Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 475 (2003) 99–106
www.elsevier.com/locate/ejphar

Protein kinase Cβ inhibition and aorta and corpus cavernosum function in streptozotocin-diabetic mice

Matthew R. Nangle, Mary A. Cotter, Norman E. Cameron *

Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland, UK

Received 10 April 2003; received in revised form 7 July 2003; accepted 15 July 2003

Abstract

Increased activity of the β-isoform of protein kinase C (PKC) has been linked to the vascular and neural complications of diabetes mellitus. Treatment with the PKCβ inhibitor, (*s*)-13-[(dimethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione, (LY333531), improves somatic nerve function and blood flow in diabetic rats. The aim was to assess whether LY333531 treatment could prevent nitric oxide-dependent autonomic nerve and vascular dysfunction in a diabetic mouse model. Diabetes was induced by streptozotocin; duration was 4 weeks. Aorta and corpus cavernosum were isolated and mounted in organ baths and agonist or electrical stimulation-evoked nerve-mediated tension responses were examined. Maximum nitric oxide-mediated endothelium-dependent relaxation of phenylephrine-precontracted aorta and cavernosum to acetylcholine were more than 30% reduced by diabetes. LY333531 treatment (10 mg kg^{-1} day^{-1}) completely prevented the diabetic deficit in cavernosum, and 75% prevented the deficit in aorta. Maximum nitric oxide-dependent non-adrenergic, non-cholinergic (NANC) nerve-mediated relaxation of phenylephrine-precontracted cavernosum was approximately 43% reduced by diabetes; LY333531 attenuated the deficit by 44%. For diabetic aorta, but not cavernosum, sensitivity (EC_{50}) to phenylephrine-mediated contraction was increased by approximately 0.85 $\log_{10}$ M units; LY333531 treatment completely prevented this effect. Thus, PKCβ activation contributes to nitric oxide-dependent vascular and autonomic nerve dysfunction in diabetic mice and could prove suitable for further study in clinical trials of diabetic autonomic neuropathy and vasculopathy.

Keywords: Protein kinase C; Diabetes mellitus; Endothelium; Smooth muscle; NANC (non-adrenergic, non-cholinergic) nerve; Nitric oxide (NO); Aorta; Corpus cavernosum

1. Introduction

Hyperglycaemia associated with diabetes mellitus increases de novo synthesis of diacylglycerol, which, in turn, increases activation of the protein kinase C (PKC) signal transduction pathway. This process has been demonstrated in multiple vascular beds including aortic endothelial and smooth muscle cells (Tesfamariam et al., 1991; Inoguchi et al., 1994; Xia et al., 1994), affecting vascular permeability, proliferation and contractility (Koya and King, 1998). Of the various PKC isoforms, the conventional Ca^{2+}-dependent PKC-β and -δ appear to be preferentially activated in diabetes (Inoguchi et al., 1992).

The bisindolylmaleimide compound, (*s*)-13-[(dimethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione, (LY333531), is a selective inhibitor of the βI and βII isoforms of PKC (Ishii et al., 1996). Vascular complications play a pivotal role in the development of diabetic neuropathy in animal models and man (Cameron et al., 2001). LY333531 treatment prevented the development of retinal and renal haemodynamic deficits in diabetic rats (Ishii et al., 1996), and PKC inhibition corrected somatic peripheral nerve conduction and blood flow deficits (Cameron et al., 1999; Jack et al., 1999; Nakamura et al., 1999; Cameron and Cotter, 2002; Cotter et al., 2002).

Nitric oxide-mediated endothelium-dependent relaxation is depressed in aorta from diabetic animals (Durante et al., 1988; Pieper, 1998; Piercy and Taylor, 1998); similar deficits have been reported in man (Johnstone et al.,

* Corresponding author. Tel.: +44-1224-555713; fax: +44-1224-555719.
E-mail address: n.e.cameron@abdn.ac.uk (N.E. Cameron).

0014-2999/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0014-2999(03)02113-7

1993; McVeigh et al., 1992). Furthermore, defects in nitric oxide-mediated endothelial- and non-adrenergic, non-cholinergic (NANC) nerve-dependent smooth muscle relaxation are evident in corpus cavernosum from animals and man (Azadzoi and Saenz de Tejada, 1992; Gocmen et al., 2000; Keegan et al., 1999; Saenz de Tejada et al., 1989). The NANC nerves provide the majority of nitric oxide during the erectile process, however, endothelium-derived nitric oxide may also have a physiological role (Andersson and Wagner, 1995; Bivalacqua et al., 2000).

