Effects of the protein kinase C\(\beta\) inhibitor LY333531 on neural and vascular function in rats with streptozotocin-induced diabetes

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Abstract

Elevated protein kinase C activity has been linked to the vascular and neural complications of diabetes. The aim of the present study was to examine the involvement of the \(\beta\)-isoform of protein kinase C in abnormalities of neuronal function, neural tissue perfusion and endothelium-dependent vasodilation in diabetes, by treatment with the selective inhibitor LY333531 (10 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) day\(^{-1}\)). Diabetes was induced in rats by streptozotocin; the duration of diabetes was 8 weeks. Nerve conduction velocity was monitored, and responses to noxious mechanical and thermal stimuli were estimated by the Randall–Sellito and Hargreaves tests respectively. Sciatic nerve and superior cervical ganglion blood flow were measured by microelectrode polarography and hydrogen clearance. Vascular responses were examined using the in vitro mesenteric bed preparation. An 8-week period of diabetes caused deficits in sciatic motor (20\%) and saphenous nerve sensory (16\%) conduction velocity, which were reversed by LY333531. Diabetic rats had mechanical and thermal hyperalgesia. LY333531 treatment did not affect mechanical thresholds, but corrected thermal hyperalgesia. Sciatic nerve and superior cervical ganglion blood flow were both reduced by 50\% by diabetes; this was almost completely corrected by 2 weeks of LY333531 treatment. Diabetes caused a 32\% reduction in vasodilation of the mesenteric vascular bed in response to acetylcholine, mediated by nitric oxide and endothelium-derived hyperpolarizing factor. When the former was abolished during nitric oxide synthase inhibition, an 80\% diabetic deficit in the remaining relaxation was noted. LY333531 treatment attenuated the development of these defects by 64\% and 53\% respectively. Thus protein kinase C\(\beta\) contributes to the neural and vascular complications of experimental diabetes; LY333531 is a candidate for further study in clinical trials of diabetic neuropathy and vasculopathy.

Introduction

Several metabolic sequelae of hyperglycaemia cause or contribute to diabetic complications, including oxidative stress, polyol pathway activation, advanced glycation, essential fatty acid dysmetabolism and altered diacylglycerol (DAG)\{protein kinase C (PKC) activity\[1,2\]. In blood vessels and tissues prone to microvascular compli-
cations, such as retina, kidney and heart, DAG/PKC activity is elevated. The β isoform of PKC appears to be particularly important in vascular tissues, with demonstrations of up-regulation and membrane translocation in diabetes, and the development of ‘diabetes-like’ complications in normoglycaemic mice overexpressing PKCβ. Treatment with LY333531, a specific inhibitor of the PKCβ isoform, prevented the development of impaired retinal blood flow, renal glomerular hyperfiltration and microalbuminuria in diabetic rats [2].

In contrast with the renal and retinal microvascular complications of diabetes, a neurochemical cause for peripheral nerve complications has been advanced. This involves reduced DAG/PKC activity consequent upon impaired phosphoinositide turnover and myo-inositol metabolism [3]. Studies have shown that treatment with DAG or PKC inhibitors can prevent or correct motor nerve conduction velocity (NCV) defects in diabetic rats [4–6], suggesting that this neurochemical mechanism is not crucial. Nerve blood flow and oxygen tension measurements and studies using peripheral vasodilators in patients and animal models [2,7–9] suggest that microvascular alterations and ischaemia make major contributions to diabetic neuropathy. Thus, in common with the situation in the kidney and retina, it is plausible that diabetes-induced PKCβ activation of the neural vasculature affects nerve function deleteriously [4–6]. The precise details of PKCβ-mediated vascular effects in diabetes are unclear. However, experiments using a 6-h hyperglycaemic clamp in humans showed that depression of forearm blood flow responses to methacholine was prevented by LY333531 treatment. This suggests that PKCβ activation causes dysfunction of endothelium-dependent relaxation, including the nitric oxide (NO) system [10].

The aim of the present study was to elucidate further the role of PKCβ in vascular and neural dysfunction in experimental diabetes. To this end, experiments examined the effects of chronic treatment of diabetic rats with the PKCβ isoform-specific inhibitor LY333531 in vivo on sciatic nerve and superior cervical ganglion (SCG) blood flow, large nerve fibre function (motor and sensory NCV) and small sensory fibre systems (thermal and mechanical nociceptive responses). Further experiments used the in vitro mesenteric bed preparation to study detailed vascular changes.

