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Deficient nitric oxide responsible for reduced nerve blood flow in diabetic rats: effects of L-NAME, L-arginine, sodium nitroprusside and evening primrose oil

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1 This study examined the potential role of impaired nitric oxide production and response in the development of endoneurial ischaemia in experimental diabetes. Rats were anaesthetized (Na pentobarbitone 45 mg kg^{-1}, diazepam 2 mg kg^{-1}) for measurement of sciatic nerve laser Doppler flux and systemic arterial pressure. Drugs were administered into the sciatic endoneurium via a microinjector attached to a glass micropipette.

2 In two separate studies comparing diabetic rats (streptozotocin-induced; 8-10 wk duration) with controls, nerve Doppler flux in diabetic rats (Study 1, 116.6 ± 40.4 and Study 2, 90.1 ± 34.7 (s.d.) in arbitrary units) was about half that measured in controls (219.6 ± 52.4 and 212.8 ± 95.5 respectively; $P<0.005$ for both). There were no significant differences between the two in systemic arterial pressure.

3 Inhibition of nitric oxide production by microinjection of 1 nmol L-NAME into the endoneurium halved flux in controls (to 126.3 ± 41.3 in Study 1 and 102.1 ± 38.9 in Study 2; both $P<0.001$), with no significant effect in diabetic rats, indicating markedly diminished tonic nitric oxide production in the latter. D-NAME was without effect on nerve Doppler flux.

4 L-Arginine (100 nmol), injected after L-NAME, markedly increased flux in controls (by 65.8% ($P<0.03$) and 97.8% ($P<0.01$) in the two studies) and by proportionally similar amounts in diabetic rats [75.8% ($P<0.001$) and 60.2% ($P<0.02$)]. The nitro-donor, sodium nitroprusside (SNP; 10 nmol) had similar effects to L-arginine in both groups (increases of 66.0% in controls and 77.5% in diabetics; both $P<0.002$).

5 A second diabetic group, treated with evening primrose oil performed exactly like control rats in respect of responses to L-NAME, L-arginine and SNP.

6 These findings implicate deficient nitric oxide in nerve ischaemia of diabetes and suggest correction thereof as a mechanism of action of evening primrose oil.

Keywords: blood flow; diabetes mellitus; diabetic neuropathy; nitric oxide; rat nerve; streptozotocin

Introduction

Distal symmetrical neuropathy is the commonest form of peripheral nerve damage associated with diabetes mellitus. Recent studies have suggested that ischaemia of nerve trunks may participate in its pathogenesis. Thus, nerve ischaemia and endoneurial hypoxia have been reported in association with diabetic neuropathy in patients (see Tesfaye *et al.*, 1994, for a recent review). The phenomenon has been modelled in diabetic rats and most studies on nerve blood flow or its indirect estimation via laser Doppler velocimetry report a reduction of about 50% in the sciatic nerves of rats with either streptozotocin-induced (Tuck *et al.*, 1984; Monafo *et al.*, 1988; Cameron *et al.*, 1991; Kappelle *et al.*, 1993; Stevens *et al.*, 1993b) or genetic (Stevens *et al.*, 1994) diabetes mellitus.

The cause of endoneurial ischaemia in diabetic rats remains to be established, though pharmacological studies suggest potential involvement of deficient prostacyclin (Stevens *et al.*, 1993a) or exaggerated effects of a range of vasoconstrictors (see Tesfaye *et al.*, 1994, for review). The present study was designed to examine possible involvement of deficient nitric oxide production in nerves of diabetic rats by measuring the effect of its inhibition on sciatic nerve laser Doppler flux. We used a competitive inhibitor of nitric oxide synthase, N^G-nitro-L-arginine methyl ester (L-NAME), following up this acute intervention with reversal by L-arginine or, in separate rats, activation of the nitric oxide-sensitive guanylate cyclase by microinjection of the nitro-donor, sodium nitroprusside (SNP). Since the reduced nerve blood flow in diabetic rats is known to be attenuated by treatment with evening primrose oil (Stevens *et al.*, 1993b), we also examined responses to these acute interventions in a group of diabetic rats given this treatment throughout the period of diabetes.

