**Table 1.** Experimental data from all measurements

Group	Final weight (g)	Blood glucose (mmol/l)	NPY (fmol/cm nerve)	SP (pg/cm nerve)	NGF (pg/cm nerve)	MNCV (m/s)	SNCV (m/s)
Control	496 ± 33 (11)	5.6 ± 0.7	117.7 ± 24.7 (11)	81.9 ± 25.0 (10)	73.7 ± 26.3 (9)	49.3 ± 6.8 (11)	51.5 ± 7.4 (10)
Diabetic	289 ± 29 (10)	21.7 ± 2.5	71.3 ± 16.8 (10) <sup>a</sup>	38.4 ± 15.4 (8) <sup>a</sup>	31.6 ± 6.3 (5) <sup>a</sup>	42.5 ± 3.1 (10) <sup>a</sup>	42.9 ± 7.7 (10) <sup>a</sup>
Diabetic BHT	291 ± 29 (10)	23.1 ± 2.8	77.2 ± 11.1 (9) <sup>a</sup>	45.6 ± 16.8 (10) <sup>a</sup>	40.1 ± 18.5 (5) <sup>b</sup>	40.7 ± 1.6 (10) <sup>a</sup>	45.0 ± 4.7 (9) <sup>a</sup>
Diabetic LA	309 ± 19 (10)	24.0 ± 2.5	86.9 ± 27.3 (9) <sup>a</sup>	44.6 ± 18.7 (8) <sup>a</sup>	49.2 ± 24.7 (8)	41.0 ± 7.0 (10) <sup>a</sup>	44.3 ± 6.7 (9) <sup>a</sup>
Diabetic GLA-LA	332 ± 19 (10)	20.7 ± 3.0	92.4 ± 13.7 (10) <sup>ac</sup>	63.0 ± 20.2 (10) <sup>bc</sup>	59.6 ± 29.1 (7)	45.1 ± 3.9 (10)	48.6 ± 4.2 (10)

Data are mean ± 1 SD; animal numbers in brackets (numbers for blood glucose were the same as for body weight). Statistical comparisons were made by one-way ANOVA with Duncan's

multiple range tests where  $F < 0.05$ . Levels were <sup>a</sup> =  $p < 0.01$  vs controls, <sup>b</sup> =  $p < 0.05$  vs controls, <sup>c</sup> =  $p < 0.05$  vs diabetic

cantly increased the NGF content of sciatic nerve and produced small arithmetic increases in both SP and NPY, which did not attain significance. There was no effect on either nerve conduction velocity. Treatment with the gamma linolenic acid-lipoic acid (GLA-LA) conjugate increased sciatic nerve levels of both SP and NPY, which were higher than those measured in nerves from untreated diabetic rats ( $p < 0.05$ ), although they remained lower than those of controls ( $p < 0.05$  and  $0.01$ , respectively). The conjugate also increased NGF levels and both MNCV and SNCV so that these values were not different from those of control rats. The potential dependence of the neuropeptide changes on NGF levels was examined by regression analyses and the scatterplots are shown in Figure 1. For the dependence of SP on NGF  $r^2$  was

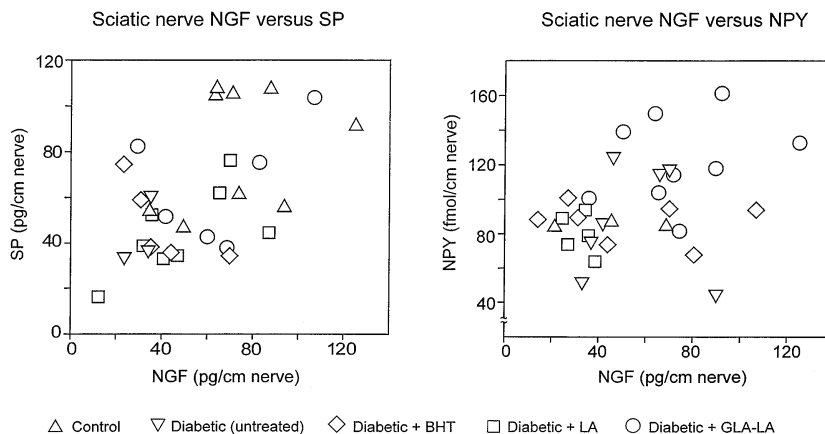
0.257 ( $p < 0.005$ ), but the levels of NPY were less closely related to those of NGF, where  $r^2$  was 0.145 and the regression was barely significant ( $p < 0.05$ ).