METHODS

Experiments were performed in accordance with regulations specified by the U.K. Animal Procedures Act, 1986, and the National Institutes of Health Principles of Laboratory Animal Care, 1985 revised version.

Experimental groups and induction of diabetes

Male Sprague–Dawley rats (Aberdeen University colony), 19 weeks old at the start of the study, were used. Diabetes, induced by intraperitoneal streptozotocin (Astra-Zeneca; Alderley Edge, Cheshire, U.K.) at 40–45 mg·kg⁻¹ freshly dissolved in sterile 0.9% NaCl solution, was verified 24 h later by estimating hyperglycaemia and glycosuria (Visidex II and Diastix; Ames, Slough, U.K.). Blood samples were taken from the tail vein or by cardiac puncture (mesenteric bed study) for plasma glucose determination (GOD-Perid method; Boehringer Mannheim, Mannheim, Germany) just before final experiments.

Three investigations were undertaken, in which the basic duration of diabetes was 8 weeks. The first set of experiments used a reversal paradigm to examine the effects of LY333531 on sciatic endoneurial blood flow and large-nerve-fibre-mediated motor and sensory NCV: treatment was given for 2 weeks, following 6 weeks of untreated diabetes. The longevity of neurovascular effects was tested further in 24-week-diabetic rats, treated with LY333531 for 2 weeks. In a second set of experiments, also using the 8-week reversal paradigm, rats were subjected to sensory testing before and after LY333531 treatment to estimate the function of small-fibre-mediated nociceptive systems. These rats were then used for measurements of SCG blood flow. In the third set of experiments, the effects of diabetes and LY333531 treatment over 8 weeks were examined using the in vitro mesenteric vascular bed preparation. In all studies, groups comprised non-diabetic control, untreated diabetic and treated diabetic rats. LY333531 is a competitive inhibitor at the catalytic site of PKC, and exhibits 76- and 61-fold selectivity for inhibition of PKCβII and PKCβI respectively compared with PKCα. Selectivity is also >40-fold compared with the γ, δ, ε, and ζ PKC isoforms [2]. LY333531 was given as a dietary supplement, resulting in a daily dose of approx. 10 mg·kg⁻¹, which was based upon maximal effectiveness in dose-ranging pilot studies on sciatic motor NCV.

Nerve function tests

For NCV, rats were anaesthetized with thiobutabarbital (Astra-Zeneca; 50–100 mg·kg⁻¹), by intraperitoneal injection. The sciatic nerve was exposed between sciatic notch and knee, and motor NCV was measured as described previously [11] in the nerve branch to the tibialis anterior muscle, which is representative of the whole sciatic nerve in terms of susceptibility to diabetes and treatment effects. Saphenous nerve sensory NCV was measured between groin and calf. Rectal and near-nerve temperatures were monitored and regulated between 36.5 and 37.5 °C with radiant heat.
Nociceptive thresholds for mechanical stimulation were measured by the Randall–Sellito test [12], and latencies for withdrawal reflexes to noxious thermal stimulation of the foot were estimated by the Hargreaves plantar test [13] using commercially available equipment (Ugo-Basile, Comerio, Italy). Briefly, tests were carried out in a constant-temperature room at the same time each day, and rats were given a 3-day period for familiarization with handling, the environment and equipment, and the measurement procedure. Mechanical pressure thresholds were then estimated twice per day for each foot over a 3-day period before LY335531 treatment commenced. After 12 days of treatment, thresholds were again determined over 3 days. Data from the three pretreatment and the three end-of-treatment days were averaged to give pressure threshold values. On each day following mechanical testing, rats were placed in the thermal testing apparatus, which consisted of a Perspex enclosure with a glass base, in which they were free to move. After 30 min of acclimatization, a constant-power IR stimulus was focused through the glass base on to the sole of the foot, and the latency for reflex foot withdrawal was recorded automatically via a photoelectric monitor. For each session, four measurements were obtained, two from each foot, the average being taken as the final withdrawal latency. As with the mechanical estimates, there was a 3-day run-in period, followed by 3 days’ pre-drug and 3 days’ end-of-drug testing.

