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Oxidative stress participates in the breakdown of neuronal phenotype in experimental diabetic neuropathy

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Abstract

Aims/hypothesis. This study compared the effects of streptozotocin-induced diabetes in rats with those of two pro-oxidant interventions; a diet deficient in vitamin E and treatment with primaquine.
Methods. Measurements were made by the classic motor and sensory conduction velocity deficits and by indicators of the breakdown of small fibre phenotype i.e., sciatic nerve content of nerve growth factor and the neuropeptides, substance P and neuropeptide Y.
Results. As with diabetes, the pro-oxidant interventions decreased conduction velocities (though the effect of vitamin E deficiency was not significant), the sciatic nerve content of nerve growth factor and the neuropeptides (all percentages refer to the mean value for the appropriate control groups). In diabetes, nerve growth factor was depleted to 50 % in the control rats ($p < 0.05$); oxidative stress depleted nerve growth factor to 64 % (primaquine; $p < 0.05$) and 81 % (vitamin E deficient; not significant) of controls. Substance P was depleted to 51 % in the control rats ($p < 0.01$) with depletions to 74 % and 72 % (both $p < 0.01$) by oxidative stress; equivalent depletions for neuropeptide Y were 38 % controls in diabetes ($p < 0.001$) and 67 % (primaquine; $p < 0.001$) and 74 % (vitamin E deficient; $p < 0.05$) for oxidative stress.
Conclusion/interpretation. The relative magnitudes of these changes suggest an effect in diabetes of oxidative stress, coupled with some other cellular event(s). This is supported by the effects of a diester of γ-linolenic acid and α-lipoic acid, which completely prevented the effects on the pro-oxidant interventions on conduction velocity, nerve growth factor and neuropeptide contents, but was only partially preventative in diabetes. [Diabetologia (2001) 44: 424–428]

Keywords Primaquine, vitamin E deficiency, oxidative stress, γ-linolenic acid, α-lipoic acid, nerve growth factor, neuropeptide Y, substance P, nerve conduction velocity, diabetic neuropathy.

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Abbreviations: CPRG, Chlorophenolred-β-D-galactopyranoside; DRG, dorsal root ganglia; DTT, dithiothreitol; GLA^^-LA, γ-linolenic acid, α-lipoic acid diester; GSH, reduced glutathione; GSSG, oxidised glutathione; LG, lauryl glycol; MCT, medium chain triglyceride; MNCV, motor nerve conduction velocity, NGF, nerve growth factor; NPY, neuropeptide Y, ROS, reactive oxygen species; SCG, superior cervical ganglia; SNCV, sensory nerve conduction velocity, SP, substance P.

Oxidative stress has been suggested to be a causal factor of diabetic complications [1–3]. Diabetes compromises natural antioxidant defence systems, lowering tissue GSH (reduced glutathione), ascorbic acid, vitamin E and antioxidant enzyme activities [4–7]. Experiments have shown that antioxidants, including probucol [8], butylated hydroxytoluene [9], vitamin E [8] and GSH [10], have some capacity in preventing or reversing nerve dysfunction in experimental diabetes. The mechanisms by which reactive oxygen species (ROS) cause neurological dysfunction in diabetes have not been determined, but increased free radical production in diabetes interferes with normal en-

dothelial function compromising blood flow [11–13]. Therefore oxidative stress might affect nerve function by causing damage to endoneurial endothelium.

These and other studies used antioxidants or radical scavengers to attenuate biochemical and functional deficits and in experimental diabetes reveal effects of oxidative stress. We aimed to compare this approach with an attempt to mimic diabetes-associated deficits by creating oxidative stress in non-diabetic animals. The interventions selected were primaquine treatment and vitamin E deficiency and we compared the effect of a potent antioxidant on these interventions with its effect on diabetes.

Short-term conduction velocity deficits in diabetic rats have been widely studied in this and other contexts but there is no irrefutable relation between these and the development of true diabetic neuropathy. We have argued that neurotrophic deficits and their related disturbances in neuronal phenotype are more meaningful defects, for the study of the pathogenesis of neuropathy in experimental diabetes. Accordingly, this study concentrates on the following measurements: nerve growth factor (NGF) and the neuropeptides substance P (SP) and neuropeptide Y (NPY). In continuity with other work we also included measurements of motor and sensory nerve conduction velocity (MNCV and SNCV).

Materials and methods

Experimental organisation and treatments. This study comprised three self-contained experiments, all using male Wistar rats. The first experiment studied effects of diabetes and treatment with the diester of α-lipoic acid and γ-linolenic acid (GLA^^LA). The second experiment studied effects of primaquine with and without GLA^^LA and the third experiment studied vitamin E deficiency with and without GLA^^-LA. Each experiment had its own non-diabetic control group. These groups were given the appropriate vehicle for the treatment.