## Discussion

The changes seen in untreated diabetic rats have all been reported before [15, 19, 24]; indeed the conduction deficits are classical. The reduced levels of NGF and SP correlate reasonably well in this, as in other studies, and the dependence of the latter on the former is well established. Treatment of normal or diabetic rats with NGF raises SP in the sciatic nerve [25, 26] and it is established that the promoter of the gene coding for the precursor of SP (preprotachykinin A) has a response element sensitive to NGF [27]. Thus the deficit in sciatic nerve SP in diabetic rats is secondary to reduced axonal transport [28] of NGF and the latter relates both to reduced production of NGF by the target tissues [28,29] and to reduced production of receptors by the neurone [30].

The explanation of NPY deficit is not so clear. NPY in the sciatic nerve may derive from mixed fibre populations – some must be in sympathetic postganglionic fibres [31, 32] – but there could also be some in somatic sensory fibres. The expression in somatic

**Fig. 1.** Scatterplots showing the associations between sciatic nerve neuropeptides (substance P (SP), left-hand plot, neuropeptide Y (NPY), right-hand plot) and nerve growth factor (NGF). SP and NPY were measured in the same extracts made from one segment of nerve, NGF was measured in the adjacent segment. There was an association between SP and NGF ( $r^2 = 0.257$ ), but not between NPY and NGF ( $r^2 = 0.145$ ) and the intercept for the latter was clearly not zero on the NPY axis



afferents is low, however, unless they are damaged, when it increases [33]; after axotomy, this increased expression may be reduced by either NT-3 or NGF [34, 35]. Expression of NPY by the sympathetic phenotype is clearly stimulated by NGF [36] and NGF-responsive elements have been identified on the NPY promoter [37]. Thus, our findings could be explained by the proposition that the NPY deficit in sciatic nerves of diabetic rats is also derived from reduced NGF neurotrophic support. Our previous study, however, showed that treatment of diabetic rats with NGF, returned SP in sciatic nerve to normal but did not affect the NPY deficit [19]. Thus, there may be control of NPY expression in these fibres by another neurotrophin with NGF response elements becoming functional only when other influences are removed. This could explain the NGF effects on NPY *in vitro* [36, 38] and increases in NPY after axotomy *in vivo* [33, 39]. Thus, the evolution of the decrease in NPY expression in our diabetic rats cannot be explained as yet, though the deficit clearly responds to treatment with the GLA-LA conjugate and, in our previous study, also responded to  $\alpha$ -lipoic acid [19]. We suggest an examination of the effects of this conjugate on other neurotrophic deficits in nerve of diabetic rats would pay dividends. Of particular note is the range of deficits which was attenuated by the conjugate in this study applying to both electrophysiological and neurochemical deficits in the nerves of diabetic rats.

The effect of the GLA-LA conjugate is also remarkable when related to the current effects of  $\alpha$ -lipoic acid and previously reported effects of evening primrose oil. Approximate calculations suggest that the dose of GLA-LA used here, could deliver about  $14 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$   $\alpha$ -lipoic acid and  $18 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$   $\gamma$ -linolenic acid.  $\alpha$ -Lipoic acid must be given at least  $50 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  to influence nerve conduction [18] and in the present and previous [19] studies had little effect on sciatic nerve NGF levels at  $100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ . Assuming an approximate content of 10%  $\gamma$ -linolenic acid in evening primrose oil [40], a daily consumption of about 180 mg oil would be required to match the current dose of conjugate. A previous study demonstrated no effect of evening primrose oil at about  $3.5 \text{ g} \cdot \text{rat}^{-1} \cdot \text{day}^{-1}$  (equivalent to about  $10 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ) on the deficit in sciatic nerve SP in diabetic rats [16]. It is, therefore, clear that the properties of the conjugate are significantly greater than those of its constituent molecules and it may be that the conjugate maintains the antioxidant properties of  $\alpha$ -lipoic acid [17, 41] in a particularly influential part of the cell. We suspected that a membrane localisation might be important and that was why BHT, a lipid soluble antioxidant, was included in this study; but it showed no efficacy indicating that simple membrane sequestration of  $\alpha$ -lipoic acid does not provide an explanation for the efficacy of

the conjugate. Furthermore, although NGF treatment of diabetic rats does not affect nerve conduction deficits, the conjugate had multiple protective effects on different fibre groups, i. e. increasing nerve NGF, stimulating NPY expression by a different mechanism and boosting deficient conduction velocities. Clearly, this molecule shows clinical potential.

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