Essential fatty acid treatment – effects on nerve conduction, polyol pathway and axonal transport in streptozotocin diabetic rats

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Summary. This study was designed to examine the effect of dietary supplementation with essential fatty acids (evening primrose oil – 5% weight:weight added to the diet) on acute neurophysiological and neurochemical defects in streptozotocin-diabetic rats. Diabetic rats, which were not given evening primrose oil, showed highly significant elevations of nerve sorbitol and fructose combined with a depletion of nerve myo-inositol. In those animals there was also a 40% reduction ($p < 0.02$) in the accumulation of axonally transported substance P-like immunoreactivity proximal to a 12 h sciatic nerve ligature together with reduced motor nerve conduction velocity (13% [$p < 0.001$] and 20% [$p < 0.001$] in two separate

experiments). Treatment of other diabetic rats with evening primrose oil prevented completely the development of the motor nerve conduction velocity deficit without affecting sorbitol, fructose or myo-inositol levels or the deficit in axonal transport of substance P. In a second experiment, treatment of diabetic rats with evening primrose oil was associated with significant attenuation of the conduction velocity deficit, but not complete prevention.

Key words: Diabetes mellitus, diabetic neuropathies, essential fatty acids, axonal flow, substance P, nerve conduction, myo-inositol, sorbitol, streptozotocin, rat.

The availability of potent, selective inhibitors of aldose reductase has enabled simple experiments to determine whether various defects of nerve function, characteristic of experimental diabetes, are associated with exaggerated flux through the sorbitol pathway. In some instances this association has been refined to indicate an involvement of myo-inositol depletion, itself derived from polyol pathway flux. Thus, the acute slowing of nerve conduction, which is a reproducible phenomenon in diabetic rats, may be prevented by aldose reductase inhibitors [1–3] or by treatment with myo-inositol [4–6]. In either case the treatment maintains levels of nerve free myo-inositol in the normal range. The only other reports of prevention of the conduction deficit in diabetic rats have used intensive insulin treatment and again, where measured, nerve myo-inositol levels were normalised [4]. This defect, therefore, seems to be related to the availability of adequate levels of free myo-inositol in the nerve, with a possible involvement of impaired synthesis and turnover of inositol-containing membrane lipids [7, 8].

Other defects do not seem to be clearly related to myo-inositol or to sorbitol pathway metabolites. Pragmatic assessment, by testing the effect of aldose reduc-

tase inhibitors on particular defects, has led to the following conclusions. Slow anterograde axonal transport of 6-phosphofructokinase activity is deficient in diabetic rats, this deficit is prevented by intensive insulin treatment [9], but is unaffected by treatment with either of two structurally-unrelated aldose reductase inhibitors [9, 10]. Fast anterograde axonal transport of substance P is also deficient in rats with streptozotocin-induced diabetes mellitus of either 3 weeks' [11] or 9 months' [12] duration. In both cases aldose reductase inhibition attenuated, but did not prevent the deficit, however the deficit was absent in diabetic rats treated with both insulin and an aldose reductase inhibitor [11].

It appears, therefore, that some dysfunctions are entirely related to the sorbitol pathway, others entirely unrelated and one defect of established mixed aetiology.

A search of the literature for other potentially influential disorders of biochemistry in diabetes indicates a possible importance for defects of metabolism of essential fatty acids, with particular reference to gamma-linolenic acid and its metabolic products. Thus, there are abnormalities of essential fatty acids in various tissues in both clinical and experimental diabetes [13–15]. Furthermore, treatment of patients [16] or diabetic rats

Table 1. Experiment 1 – body weight changes, final plasma glucose, motor nerve conduction velocity (MNCV) and axonal transport of substance P-like immunoreactivity (SPLI)

Group [n]	Body weight [g]		Blood glucose [mmol/l]	MNCV [m/s]	SPLI [pg]	
	Start	End			Background	Proximal accumulation
Control ^a Rats (7)	288 ± 3	312 ± 6] _d	4.8 ± 0.1] _d	49.3 ± 1.2] _d	303 ± 41	1733 ± 179] _b
Diabetic ^a Rats (8)	288 ± 3	225 ± 4] _d	25.4 ± 0.6] _d	42.9 ± 0.7] _d	245 ± 27	1055 ± 166] _b
Control Rats GLA treated (7)	288 ± 4	322 ± 7] _d	5.3 ± 0.2] _d	48.7 ± 1.4] _d	322 ± 42	1845 ± 184] _c
Diabetic Rats GLA treated (8)	292 ± 3	241 ± 11] _d	22.0 ± 1.2] _d	47.2 ± 1.7] _d	256 ± 48	1144 ± 140] _c