Methods

Experimental organisation

This investigation comprised two separate studies, in which all measurements of one study were made by different individuals from those performing the other. Study 1 compared control and diabetic rats and Study 2 repeated this comparison with the addition of a third diabetic group treated with evening primrose oil. Diabetes was induced with streptozotocin (60 mg kg^{-1}, i.p.) and confirmed from tail vein blood glucose three days later. In Study 2, the rats of one diabetic group were given evening primrose oil (EPO) by dietary admixture (5% diet w:w), beginning on day three and maintained throughout the remainder of the protocol (8-10 wk). Detailed methods, outlined below, have been described in our previous publications (Stevens & Tomlinson, 1993; 1995; Karasu *et al.*, 1995)

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and differ only in the choice of anaesthetic (Na pentobarbitone 45 mg kg^{-1}, diazepam 2 mg kg^{-1}, i.p.) and the selection of a 0.8 mm probe for the Laser Doppler monitor. Extra anaesthetic (mixture of Na pentobarbitone 15 mg ml^{-1} and diazepam 1.25 mg ml^{-1}) was administered as required via a jugular cannula, with systemic arterial pressure being monitored with a standard pressure transducer (Bell & Howell, Type 4-327 L221) connected to a carotid arterial cannula. A section of the sciatic nerve just below the sciatic notch was exposed by careful dissection of the overlaying muscle layers and the laser probe then positioned at the nerve surface. Sciatic nerve laser Doppler flux (LDF) was monitored on a laser Doppler flow monitor (Type MBF3D; Moor instruments). Body temperature was maintained at 37°C via a rectal temperature probe connected to a homeothermic blanket throughout. The reduced tip diameter explains the numerically smaller values for LDF in this study compared with our earlier publications (see above). LDF values are presented in arbitrary units.

At the end of all measurements blood was collected for plasma glucose measurement by a glucose oxidase assay kit, as defined elsewhere (Stevens & Tomlinson, 1993; 1995; Karasu *et al.*, 1995).

Endoneurial drug administration

A glass micropipette (tip diameter 10–15 μm) was advanced into the endoneurium of the sciatic nerve using a X–Y–Z micropositioner; this pipette was connected to a Drummond Microdispenser (Laser Labs, Southampton, U.K.). The sciatic nerve was impaled about 1–2 mm proximal to the site at which LDF was monitored. Resting cardiovascular parameters were then recorded in steady-state. In some rats, saline (1 μl) was injected to demonstrate that the physical effect of injection was insignificant and short-lasting (<5 min). Infusion of L-NAME (1 nmol in 1 μl) was made over 5 min to inhibit the production of nitric oxide. Approximately 30 min later L-arginine (100 nmol in 1 μl) was infused in some of the rats to reverse the effect of L-NAME. In the remaining animals, L-NAME was followed after about 30 min with sodium nitroprusside (10 nmol in 1 μl) to examine the response of the guanyl cyclase system to a nitro-donor. Both of these infusions were also made over 5 min.

Statistical analysis

All data are presented as means ± 1 standard deviation. Acute drug effects were evaluated by comparison within animal (effect versus baseline) using paired *t* tests. Single comparisons between groups used unpaired *t* tests (Study 1) or one-way ANOVA with Duncan's multiple range tests where $F<0.05$ (Study 2).

Drugs

All drugs were obtained from Sigma Chemical Company (UK), except for evening primrose oil (Scotia Pharmaceuticals, UK), pentobarbitone sodium (May & Baker, UK) and diazepam (Dumex, UK).

Results

Background data are presented in Table 1. Diabetic rats were hyperglycaemic and lost body weight; neither of these changes was altered by evening primrose oil. Untreated diabetic rats were slightly hypotensive compared to controls, as we have seen in most of our previous studies (Stevens & Tomlinson, 1993; 1995; Karasu *et al.*, 1995) as have others (see for example, Cameron *et al.*, 1991). EPO-treated diabetic rats had a systemic diastolic pressure that was significantly higher than that of untreated diabetic rats, but none of the systemic arterial pressure indices in EPO-diabetic rats were different from those of untreated controls.