**Sciatic nerve and SCG blood flow**

Rats were anaesthetized with thiobutabarbital (AstraZeneca; 50–100 mg kg$^{-1}$), by intraperitoneal injection. The trachea was cannulated for artificial ventilation. A cannula in the right carotid artery was used to monitor mean systemic blood pressure. Core temperature was monitored and regulated at 37–38 °C, using a rectal probe and radiant heat.

Blood flow was estimated by microelectrode polarography and hydrogen clearance as previously described [14]. For peripheral nerve recordings, the sciatic nerve was exposed between sciatic notch and knee. For ganglion recordings, the left SCG was located in the vicinity of the carotid bifurcation. In both cases, the skin around the incision was used to form a pool that was filled with paraffin oil maintained at 35–37 °C by radiant heat during measurements. A glass-insulated platinum microelectrode was inserted into the appropriate neural structure and polarized at 0.24 V with respect to a subcutaneous reference electrode. H$_2$(10%) was added to the inspired gas, the proportions of O$_2$ and N$_2$ being adjusted to 20% and 70% respectively. When the H$_2$ current recorded by the electrode had stabilized, indicating equilibrium with arterial blood, the H$_2$ supply was shut off and N$_2$ delivery was increased appropriately. The H$_2$ clearance curve was recorded until baseline, which was defined as no systematic decline in electrode current over 1 min for SCG or 5 min for sciatic nerve recordings. This procedure was then repeated at another site. Clearance curves were digitized, and mono-exponential or bi-exponential curves were fitted off-line by computer using non-linear regression, the Marquardt algorithm and the least-squares method for optimizing goodness-of-fit (Prism; Graphpad, San Diego, CA, U.S.A.). The slow exponent was taken to reflect nutritive flow [14].

**Mesenteric vascular bed**

The mesenteric vascular bed was used as previously described [15]. Briefly, under 5% halothane anaesthesia, the superior mesenteric artery was cannulated and the mesenteric bed was freed from the small intestine and placed in a tissue chamber at 37 °C. The preparation was perfused with modified Krebs–Ringer solution (mmol 1$^{-1}$: 144.0 Na$^+$, 5.0 K$^+$, 1.25 Ca$^{2+}$, 1.1 Mg$^{2+}$, 25.0 HCO$_3^-$, 1.1 PO$_4^{3-}$, 1.1 SO$_4^{2-}$, 5.5 glucose) at 37 °C and gassed continuously with 95% O$_2$/5% CO$_2$ (pH 7.35). The flow rate was 5 ml min$^{-1}$. Perfusion pressure and drug effects were monitored using a pressure transducer. At least 30 min equilibration was allowed. Preparations were first contracted with 100 μmol 1$^{-1}$ phenylephrine (PE) and then allowed to relax for 30 min. Preparations were then precontracted with a dose of PE (1–100 μmol 1$^{-1}$) adjusted to give a similar increase in perfusion pressure of 25–60 mmHg in all groups under the different preincubation conditions. Cumulative concentration–response curves were estimated in the presence of the cyclo-oxygenase inhibitor flurbiprofen (3 μmol 1$^{-1}$). Endothelium-dependent depressor responses were recorded for acetylcholine (ACh) alone, and after preincubation with and co-perfusion of a high dose (3–30 mmol 1$^{-1}$) of N$^\omega$-nitro-L-arginine (l-NNA) to ensure maximal blockade of NO synthase. Endothelium-independent vasorelaxation to the NO donor sodium nitroprusside (SNP) in the presence of l-NNA was then assessed. Pressor responses to cumulatively increasing PE concentrations were also determined before and after preincubation with 3 mmol 1$^{-1}$ l-NNA.

**Statistical analysis**

Results are expressed as means±S.E.M. Data were subjected to Bartlett’s test for homogeneity of variances, followed by logarithmic transformation if necessary before one-way ANOVA. Where significance (P < 0.05) was reached, between-group differences were established using the Student–Neuman–Keuls or Bonferroni multiple-comparison test. If variances were not homogeneous, data were analysed by appropriate non-parametric tests, such as Kruskal–Wallis one-way ANOVA followed by Dunn’s multiple-comparison test. Within-group serial comparisons were made using
paired Student’s *t* tests. Concentration–response curves were fitted by sigmoid curves using the least-squares method to estimate EC\(_{50}\) (Graphpad).