^a These groups received an olive oil supplement (see Methods). GLA = Gamma-linolenic acid. Data are mean ± SEM. Significance of differences was assessed by unpaired t tests; ^b $p < 0.02$, ^c $p < 0.01$, ^d $p < 0.001$

[17] with a preparation rich in gamma-linolenic acid is reported to ameliorate various aspects of diabetic neuropathy. The present study was, therefore, designed to examine the effect of a dietary supplement of essential fatty acids on defects of nerve biochemistry and function in diabetic rats. The supplement comprised evening primrose (*Oenothera biennis*) oil. The defects studied were motor nerve conduction velocity (as an example of sorbitol-related dysfunction), axonal transport of substance P (as an example of mixed aetiology) and nerve levels of sorbitol pathway metabolites and myo-inositol.

Materials and methods

Experimental organisation

This study comprised two self-contained experiments. Both used male Wistar rats (weight range – 300 to 320 g; age 16–18 weeks) obtained from, and bred in, the Animal Unit, Queen's Medical Centre, Nottingham, U.K. In designated groups diabetes was induced by intraperitoneal streptozotocin (50 mg/kg in saline; 0.09% NaCl), administered after an overnight fast. Two days later the streptozotocin-treated rats were monitored for blood glucose by tail prick and a strip-operated reflectance meter ('Reflux II'; Boehringer, Mannheim, FRG). Rats with blood glucose values < 15 mmol/l were rejected from the study. Age-matched controls were given contemporary saline (0.09% NaCl) injections and maintained under conditions identical to the diabetic rats. All animals were studied at week 4 of diabetes duration.

In the first experiment 4 groups were studied; two diabetic and two non-diabetic. One group of each was fed a diet containing evening primrose oil ("Efamol"; Efamol Ltd., Guildford, Surrey, U.K.), which was mixed with powdered diet (Heygates 41B (Modified); L.A.

Pilsbury, Northampton, U.K.) to comprise 5% by weight. The other two groups were given the same rat diet with added olive oil (to 5%) to control for the increased fat intake. In each case the oil-enriched diet was mixed thoroughly and pelleted. Feeding with these diets was begun on the day of injection of streptozotocin and maintained throughout the protocol.

On the penultimate day of the protocol all rats were anaesthetised with halothane (2–4% in O₂) for measurement of distal motor latency followed by constriction of the left sciatic nerve to arrest axonal transport of substance P (see below for details). Twelve hours later the rats were killed and both sciatic nerves removed for assay of substance P-like immunoreactivity and measurement of sugars and polyols in the unconstricted nerve (see below).

The second experiment was a repeat of part of the first. It comprised three groups – untreated control rats, untreated diabetic rats (both fed a normal diet) and diabetic rats fed a diet containing 5% evening primrose oil; olive oil was not used. In this study measurements were restricted to motor nerve conduction velocity and nerve contents of glucose, sorbitol, fructose and myo-inositol.

Motor nerve conduction velocity

The method for measurement of distal motor latency is described in detail elsewhere [5]. In the present study this method was adopted exactly as previously described except that a microthermocouple, connected to a digital electronic thermometer (Comark Electronics, Rustington, Sussex, U.K.) was inserted into the sciatic fossa to measure the temperature of the mid-femoral portion of the nerve. The rat was warmed with infra-red lamp and distal motor latency measured at 36°C. Nerve length, separating the two cathodal stimulation points (sciatic notch and Achilles tendon) was measured on the cadaver, after exposure of the whole nerve, using Vernier calipers.

Axonal transport of substance P

The method is described exactly elsewhere [11, 12] and was used here without modification. In previous studies the velocity of axonal transport of substance P was found to be unaltered in diabetic rats, whilst the amount in transit was reduced. The latter was reflected as a decrease in the material accumulating proximal to a 12 h constriction applied to the sciatic nerve. Thus, the present study measured this accumulation. The portions of nerve proximal to the constrictions were divided into 5 mm segments, each of these was extracted and assayed for substance P-like immunoreactivity by radioimmunoassay exactly as described elsewhere [11, 12].

Statistical analysis

All data are presented as arithmetic mean ± SEM. Group means were compared by unpaired t-tests as is indicated in the tables.