Figure 1 shows typical traces illustrating responses to L-NAME followed by L-arginine in control and diabetic rats. The modest brief effect of the injection on systemic arterial pressure is shown. The changes in nerve Doppler flux attributable to manipulation of nitric oxide synthase are slower in onset. Group data are presented in Tables 1 and 2. Diabetic rats had resting sciatic nerve LDF that was about half that of control rats (Study 1, $P<0.001$; Study 2, $P<0.005$). Blockade of nitric oxide production with L-NAME in control rats reduced sciatic nerve LDF by 42% or 49% (both $P<0.001$), whilst similar treatment in diabetic rats had a much more modest effect in Study 1 and was without significant effect in Study 2. Thus after L-NAME, sciatic nerve LDF in control rats was similar to the initial resting value in diabetic rats. In 2 control rats and 2 diabetic rats we injected D-NAME, which does not inhibit nitric oxide production (Graves & Poston, 1993), with no effect on sciatic nerve LDF (individual values before and 30 min after D-NAME changed from 253 to 225 and 178 to 176 for controls and for diabetics 89 to 71 and 112 to 111).

In control rats reversal of the effect of L-NAME with L-arginine increased (Study 1, $P<0.03$; Study 2 $P<0.01$) LDF to a level that was not different from the mean starting value (i.e. before L-NAME). In contrast, sciatic nerve LDF in diabetic rats increased (Study 1, $P<0.001$; Study 2 $P<0.02$) with L-arginine to a mean value that was significantly higher ($P<0.05$) than the initial resting value for this group, but did not attain the value seen after L-arginine in controls. The changes in response to SNP were similar to those seen with L-arginine, namely a return to the initial value in controls and a similar absolute increase in diabetic rats, though with the latter attaining a value higher ($P<0.05$) than the initial resting value

Table 1 General characteristics of diabetes and resting systemic arterial pressure in all groups

	Body weight (g)		*Final plasma glucose*	*Systemic arterial pressure* (mmHg)		
	Start	*End*	(mmol l^{-1})	*Systolic*	*Diastolic*	*Mean*
Study 1						
Controls (11)	313±15	520±40	6.9±0.6	126±6	93±6	105±8
				p<0.001	p<0.005	p<0.01
Diabetic untreated (14)	318±12	287±30	35.2±3.9	109±6	82±7	96±6
Study 2						
Controls (10)	332±11	548±42	8.0±1.2	126±11	100±11[a]	111±12
Diabetic untreated (8)	327±21	343±38	41.0±7.6	118±13	85±17[b]	100±17
Diabetic EPO (9)	331±20	366±29	38.0±3.4	133±15	102±15[a]	118±15

Data are mean ± 1 s.d. In Study 1, the two groups were compared by unpaired *t*-tests; in Study 2, data were analysed by one-way ANOVA with Duncan's multiple range tests, data superscripted [a] differed from [b] at $P<0.05$. Massive differences in weight and plasma glucose were not tested.