**RESULTS**

Body weights and blood glucose concentrations are given in Table 1. Diabetes caused an approx. 5-fold elevation in plasma glucose and an approx. 20% weight loss. These parameters were not affected significantly by LY333531 treatment.

**NCV and sciatic endoneurial blood flow**

Sciatic motor NCV (Figure 1A) was reduced by 20.1 ± 1.2% and 21.4 ± 1.0% by 8 and 24 weeks of diabetes respectively (*P* < 0.001, both time points). These deficits were corrected by 92.4 ± 4.0% and 91.5 ± 8.3% respectively (*P* < 0.001) by 2 weeks of LY333531 treatment. Similarly, saphenous sensory NCV (Figure 1B) was decreased by 15.6 ± 1.0% (8 weeks) and 19.7 ± 1.7% (24 weeks) by diabetes (*P* < 0.001). LY333531 treatment completely corrected the deficit at 8 weeks, and gave 91.6 ± 5.8% reversal at the 24-week time point (*P* < 0.001). There were no significant differences in the size of the NCV deficits or the effectiveness of LY333531 treatment between 8 and 24 weeks of diabetes.

Sciatic nutritive endoneurial blood flow (Figure 2A) was reduced by 41.1 ± 5.2% after 8 weeks and by 45.6 ± 3.6% after 24 weeks of diabetes (*P* < 0.001). Treatment with LY333531 for the final 2 weeks of the experimental period largely corrected these deficits (*P* < 0.001), with blood flow showing a trend towards being supernormal at the 8-week time point; this was not statistically significant compared with the non-diabetic group, but flow was greater than in 24-week LY333531-treated diabetic rats (*P* < 0.05). Systemic blood pressure (Figure 2B) during the hydrogen clearance measurements varied between groups and tended to be reduced by diabetes, although this was not statistically significant. When the flow data were corrected for these variations in perfusion pressure, expressing results in terms of vascular conductance (Figure 2C), then the 30.6 ± 5.0% (8 weeks; *P* < 0.05) and 35.4 ± 4.7% (24 weeks; *P* < 0.01) diabetic deficits were completely corrected by LY333531 treatment. For the 8-week LY333531-treated group, conductance was 50.4 ± 15.6% supernormal (*P* < 0.05), and also exceeded that of the 24-week LY333531-treated group (*P* < 0.05).

### Table 1 Body weights and plasma glucose concentrations

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Start</th>
<th>End</th>
<th>Plasma glucose (mmol · l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26</td>
<td>–</td>
<td>451 ± 5</td>
<td>8.1 ± 0.3</td>
</tr>
<tr>
<td>Diabetic (8 weeks)</td>
<td>26</td>
<td>459 ± 4</td>
<td>346 ± 10</td>
<td>41.8 ± 1.9</td>
</tr>
<tr>
<td>Diabetic (24 weeks)</td>
<td>6</td>
<td>460 ± 13</td>
<td>321 ± 5</td>
<td>44.0 ± 1.5</td>
</tr>
<tr>
<td>Diabetic (8 weeks) + LY333531</td>
<td>26</td>
<td>453 ± 4</td>
<td>342 ± 10</td>
<td>42.6 ± 1.7</td>
</tr>
<tr>
<td>Diabetic (24 weeks) + LY333531</td>
<td>6</td>
<td>466 ± 4</td>
<td>330 ± 10</td>
<td>42.7 ± 2.3</td>
</tr>
</tbody>
</table>
Small-fibre-mediated nociceptive responses and SCG blood flow

The latency for reflex withdrawal to noxious thermal stimulation of the sole of the foot (Figure 3A) was reduced by 28.9 ± 4.4% by diabetes ($P < 0.001$), indicating thermal hyperalgesia. Treatment of the same diabetic rats with LY333531 for 2 weeks caused a marked increase ($P < 0.0001$) in latencies, such that they were within the non-diabetic range. Mechanical thresholds (Figure 3B) for foot withdrawal in these rats were also reduced by $21.4 \pm 5.8\%$ by diabetes ($P < 0.01$), providing evidence of mechanical hyperalgesia. However, in this case LY333531 treatment was without effect.