Diabetes was induced in rats weighing 316 to 400 g after an overnight fast by a single intraperitoneal (i.p.) injection of streptozotocin freshly dissolved in saline at a dose of 50 mg/kg and verified 72 h later by measuring tail vein blood glucose (Boehringer strip-operated reflectometer); animals with a blood glucose of less than 15 mmol/l were excluded from the study.

Before primaquine was given, rats were studied for 1 week during their quarantine period to monitor their daily water intake. Rats were housed three or two to a cage and the average water consumption per rat per cage was calculated on a daily basis and a weekly mean was calculated. This was done to ensure that an adequate dose of primaquine would be provided on the day that the study started with primaquine. Water intake and rat body weight was monitored continuously and doses changed when appropriate. Primaquine (N^4[6-methoxy-8-aminoquinolinyl]-1,4 pentanediamine; Sigma Chemical, Poole, Dorset, UK) was added to the drinking water to provide an average non-diabetic rat with a daily dose of approximately 1.0 mg/kg. This experiment began with rats at 300–344 g body weight.

Weanling rats (21 days old, weight range 94–114 g) were depleted of vitamin E by a diet completely deficient in vitamin E (Special Diet Services, Witham, Essex); control rats were fed regular powdered laboratory chow.

All chow diets were powdered and mixed with either vehicle alone or vehicle containing GLA^^LA. In the first experiment, GLA^^LA was blended at 5% w/w GLA^^LA with lauryl glycol as a vehicle and food made by adding 2 g of the GLA^^LA mix to 100 g powdered rat food. A GLA^^LA supplemented diet was made freshly at three day intervals and stored in nitrogen flushed bags at 4 °C. In the second and third experiments GLA^^LA was blended and supplied as a 50% mixture with medium chain triglycerides (MCTs), rather than laurylglycol, which contains substantial amounts of α-tocopherol (vitamin E) whereas medium chain triglycerides (MCTs) do not. These differences were reflected in the vehicle treatment for control and diabetic groups that did not receive the drug. In all three experiments the dietary content of GLA^^LA was identical.

The last dose of any treatment was given 24 h before death. Rats were killed while under surgical anaesthesia by exsanguination following cardiac puncture immediately after NCV measurements. Tissues were removed and frozen in liquid nitrogen for subsequent extraction and assay. All animal procedures were in full accordance with the Project Licence issued by the United Kingdom Home Office to cover this work.

Measurement of motor and sensory nerve conduction velocity. The procedure is described in detail elsewhere [14].

Neuropeptide assays. A segment of sciatic nerve (1 cm) or the pair of superior cervical ganglia (SCG) or one pair of L4/L5 dorsal root ganglia (DRG) were extracted and assayed for substance P or neuropeptide Y as described [15].

Nerve growth factor ELISA. Nerve growth factor ELISA was carried out exactly as described previously [16].

Statistical analysis. Data are expressed as the arithmetic mean ± 1 SD. Significance mapping was by one-way ANOVA followed by Student-Newman-Keuls post-hoc test to allow for multiple comparisons. Data showing a significant lack of homogeneity of variances was subjected to a Kruskal-Wallis non-parametric ANOVA followed by Dunn's test. A p value of less than 0.05 was considered statistically significant.

Results

Terminal body weights are shown in Table 1 along with glycaemia data for the first experiment. Diabetic rats lost weight and were hyperglycaemic; GLA^^-LA treatment did not alter these indices of diabetes. None of the treatments altered body weight.

Nerve conduction velocities (Table 2). Diabetes caused a significant reduction in MNCV ($p < 0.01$ compared with controls) that was partially prevented by 5% GLA^^LA treatment ($p < 0.05$ compared with diabetic + LG). Diabetes also caused a significant reduction in sensory nerve conduction velocity (SNCV) ($p < 0.01$ compared with controls) and treatment with 5% GLA^^LA treatment increased SNCV ($p < 0.05$). Primaquine treatment caused a sig-

Table 1. Body weight data for all experiments and blood glucose values for the diabetes study

Group	*n*	Body weight (g)		Final blood glucose (mmol/l)
		Start	End	
Experiment 1				
Control LG	12	360 ± 19	478 ± 23	4.7 ± 0.7
Diabetic LG	12	361 ± 18	286 ± 35[a]	23.3 ± 2.2[b]
Diabetic GLA^^LA	12	367 ± 23	310 ± 52[a]	22.5 ± 1.8[b]
Experiment 2				
Control MCTs	10	368 ± 19	459 ± 27	N.M.
Primaquine MCTs	10	382 ± 25	480 ± 51	N.M.
Primaquine GLA^^LA	10	392 ± 20	464 ± 32	N.M.
Experiment 3				
Control MCTs	10	103 ± 5	364 ± 28	N.M.
Vitamin E (–) MCTs	10	104 ± 6	367 ± 19	N.M.
Vitamin E (–) GLA^^LA	10	103 ± 6	374 ± 14	N.M.