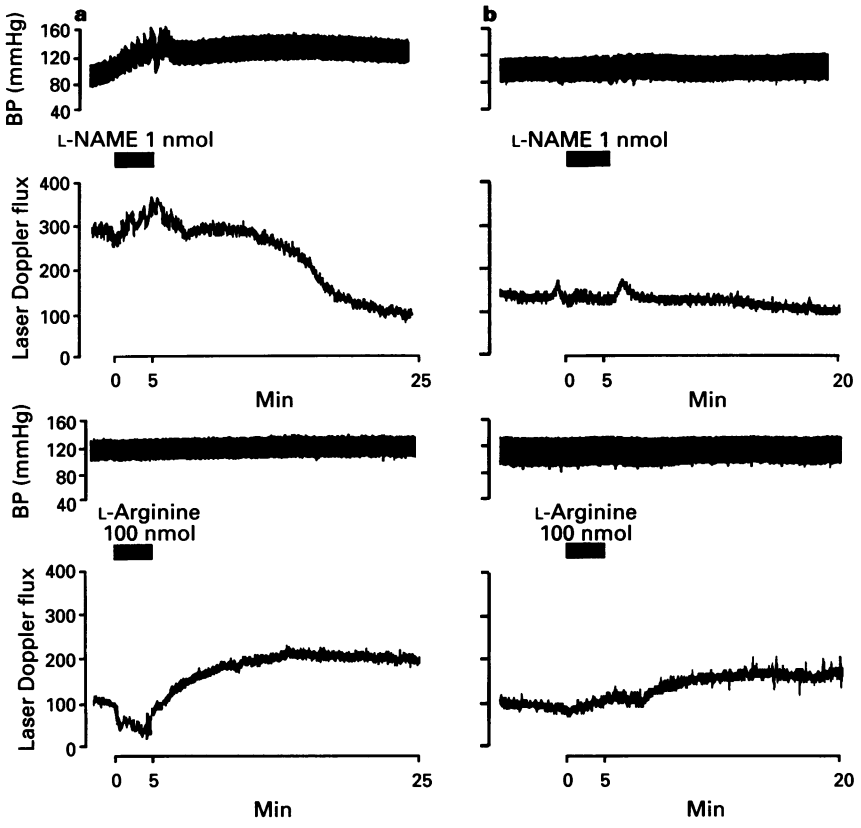

Figure 1 Typical traces showing the effects of L-NAME (upper traces), in a control (a) and a diabetic (b) rat, on systemic arterial pressure and sciatic nerve laser Doppler flux (arbitrary units). Below are shown the responses of the same animals to L-arginine.

Table 2 Sciatic nerve blood flow changes after interruption of nitric oxide synthesis (L-NAME) and reversal of L-NAME with L-arginine

	Sciatic nerve laser Doppler flux (arbitrary units)							
	Resting		*After L-NAME*	*(% fall)*	*Before L-arginine*		*After L-arginine*	*(% increase)*
Study 1								
Controls (9)	220±52	$P<0.001$	126±41	42±16	133±53	$P<0.03$	206±54	66±56
	$P<0.005$							
Diabetic untreated (12)	117±40	NS	93±34	18±28	83±18	$P<0.001$	145±32	76±29
Study 2								
Controls (10)	213±96	$P<0.002$	99±35	49±15	100±41	$P<0.01$	183±65	98±54
Diabetic untreated (8)	90±35¶	NS	81±33	13±45	84±25	$P<0.02$	129±28	60±52
Diabetic EPO (9)	203±65	$P<0.002$	101±53	49±24	103±58	$P<0.05$	217±45	110±37

Data are mean±1 s.d. For Study 1, control and diabetic groups were compared by unpaired *t*-tests; in Study 2 groups were compared by one-way ANOVA with Duncan's multiple range tests and ¶ indicates $P<0.01$. Changes within animal were assessed by paired *t*-test, comparing before treatment with after treatment; *P* values are given between columns. Numbers of rats in parentheses.

but not reaching the value seen in controls after SNP. Final values following SNP were 239±72 ($n=7$) and 157±86 ($n=8$) for control and diabetic groups respectively.

EPO-treated diabetic rats resembled control rats in their changes in sciatic nerve LDF. Thus, they had a similar resting value to controls, LDF was halved by L-NAME followed by returns to the pretreatment value with either L-arginine or SNP.

None of these acute changes in sciatic nerve LDF was accompanied by an alteration in systemic arterial pressure.

Discussion

In this study there were clear effects of several drugs on nerve Doppler flux when these were injected into the endoneurium. We cannot be specific about the compartmental distribution of these agents within the nerve. Pilot studies using dye show that the injectate diffuses throughout the endoneurium and moves slowly away from the pipette tip. The microvasculature of the rat sciatic nerve has been described in detail by others (Bell & Weddell, 1984) and microvessels containing smooth muscle are present both in the endoneurium and in the epineurium. In addition endoneurial capillaries are enclosed by pericytes, which may also be contractile. Thus, we cannot discriminate between possible effects on these different types of vessel. However, irrespective of cellular targets, the effects of the drugs used appear to be clear in terms of gross changes in the nerve Doppler signal.