SCG nutritive blood flow (Figure 4A) was approx. 4-fold greater than sciatic endoneurial perfusion. Diabetes for 8 weeks caused a $49.7 \pm 3.7\%$ reduction ($P < 0.001$) in SCG flow, which was corrected ($P < 0.001$) by 2 weeks of LY333531 treatment. Data for vascular conductance (Figure 4B) showed very similar trends.

Mesenteric vascular bed

Against a background PE precontraction, concentration–response curves for ACh (Figure 5A) showed that diabetes impaired vasodilation ($P < 0.01$) at ACh concentrations $\geq 3\ \text{nmol}\cdot\text{l}^{-1}$. However, with preventive LY333531 treatment from diabetes induction, there was greater vasorelaxation ($P < 0.05$) for ACh concentrations $\geq 30\ \text{nmol}\cdot\text{l}^{-1}$ compared with untreated diabetes, although significant deficits ($P < 0.01$) remained compared with the non-diabetic group for low ACh concentrations.
Figure 4 Effects of diabetes and PKCβ inhibition on SCG nutritive blood flow (A) and vascular conductance (B)

Groups: N, non-diabetic controls (n = 8); D, 8-week diabetic controls (n = 8); LY, 8-week diabetic rats treated with 10 mg · kg⁻¹ · day⁻¹ LY333531 for the final 2 weeks (n = 10). Data are group means ± S.E.M. Statistics (flow and conductance): N or LY compared with D, P < 0.001. All other comparisons were not significant.

concentrations (3–30 nmol · l⁻¹). Diabetes reduced maximum vasodilation by 32.4 ± 4.7% (P < 0.001), which was partially (64.0 ± 9.7%; P < 0.05) attenuated by LY333531.

After preincubation with L-NNA and precontraction to PE, endothelium-derived hyperpolarizing factor (EDHF)-dependent responses to ACh were revealed (Figure 5B). Diabetes caused deficits (P < 0.01) for ACh doses ≥ 30 nmol · l⁻¹. Responses were improved with LY333531 treatment (≥ 300 nmol · l⁻¹ ACh; P < 0.05).

Maximum vasodilation was decreased by 80.0 ± 4.8% by diabetes (P < 0.001); this was partially (53.3 ± 12.6%; P < 0.001) prevented by LY333531 treatment, although a deficit remained (P < 0.01) compared with the non-diabetic group.

Diabetes and L-NNA affected sensitivity to ACh, as indicated by −log EC₅₀ values (Figure 6). Thus, in the absence of L-NNA, −log EC₅₀ values were depressed by 0.77 log unit by diabetes (P < 0.001). With LY333531 treatment, this fall in sensitivity was markedly attenuated (P < 0.001), although a deficit of 0.32 log unit (P < 0.01) remained compared with the non-diabetic group. With L-NNA preincubation and co-perfusion, −log EC₅₀ for ACh was reduced by 0.83 log unit (P = 0.009) in the non-diabetic group, indicating that responses to lower ACh concentrations are normally dominated by the NO mechanism. In mesenteric beds from LY333531-treated diabetic rats there was a similar reduction in sensitivity to ACh with L-NNA preincubation (0.66 log unit; P = 0.002). For the diabetic group, however, L-NNA did not cause a significant shift in ACh sensitivity. All groups had similar −log EC₅₀ values under these conditions.

Endothelium-independent vasodilation in response to the NO donor SNP (Figure 7) was examined after precontraction to PE and in the presence of L-NNA to inhibit spontaneous and flow-induced endogenous NO release. There were no significant differences in maximum relaxation or −log EC₅₀ (Figure 7, inset).

Pressor concentration–response curves for PE (Figure 8A) revealed a trend for reduced responses in the diabetic group. While this was not significant for the maximum pressor response, it was apparent in the mid-range (30 nmol · l⁻¹ PE; P < 0.01) and was confirmed for the...
Figure 6  Effects of diabetes and PKCβ inhibition on ACh \(-\log EC_{50}\) values for endothelium-dependent vasodilation of the PE-preconstricted mesenteric vascular bed in the absence (−) and presence (+) of the NO synthase inhibitor L-NNA

Groups: N, non-diabetic controls (n = 12); D, 8-week diabetic controls (n = 12); LY, 8-week diabetic rats treated with 10 mg \cdot kg\(^{-1}\) \cdot day\(^{-1}\) LY333531 for the final 2 weeks (n = 9). Data are group means ± S.E.M. Statistics: *P < 0.01, **P < 0.001, †P < 0.01 for the effect of L-NNA.