PKC can reduce endothelial nitric oxide synthase activity via phosphorylation (Hirata et al., 1995). Furthermore, PKC inhibition increases endothelial nitric oxide synthase mRNA and protein expression (Ohara et al., 1995). This could improve endothelial function in diabetes, particularly as diabetic vasculopathy is often associated with reduced nitric oxide synthase activity and/or increased nitric oxide inactivation by reactive oxygen species (Cai and Harrison, 2000). However, hyperglycaemia-driven PKC activation has not been observed in peripheral nerve from diabetic rats (Cameron et al., 1999). Thus, it is not known whether PKC inhibition could modulate neuronal nitric oxide synthase activity in NANC nerves supplying corpus cavernosum, although this has clear implications for diabetic impotence. Therefore, the aim study was to examine whether LY333531 treatment could protect nitric oxide-mediated cavernosum NANC nerve function and to further clarify the effects of PKCβ inhibition on nitric oxide-dependent vascular function in diabetic mice.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice from the University of Aberdeen breeding colony were aged between 4 and 6 months on the day of experimentation. All mice received standard laboratory chow and had access to water ad libitum. Experiments were performed in accordance with regulations specified by the United Kingdom ‘Animal Procedures Act, 1986’ and the National Institutes of Health ‘Principles of Laboratory Animal Care, 1985 revised version’. Unless otherwise stated, all chemicals were obtained from Sigma (Poole, Dorset, UK).

2.2. Anaesthesia and diabetes induction

Diabetes was induced by 125 mg kg^{-1} i.p. streptozotocin (AstraZeneca, Macclesfield, Cheshire, UK) dissolved in sterile 154 mM saline solution. After 48 h, diabetes was verified by the presence of hyperglycaemia and glycosuria (Visidex II and Diastix, Ames, Slough, UK) in nonfasted mice. Diabetic mice were weighed daily and were rejected if blood glucose levels were less than 20 mM. Nonfasting plasma glucose concentrations were more accurately estimated from samples taken from the heart on the day of experimentation, by means of colorimetric spectrophotometry using a standard test kit (Sigma). Diabetes duration was 4 weeks. In addition to untreated nondiabetic and diabetic control groups, one group of diabetic mice received preventative treatment with LY333531 (Eli-Lilly, Indianapolis, IN, USA) at a dose of 10 mg kg^{-1} day^{-1} as a dietary supplement. This dose was previously shown to have optimal effects for restoration of nerve conduction velocity in diabetic rats (Cameron and Cotter, 2002). On the final day of experiments, mice were anaesthetised (5% halothane in air, with 0.1 ml 10% urethane in 154 mM saline solution per 10 g body weight i.p.) before removal of tissues.

2.3. Aorta experiments

The thoracic aorta was removed between the aortic arch and diaphragm. It was cleared of connective tissue and two sections cut into 1 mm lengths were mounted as ring preparations, using 40 μm tungsten wire, into 5 ml organ baths in a small-vessel myograph (Linton Instrumentation, Diss, Norfolk, UK) for measurement of isometric tension as previously described (Mulvany and Halpern, 1976). Aortas were bathed in carboxygenated (95% O_2/5% CO_2) modified Krebs–Ringer solution (144 NaCl, 5 KCl, 1.1 $MgSO_4$, 25 $NaHCO_3$, 1.1 NaH_2PO_4, 1.25 CaCl and 5.5 glucose; in mM) at 37 °C (pH 7.35). Resting tension was maintained at 0.75 g. Tissues were left to equilibrate for 1 h, with frequent changing of bathing solution. Tissue viability was assessed with a priming 300 nM phenylephrine and 100 nM acetylcholine contraction/relaxation cycle. Following a 45–60 min washout and recovery period, cumulative concentration–response curves to phenylephrine, and acetylcholine and sodium nitroprusside (against an approximate 80% maximal phenylephrine contraction), were determined. In some experiments, concentration–response curves were repeated following a 30-min incubation period with 10 μM of the nitric oxide synthase inhibitor, N^G-nitro-L-arginine (L-NNA).

2.4. Corpus cavernosum experiments

The penis was excised at its base with removal of the glans penis and connective and adventitial tissues along the shaft, and two corpus cavernosum strips were obtained. These were mounted in 10 ml organ baths and bathed in Krebs–Ringer solution as for aorta. Tension was monitored by isometric transducers and resting tension was set at 0.5 g. Tissues were left to equilibrate for 1 h before determination of cumulative concentration–response curves for phenylephrine, and for sodium nitroprusside against an approximately 80% maximal phenylephrine contraction. Noncumulative concentration–response curves for acetylcholine were established against individual 80% maximal phenylephrine contractions. Tissues were washed and

allowed to recover for 20–30 min between response curve determination.