Table 2. Experiment 1 – sciatic nerve contents of monosaccharides and polyols

Group [n]	Sciatic nerve content [nmol/mg wet nerve]			
	Glucose	Sorbitol	Fructose	myo-Inositol
Control ^a Rats (7)	1.2 ± 0.2] _c	0.3 ± 0.1] _c	0.1 ± 0.1] _c	4.1 ± 0.5] _b
Diabetic ^a Rats (8)	11.5 ± 1.1] _c	3.0 ± 0.1] _c	5.9 ± 0.4] _c	2.5 ± 0.1] _b
Control Rats (7)				
GLA treated	1.6 ± 0.2] _c	0.2 ± 0.1] _c	0.1 ± 0.1] _c	4.2 ± 0.4] _b
Diabetic Rats (8)				
GLA treated	9.9 ± 0.9] _c	2.3 ± 0.2] _c	5.8 ± 0.7] _c	2.5 ± 0.2] _b

^a These groups received an olive oil supplement. GLA = Gamma-linolenic acid. Data are mean ± SEM. Significance of differences (unpaired t tests) were ^b $p < 0.01$; ^c $p < 0.001$

Results

General state of the rats

One of the streptozotocin-treated rats showed sub-threshold hyperglycaemia and was rejected after primary blood glucose screening (see Methods). The remainder were adequately diabetic and one animal died of suspected ketacidosis 3 days after treatment.

All diabetic rats showed marked weight loss and hyperglycaemia at the end of the protocol (Tables 1 and 3). In neither experiment was there any indication of an effect of treatment with evening primrose oil on body weight or blood glucose. We conclude that this treatment did not attenuate the general severity of diabetes mellitus.

Nerve sugars and polyols

Data are shown in Tables 2 and 4. Diabetic rats showed characteristic elevations of nerve glucose, sorbitol and fructose together with depletions of myo-inositol. None of these changes was altered in the diabetic rats fed with evening primrose oil. Likewise, there was no indication of an effect of this treatment on the levels of sugars or polyols measured in the nerves of non-diabetic rats.

Motor nerve conduction velocity

In both experiments diabetic rats, whether treated with olive oil or fed an unadulterated diet, showed significant reductions in motor nerve conduction velocity. In experiment 1, the deficit in conduction velocity was absent in the group of diabetic rats treated with evening primrose oil; in experiment 2, the deficit was significantly attenuated in the diabetic group given evening primrose oil. (see Tables 1 and 3).

Axonal transport of substance P

The accumulation of substance P-like immunoreactivity proximal to 12 h sciatic nerve constrictions was significantly reduced in olive oil-treated diabetic rats compared to their controls; this accumulation was also reduced in the diabetic rats given evening primrose oil when compared with their controls (see Table 1). The content of substance P-like immunoreactivity in segments of unconstricted nerve was also numerically reduced in both diabetic groups, but these changes did not attain statistical significance.

Discussion

The findings of this study are clear. Diabetic rats showed a series of peripheral nerve changes which have been well characterized in other studies (see [1–6, 11, 12]); these were accumulation of sorbitol pathway me-

Table 3. Experiment 2 – body weight changes, final plasma glucose and motor nerve conduction velocity (MNCV)

Group [n]	Body weight [g]		plasma glucose [mmol/l]	MNCV [m/s]
	Start	End		
Control Rats (8)	320 ± 6	353 ± 7	5.6 ± 1.7	53.4 ± 1.4
Diabetic Rats (7)	318 ± 5	259 ± 11	32.8 ± 2.0	42.4 ± 0.5
Diabetic Rats (9)				
GLA-treated	323 ± 5	255 ± 9	34.8 ± 3.0	48.6 ± 1.4

Data are mean ± SEM. GLA = Gamma-linolenic acid. Significance of differences (unpaired t-tests), ^a $p < 0.001$ (massive differences not tested)

Table 4. Experiment 2 – sciatic nerve contents of monosaccharides and polyols

Group [n]	Sciatic nerve content [nmol/mg wet nerve]			
	Glucose	Sorbitol	Fructose	myo-Inositol
Control Rats (8)	2.2 ± 0.3	0.3 ± 0.1	0.9 ± 0.3	4.5 ± 0.4
Diabetic Rats (7)	12.2 ± 2.0 ^a	2.8 ± 0.2 ^a	5.4 ± 0.4 ^a	2.6 ± 0.2 ^a
Diabetic Rats (8)				
GLA-treated	13.6 ± 1.3 ^a	3.2 ± 0.3 ^a	6.4 ± 0.6 ^a	2.9 ± 0.3 ^a

Data are mean ± SEM. GLA = Gamma-linolenic acid. Significance of differences (unpaired t-tests) were ^a $p < 0.001$ vs equivalent control mean

tabolites, depletion of myo-inositol, reduced accumulation of axonally transported substance P-like immunoreactivity and a deficit in motor nerve conduction velocity. Before discussing the effect of the treatment studied, it is important to consider the possibility that defects in the diabetic rat may be related to an effect of streptozotocin which is distinct from the diabetes it generates. This possibility can be evaluated pragmatically, by examining the extent to which the neurological defects in diabetic rats can be reversed by fine control of glycaemia using insulin. As is indicated in the Introduction, all the defects studied here have been prevented by either insulin alone or by a combination of insulin and an aldose reductase inhibitor (see [4, 5, 11, 18]).