Figure 7  Effects of diabetes and PKCβ inhibition on SNP concentration–response data for endothelium-independent vasodilation of the PE-preconstricted mesenteric vascular bed in the presence of NO synthase inhibition

The inset histogram shows \(-\log EC_{50}\) values. Groups: ○, non-diabetic controls (N; n = 10); ●, 8-week diabetic controls (D; n = 10); ■, 8-week diabetic rats treated with 10 mg \cdot kg\(^{-1}\) \cdot day\(^{-1}\) LY333531 for the final 2 weeks (LY; n = 10). Data are group means ± S.E.M. There were no significant differences between the groups.

Thus the maximum developed pressure was attenuated by 71.7 ± 5.0% (P < 0.001) and 64.1 ± 6.4% (P < 0.001) compared with the non-diabetic and diabetic groups respectively.

After preincubation with L-NNA, maximum pressure development with PE (Figure 8B) was approximately doubled in the non-diabetic (P < 0.0001) and diabetic (P < 0.0004) groups. However, in the LY333531-treated diabetic group, pressor responses to PE showed a 4.4-fold increase (P < 0.0001) in the presence of L-NNA, the resulting value being not significantly different from that of the diabetic group, although a deficit was still apparent compared with non-diabetic rats (P < 0.01). The maximum pressor response was not significantly different between the non-diabetic and diabetic control groups.

Sensitivity to PE, gauged by \(-\log EC_{50}\) values, did not differ significantly between groups, being 4.89 ± 0.09, 4.69 ± 0.15 and 4.69 ± 0.09 for the non-diabetic, diabetic
and LY333531-treated diabetic rats respectively. Pre-incubation with l-NNA increased sensitivity to PE in the non-diabetic (5.37 ± 0.16; \( P = 0.004 \)), diabetic (5.08 ± 0.12; \( P = 0.01 \)) and LY333531 (4.97 ± 0.10; \( P = 0.02 \)) groups.

**DISCUSSION**

The data presented here show that both diabetes and inhibition of PKC\(\beta\) have profound neural and vascular effects. The diabetic deficit in the endothelium-dependent relaxation of mesenteric vessels is in agreement with several studies on this tissue [15,16], as well as on other vessels and vascular beds [17–20], including peripheral nerves [21]. Diabetic defects in vasodilation affected both the NO and EDHF systems; this was evident for the former in terms of sensitivity to ACh-induced responses, and for the latter when the NO and prostanoid systems were blocked. In previous studies on responses of mesenteric preparations from diabetic and non-diabetic rats, vasodilation in the presence of NO and cyclooxygenase blockade was abolished by high extracellular K\(^+\), the K\(^+\)-channel blocker tetraethyl ammonium, or a combination of the K\(^+\)-channel blockers apamin and charybdotoxin, which is consistent with the pharmacological properties of EDHF in this tissue [15]. This is the first report showing that chronic treatment with a PKC inhibitor markedly attenuates the development of these abnormalities of endothelium-dependent vasodilation. The relative selectivity of LY333531 [3] implicates PKC\(\beta\) in the pathogenesis of diabetic endothelial dysfunction for both the NO and the EDHF systems.

The chemical identity of EDHF has not been established, and there may be more than one mediator. Various, EDHF has been suggested to be a cytochrome P450-derived arachidonic acid metabolite, an endocannabinoid or K\(^+\); EDHF action involves the opening of several types of K\(^+\) channel, and its effects may be propagated via gap junctions [22–25]. The present data do not further elucidate the nature of EDHF. The precise role of PKC\(\beta\) in the diabetic deficit cannot be deduced from the available evidence; putative actions at the level of EDHF synthesis/release, gap junction integrity, transduction and K\(^+\)-channel modulation require further investigation. Nonetheless, the diabetic EDHF deficit is profound [16]. The balance between NO- and EDHF-mediated vasodilation alters as vessel size decreases, with EDHF predominating in the small resistance vessels controlling nutritive perfusion [26]. Thus PKC\(\beta\) is likely to be very important in the aetiology of diabetic microvascular complications.