N.M. = not measured. Comparisons were made by ANOVA with Student-Newman-Keuls

[a] $p < 0.05$

[b] $p < 0.01$ against an appropriate control group

Table 2. Conduction velocity data

Group	*n*	MNCV (m/s)	SNCV (m/s)
Experiment 1			
Control LG	12	56.0 ± 3.2	57.5 ± 2.8
Diabetic LG	12	44.0 ± 2.0[b,c]	42.4 ± 2.6[b,c]
Diabetic GLA^^LA	12	48.4 ± 2.2	50.0 ± 1.7
Experiment 2			
Control MCTs	10	51.5 ± 2.4	50.4 ± 4.0
Primaquine MCTs	10	45.7 ± 2.6[a,b]	44.9 ± 3.8[a,b]
Primaquine GLA^^LA	10	51.6 ± 1.8	52.2 ± 2.3
Experiment 3			
Control MCTs	10	54.2 ± 3.8	55.4 ± 4.6
Vitamin E (–) MCTs	10	52.8 ± 5.0	49.5 ± 5.8
Vitamin E (–) GLA^^LA	10	55.1 ± 7.2	55.9 ± 4.7

Within each experiment (no comparisons were made between experiments), by ANOVA with Student-Newman-Keuls

[a] $p < 0.05$ vs control group

[b] $p < 0.05$ vs GLA^^LA-treated group

[c] $p < 0.01$ vs control group

nificant reduction in motor nerve conduction velocity ($p < 0.01$ vs controls). This reduction was completely prevented by a treatment with 5% GLA^^LA ($p < 0.01$). Primaquine treatment also caused a significant reduction in sensory nerve conduction velocity ($p < 0.01$ vs controls), which was prevented by 5% GLA^^LA treatment ($p < 0.01$). Vitamin E deprivation did not reduce motor nerve conduction velocity and treatment with 5% GLA^^LA did not increase MNCV above control values. Vitamin E deprivation significantly reduced sensory nerve conduction velocity ($p < 0.05$) and treatment with 5% GLA^^LA completely prevented the decrease in SNCV ($p < 0.05$).

Nerve growth factor (Table 3). Sciatic nerve NGF was depleted by both diabetes and by primaquine treatment; these effects were significantly ($p < 0.05$) reduced by treatment with GLA^^LA, although the NGF content in the treated diabetic group was still significantly ($p < 0.05$) lower than those of the vehicle-treated controls. Vitamin E deprivation did not significantly lower sciatic nerve NGF, so that no reduction was evident with GLA^^LA.

Substance P. Sciatic nerve SP was depleted by diabetes, primaquine treatment and vitamin E deprivation and, with each of these interventions SP content was normalised by treatment with GLA^^LA (Table 3).

Neuropeptide Y. Neuropeptide Y was also depleted by all three interventions (Table 3), but with GLA^^-LA treatment of diabetic rats there was attenuation of depletion, although NPY remained lower than those of vehicle-treated controls. With the oxidative stressors, GLA^^LA gave complete protection against NPY depletion and, in rats treated with primaquine, it raised NPY content above that of vehicle-controls.

Discussion

These data give a clear indication that oxidative stress produces qualitatively similar changes to those seen in diabetic rats in all the variables measured. Furthermore, treatment with GLA^^LA, a putative antioxidant, gave protection against all the diabetes-induced or pro-oxidant-induced deficits. This latter observation confirms the antioxidant properties of this agent and strengthens the supposition that oxidative stress participates in the deficits induced by streptozotocin-induced diabetes.

There is, however, also strong evidence that oxidative stress is not the only factor at work in producing these deficits in diabetes. This conclusion comes from comparing the magnitude of the deficits in diabetes and under oxidative stress. In diabetes, NGF