Of the defects referred to above, only the conduction velocity deficit was affected by treatment with evening primrose oil, in that the two separate treated groups of diabetic rats gave motor nerve conduction velocity measurements, which were not significantly different from the values in their control groups. To our knowledge this is the first time that the acute conduction deficit has been prevented by treatment of diabetic rats when the treatment has not normalised nerve myo-inositol levels. This finding surprised us and it was this that motivated the second experiment to remove all doubt about the veracity of the findings from the first experiment. Previous manipulations to prevent the acute conduction deficit have been tight control of blood glucose [4, 18], administration of aldose reductase inhibitors [1–3, 5, 6] or administration of myo-inositol itself [4, 5]. In the latter two cases nerve myo-inositol le-

vels have been measured and their normality related to abolition of the conduction deficit [4, 5]. In the case of insulin treatment normal nerve myo-inositol levels have been registered when this measurement has been included in the study [4, 9], so that, even when the measurement has not been made, it is likely that the treatment prevented or attenuated myo-inositol depletion. This association had led to the assumption that the level of nerve myo-inositol effectively limits the maintenance of normal conduction velocity in experimental diabetes (see [7, 8], for example). Hypotheses have developed suggesting that depletion of myo-inositol restricts the synthesis of inositol-containing membrane phospholipids, this reduces the turnover of these lipids and thereby down-regulates processes which are dependent upon their second messenger function [7, 8]. The exact chain of events whereby these processes are connected to nerve conduction remains a matter for speculation. However, the experiments described in this study demand that this hypothesis be modified.

Evening primrose oil is rich in the essential fatty acids oleic, linoleic and gamma-linolenic acid [19]. Further experiments are needed to define which of these is the critical agent in the treatment, but evidence from other studies [13, 20–22] indicates that gamma-linolenic acid is of potential importance. The metabolism of gamma-linolenic acid in peripheral nerve has not been studied in either normal or diabetic animals. However, information from other tissues indicates that treatment with essential fatty acids can promote conversion of gamma-linolenic acid to arachidonic acid [14, 23, 24]. This, in turn, could boost synthesis of the lipid precursors of inositol-containing phospholipids via conversion of arachidonate to phosphatidic acid and cytosine diphosphate-diacyl glycerol [25]. Thus, the hypothesis stated earlier may be modified to suggest that synthesis of inositol-containing phospholipids may be restricted by deficiencies of both free inositol and the diacyl glyceride moiety and that supplementation of either could restore the balance. Alternatively, it is possible that arachidonate or its fatty acid precursors could activate protein kinase C [25, 26] independently of breakdown products of inositol-containing lipids. Such a mechanism could exert a tonic activation of protein kinase C, but the interaction of such an effect with any biochemical parameter of electrical activation of the nerve remains to be elucidated.

A separate possibility might be based on production of prostaglandin E₁ from gamma-linolenic acid [23, 24]. Again, this has not been demonstrated in peripheral nerves, but it is an established phenomenon in vascular tissue where the cell types responsible for the conversion remain to be identified [27]. Prostaglandin E₁ so formed might also modulate biochemical events in the nerve cell membrane favouring nerve conduction, might release other mediators with similar effects or it might act as a vasodilator in the endoneurium – again either directly or indirectly – to preclude the development of ischaemic endoneurial hypoxia [28]. A recent

study has shown that a structural analogue of prostaglandin E₁ can prevent the acute deficit in motor nerve conduction velocity when administered to streptozotocin-diabetic rats [29]. The mechanism remains to be elucidated, but the finding supports a possible involvement of prostaglandin E₁ in the effect of essential fatty acids.

Clearly, more incisive studies are warranted. The present findings receive support, in underlining the importance of future work, from clinical studies indicating potential benefits of essential fatty acids in the prevention of diabetic [16, 30] and other forms [31, 32] of neuropathy.

Acknowledgements. This work was partially funded by Scotia Pharmaceuticals. We thank Dr. David Horrobin for his interest and support and Miss Carol Brown for typing the manuscript.

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Received: 3 March 1989
and in revised form: 30 May 1989

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