First, it should be noted that the diabetic rats exhibited a proportionally similar, relative to controls, reduction in sciatic nerve laser Doppler flux to that seen in previous studies (see

Stevens *et al.*, 1994, for detail). The proportion of this change in diabetic rats is also similar to that reported by use of other methods for measurement of nerve blood flow (Tuck *et al.*, 1984; Monafo *et al.*, 1988). Second, we found complete prevention of this deficit by treatment with evening primrose oil; again confirming previous work (Stevens *et al.*, 1993b). In this and previous studies, evening primrose oil was shown to have no effect upon body weight, clearly showing this deficit to be dependent on factors other than diabetes-related changes in animal size. The new observations implicate impairment of production and response to nitric oxide in this deficit. Thus, reducing LDF by 40–50% in control rats with L-NAME indicates a powerful role for nitric oxide in the maintenance of sciatic nerve blood flow. The absence of an effect of L-NAME in diabetic rats indicates that either the production of or the responsiveness to endogenous nitric oxide – or, a combination of both – was severely compromised in the sciatic nerves of diabetic rats. It is possible to argue that the low resting LDF in the nerves of diabetic rats might be attributable to some mechanism other than failure of nitric oxide production, with the caveat that this low starting value precludes any further reduction following L-NAME. This argument cannot be refuted without data on resting nitric oxide release and we see no way of obtaining that with current technology. However, the effect of L-arginine makes the above argument less compelling. Thus, the finding that, in diabetic rats, L-arginine, administered after L-NAME, not only reversed the effect of the latter, but also established a sciatic nerve LDF which was greater than the starting value, indicates that there was a clear deficit in nitric oxide production in the resting nerve of the diabetic rats.

At first sight the failure of SNP to reinstate a level of nerve blood flow similar to that seen in controls might imply that responsiveness to nitric oxide is also impaired. However, the magnitude of the change with SNP was similar in control and diabetic rats. Thus, administration of L-arginine or SNP in diabetic rats left these animals with sciatic LDF values that were still some way short of those seen in control rats either before intervention or after L-arginine or SNP. Therefore, we suggest that the nitric oxide deficit is confined to production, but that failure of a second system, possibly of dilator prostanoids (Stevens *et al.*, 1993a), is superimposed and cannot be reversed completely by either L-arginine or SNP.

The effect of EPO is surprising, since hitherto its beneficial effects on nerve blood flow and conduction have been attributed to reinstatement of normal prostanoid release (Cameron *et al.*, 1993; Stevens *et al.*, 1993a). Clearly, such a hypothesis is incomplete and a major corrective or preventive effect on nitric oxide production must form part of the mechanism. Since evening primrose oil treatment of diabetic rats normalizes the production of prostacyclin by sciatic nerve *in vitro*, one possibility is that prostanoids support the activity of nitric oxide synthase. Interactions between the two have been reported (Gaillard *et al.*, 1992; Marotta *et al.*, 1992; Imai *et al.*, 1993; Tetsuka *et al.*, 1994; Swierkosz *et al.*, 1995), but these are restricted to the inducible form of nitric oxide synthase and we have been unable to discover any effects on the constitutive form. Since the latter is likely to be the operative enzyme in our study, such interaction remains only a theoretical suggestion. Effects of essential fatty acids that are unrelated to eicosanoid production have been considered to explain several of their functional properties and most of these derive from proposed alteration of membrane lipid composition with changes in the biophysical properties of the plasma membrane (Szamel & Resch, 1981; Bourre *et al.*, 1989; Horrobin, 1993). Since constitutive nitric oxide synthase is associated with the endothelial cell membrane, it is entirely possible that an alteration in the component lipids could influence the capacity of the enzyme to produce nitric oxide. It is also odd that EPO counteracted the hypotension of diabetes, especially if its effects on nitric oxide production were not restricted to the sciatic nerve. Clearly, further work is needed to explain this.

These observations firmly establish a failure of the nitric oxide system as a contributor to endoneurial ischaemia in experimental diabetes and make it a possible drug target for diabetic neuropathy.

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