Table 3. Nerve growth factor and neuropeptide content of the sciatic nerve

Group	*n*	NGF (pg/cm)	SP (fmol/cm)	NPY (pg/cm)
Experiment 1				
Control LG	12	79.9 ± 10.5	173.9 ± 21.4	89.6 ± 6.7
Diabetic LG	12	40.8 ± 9.2[a,b]	82.4 ± 11.4[c,d]	34.3 ± 4.6[b,e]
Diabetic GLA^^LA	12	68.0 ± 13.5[a]	140.9 ± 21.9	65.8 ± 6.0[a]
Experiment 2				
Control MCTs	10	72.7 ± 20.3	155.1 ± 12.4	73.4 ± 8.7
Primaquine MCTs	10	46.7 ± 13.8[a]	115.5 ± 10.8[c,f]	48.9 ± 5.4[e,f]
Primaquine GLA^^LA	10	57.1 ± 21.0	155.3 ± 23.3	86.6 ± 7.3
Experiment 3				
Control MCTs	10	55.0 ± 12.0	161.7 ± 22.6	71.2 ± 17.6
Vitamin E (–) MCTs	10	44.6 ± 12.1	116.6 ± 14.2[c]	52.9 ± 10.0[a,b]
Vitamin E (–) GLA^^LA	10	49.9 ± 12.1	147.6 ± 20.9	81.1 ± 21.6

Within groups, by ANOVA with Student-Newman-Keuls
[a] $p < 0.05$ vs controls
[b] $p < 0.05$ vs GLA^^LA-treated
[c] $p < 0.01$ vs controls
[d] $p < 0.01$ vs GLA^^LA-treated
[e] $p < 0.001$ vs controls
[f] $p < 0.001$ vs GLA^^LA-treated

was depleted up to 50% in control rats; oxidative stress depleted it to 64% (primaquine) and 81% (vitamin E deficient) of controls. Substance P was depleted in diabetes to 51% controls with depletions to 74% and 72% by oxidative stress; equivalent depletions for NPY were to 38% controls in diabetes and 67% (primaquine) and 74% (vitamin E deficient) for oxidative stress. Thus, the magnitude of the deficits was consistently greater in diabetes than in oxidatively stressed animals, indicating that there are factors in addition to oxidative stress at work in causing diabetes.

It could, of course be argued that the cellular compartment in which reactive oxygen species are generated by hyperglycaemia is different from primaquine or vitamin E depletion. It is also possible that the degree of oxidative stress induced by streptozotocin induced diabetes was greater than that produced by either primaquine or vitamin E deprivation but the available evidence supports the contrary. Streptozotocin induced-diabetes does not significantly reduce the concentrations of vitamin E in plasma but rather increases vitamin E content of sciatic nerve and dorsal root ganglia (DRG) [17]. In contrast, a vitamin E deficient diet for eight weeks reduced sciatic nerve vitamin E to less than 20% [18], and serum vitamin E concentration to less than 5% of normal rats [18]. Regarding effects downstream of oxidative stress, 1 month of vitamin E deficiency increased both sciatic nerve and DRG content of conjugated dienes and lipid hydroperoxides to amounts above those found in equivalent durations of streptozotocin-induced diabetes. Furthermore, the same study reported that both 1 month of vitamin E deficiency and streptozotocin-diabetes induced similar reductions in sciatic nerve reduced glutathione (GSH) [17]. In a separate study it was found that 4 months of vitamin E deficiency caused greater increases in sciatic nerve malondialdehyde and blood lipid peroxide contents than the equivalent duration of streptozotocin-diabetes [19]. In addition, vitamin E deficiency also caused a greater depletion in erythrocytes GSH than did streptozotocin-diabetes [19]. These data combined suggest that rats deficient in vitamin E suffer an oxidative insult which is greater than that imposed by streptozotocin-diabetes. A less clear-cut position is found with primaquine because no studies exist which define the degree of oxidative insult imposed by primaquine treatment.

Vitamin E depletion is associated with a progressive neurological disorder, characterised by ataxia, areflexia, loss of proprioception, opthalmoplegia and generalised muscle weakness, that is very similar in rats and humans [20, 21] and is similar to diabetic sensory polyneuropathy [22]. In studies of avitaminosis E in rats, reductions in sciatic sensory nerve conduction velocity occur after just 1 month of depletion and persist after 3 months of depletion, though motor nerve conduction is not affected [17]. This is supported by our study where the arithmetic deficit in MNCV was not significant but a statistically significant reduction in SNCV was detected. Deficits in conduction velocity in vitamin E deprivation are also associated with reductions in GSH and increases in conjugated dienes [17]. Sciatic nerve GSSG (oxidised glutathione), malondialdehyde and blood H_2O_2 are also increased after avitaminosis E [19, 23], and there seems to be no compensation for tissue depletion of vitamin E by superoxide dismutase and glutathione peroxidase in nervous tissue [18]. This confirms the association of avitaminosis E with increased oxidative stress.

In conclusion, this study strongly suggests an involvement of oxidative stress in those neurochemical deficits associated with experimental diabetes that constitute a breakdown of neuronal phenotype as well as in the extensively described conduction velocity deficits. The degree of change produced by oxida-

tive stress in the absence of diabetes was, however, consistently less than that seen with diabetes itself, indicating that other factors contribute. This provides further evidence that diabetic neuropathies are multifactorial disorders.

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