The effect of diabetes on the mesenteric NO-mediated component of ACh-induced relaxation was not caused by a reduction in the sensitivity of vascular smooth muscle to NO, because responses to SNP were unaltered. This is in agreement with findings for the majority of investigations in experimental diabetes [16–20]; however, in patients, there are also several reports of reduced responses to NO donors [27]. Thus, at least in this experimental model, the diabetic defect appears to be at the level of impaired endothelial NO production, or increased neutralization by reactive oxygen species. The possibility of ACh receptor/signalling abnormalities cannot be completely excluded, although in other preparations diabetic NO-mediated deficits were noted when different agonists (histamine, bradykinin) were used or when receptors were bypassed using Ca\(^{2+}\) ionophores [28,29].

The NO mechanism was largely protected by chronic LY333531 treatment, implicating PKC\(\beta\) in the diabetic deficit. Several studies have shown that PKC influences endothelial cell NO production. At least in culture, PKC phosphorylates endothelial NO synthase, resulting in reduced activity [30]. Moreover, PKC inhibition increases levels of NO synthase mRNA and protein [31]. In experiments on pial arteries of diabetic rats in vivo, NO-mediated vasorelaxation to ACh was impaired, and this was acutely corrected by superfusion with a non-specific PKC inhibitor [32]. PKC\(\beta\)-mediated actions on the NO system affected pressor responses to PE. Diabetes caused a modest reduction in PE pressor action, and a reduced basal pressure to constant flow in the absence of stimulation. These are probably explicable by mesenteric vascular hypertrophy, in part a response to hyperphagia [33], causing an increase in conductance. However, in the LY333531-treated diabetic group a profound inhibition of pressor responses was superimposed, which was largely abolished by l-NNA. This suggests that chronic inhibition of PKC\(\beta\) in diabetic rats promotes a marked increase in mesenteric basal/flow-induced NO release, more than compensating for a diabetic defect [34].

The endothelial effects of diabetes and LY333531 treatment probably contribute to the changes in neural tissue perfusion observed in vivo. Thus the profound diabetic deficits in sciatic endoneurial and SCG blood flow were completely corrected by PKC\(\beta\) inhibition. The sciatic blood flow data are in agreement with previous data for non-isoform-specific PKC inhibition [4,5]. Furthermore, they extend the results of a preventive study using LY333531 [6], by showing successful reversal of an established diabetic deficit. This reversal was accomplished whether LY333531 treatment was given early (8-week group) or later (24-week group) in the disease process, although the earlier effects were somewhat more profound; vascular conductance was super-normal. The mechanism is not clear, but, by analogy with the mesentery data, it could be due to a combination of improved endothelium-dependent relaxation and reduced vasa nervorum contractile responses. A similar phenomenon was noted for high-dose antioxidant treat-
ment, which was abolished by chronic low-dose l-NNA co-treatment [35]. The present data suggest that the effect is transient, disappearing by 24 weeks; however, blood flow remained in the non-diabetic range in that group, and therefore these later neurovascular changes would not be expected to adversely affect the efficacy of LY333531 in correcting nerve dysfunction.

A previous report of an SCG perfusion deficit, after a prolonged period (12 months) of diabetes, used the \(^{14}\text{C}\)iodoantipyrine autoradiographic technique [36]. The present data using \(\text{H}_2\) clearance are in agreement, and provide further information to show that ganglion blood flow defects occur relatively early after diabetes induction, in line with effects on the peripheral nerve trunk [1]. It is plausible that impaired perfusion of cell bodies could contribute to autonomic neuropathy, which probably extends to sensory neuropathy as dorsal root ganglia could contribute to autonomic neuropathy, which probably extends to sensory neuropathy as dorsal root ganglia are similarly affected [36]. This would restrict the metabolic energy supply necessary for the synthesis/transport of essential components for maintenance of axonal integrity and neurotransmission: SCG phospho-creatine levels are reduced in diabetic rats [37]. Such a hypothesis is compatible with the attenuated development of cardiac R-R interval abnormalities in diabetic rats treated with LY333531 or a vasodilator [6]. Furthermore, a ganglion perfusion defect could contribute to eventual apoptotic and necrotic cell body and fibre loss [36,38].

The motor and sensory NCV defects in diabetic rats are markers of large myelinated fibre dysfunction. These were corrected by LY333531 treatment, in agreement with non-isofrom-specific PKC inhibitor studies [4,5]. LY333531 has been shown to attenuate the development of a caudal nerve motor NCV deficit [6], and the present study extends those observations to reversal of sciatic motor and saphenous sensory NCV changes. The data also show that the efficacy of LY333531 in correcting NCV deficits was not reduced over a 24-week period of diabetes. Such large-fibre effects, together with the vascular and perfusion data, are consistent with the notion that the vasa nervorum is an important early target in the aetiology of diabetic neuropathy, and suggest that this factor does not diminish in importance in long-term experimental diabetes.

The data for nociceptive thresholds reflect responses mediated by small nerve fibre systems, although they do not discriminate between peripheral and central components. Diabetes caused mechanical hyperalgesia, which has been demonstrated in several studies [39,40]. There is less agreement on diabetes-induced changes to responses to thermal stimuli; some models, particularly using streptozotocin-induced diabetes in young rats, report hyposensitivity using the tail flick test [41]. However, when diabetes was induced in mature rats, as in the present model, thermal hyperalgesia was noted [42]. In the present study, LY333531 treatment corrected thermal hyperalgesia without having any effect on mechanical thresholds, which suggests a specific action rather than a general anti-allodynic or central analgesic effect. The thermal anti-nociceptive action could relate to increased nerve/ganglion blood flow or to improvements in skin perfusion in the vicinity of sensory nerve terminals [43], a hypothesis that is testable using peripheral vasodilator treatment. The lack of an effect on mechanical nociception thresholds, which are correctable with insulin treatment [40], suggests that a PKC\(\beta\)-mediated vascular mechanism is not critically important. Electrophysiological recordings from mechanosensitive nociceptor primary afferent fibres in diabetic rats showed altered responses to stimulation, which were acutely corrected by cutaneous injection of a non-isofrom-specific PKC inhibitor [44]. Knockout mice deficient in PKC\(\gamma\) did not develop neuropathic pain/allodynia in response to nerve damage [45]. Thus PKC could be important for mechanical nociception in rodents, but LY333531 is specific for PKC\(\beta\) and may target the wrong isofrom.

The effects of PKC\(\beta\) should be placed in context with the other altered metabolic and vascular mechanisms in diabetes. PKC stimulation may arise because of hyperglycaemia-driven \textit{de novo} DAG synthesis [2]. However, at least for peripheral nerves, this is unlikely to be the major mechanism; NCV defects are corrected by vasodilator treatment [1], which would not be expected to alter \textit{de novo} DAG production. The fact that sciatic nerve PKC activity and DAG levels themselves are unaltered in this diabetic model [5] emphasizes the vascular aetiology of neuropathy. Many of the endothelial and neural changes in diabetes identified as being PKC\(\beta\)-inhibitor-sensitive in the present study are also partially corrected by other metabolic interventions, including antioxidants, aldose reductase antagonists and advanced glycation inhibitors [1,15,17]. An important common denominator for these drugs is that they reduce the level of oxidative stress, which is elevated by diabetes. Vascular endothelium and smooth muscle PKC mechanisms are stimulated by oxidative stress and modulated by antioxidant action [46]. Furthermore, oxidative stress has been implicated in elevating the vasa nervorum endothelin 1 and angiotensin II systems, nerve dysfunction being corrected by ET\(_4\) and AT\(_2\) antagonists [1,7]. PKC acts in the signalling cascade of these vasoconstrictors [1,47]. Thus alterations in PKC can be viewed as being downstream of oxidative stress-related mechanisms and upstream of endothelial and vascular smooth muscle dysfunction in the aetiology of neurovascular complications.

In conclusion, inhibition of PKC\(\beta\) with LY333531 has been shown to have a broad range of beneficial effects on neural and vascular dysfunction in experimental diabetes. Coupled with demonstrated benefits in models of nephropathy and retinopathy [2], it is plausible that this might be reflected in the results of clinical trials on
diabetic complications, which should be the subject of in-depth inquiry.

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