Repetitive supramaximum electrical field stimulation of autonomic nerves was delivered via platinum wire electrodes placed either side of the cavernosa (train duration 30 s; frequency 2–30 Hz; pulse duration 5 ms; 90 mA; 10 V). Frequency–response curves were determined for vasoconstrictor nerves before incubation with atropine (1 μM) and guanethidine (4 μM) for 20–30 min to eliminate responses mediated by cholinergic and noradrenergic nerves, respectively. Following phenylephrine precontraction, electrical stimulation produced frequency-dependent relaxations mediated by NANC nerves. Some concentration– or frequency–response curves were repeated following 30 min preincubation with 10 μM L-NNA. At the end of experiments, tissues were lightly patted dry and weighed.

2.5. Statistical analysis

Data are expressed as means ± S.E.M. They were subjected to Bartlett's test for homogeneity of variances before one-way analysis of variance. Where significance was reached ($P<0.05$), between-group differences were established using the Newman–Keuls multiple comparison test. Otherwise, data were analysed by Kruskal–Wallis nonparametric one-way analysis of variance and Dunn's multiple comparison test. Concentration–response curves were fitted by sigmoid curves using the least squares method to calculate EC_{50}. Within-group serial comparisons were made using paired two-tailed Student's *t*-tests. All calculations used a standard statistical software package (Prism3, Graphpad, San Diego, CA, USA).

3. Results

3.1. Plasma glucose concentrations and body weights

Diabetes caused a greater than fourfold increase ($P<0.001$) in plasma glucose concentrations (Table 1). In addition, there was an approximately 20% body weight loss ($P<0.001$). These diabetic changes were unaltered by preventative LY333531 treatment.

Table 1
Body weights and plasma glucose concentrations of the mouse groups used in the study

Group	*n*	Body weight (g)		Plasma glucose (mmol l^{-1})
		Start	End	
Nondiabetic	15	32.9 ± 0.8	–	9.3 ± 1.1
Diabetic	12	32.9 ± 1.3	27.6 ± 1.0[a]	40.6 ± 0.5[b]
Diabetic + LY333531	11	30.4 ± 0.8	23.5 ± 0.8[a]	39.8 ± 0.6[b]

Data are mean ± S.E.M.
[a] $P<0.001$ vs. start weight.
[b] $P<0.001$ vs. nondiabetic control.

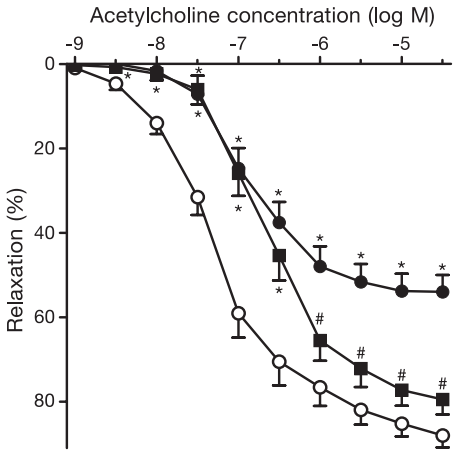

Fig. 1. Cumulative concentration–response curves for relaxation to acetylcholine of aortas from nondiabetic and diabetic mice, and the effects of preventive LY333531 treatment. Groups: nondiabetic control (○, $n=13$); 4 week diabetic control (●, $n=10$); diabetic mice treated with 10 mg kg^{-1} day^{-1} LY333531 for 4 weeks from diabetes induction (■, $n=10$). Data are mean ± S.E.M. *$P<0.05$ vs. nondiabetic control group; #$P<0.05$, effects of LY333531 treatment vs. the diabetic control group.

3.2. Aorta study

Maximum endothelium-dependent relaxation to acetylcholine (Fig. 1), following phenylephrine precontraction, was reduced by approximately 39% with diabetes compared to aortas from nondiabetic controls (54.0 ± 4.2% vs. 88.0 ± 2.9%, $P<0.001$). Treatment with LY333531 prevented the development of this deficit by approximately 75% (79.5 ± 3.7%, $P<0.001$). However, at acetylcholine concentrations of less than 1 μM, there was no significant effect of treatment. Sensitivity to acetylcholine, assessed by $(-\log)EC_{50}$, was decreased by diabetes by approximately 0.37 log units (6.89 ± 0.11 vs. 7.26 ± 0.12, $P<0.05$); this was unaffected by LY333531 treatment (6.67 ± 0.13). Preincubation with 10 μM L-NNA completely abolished acetylcholine-mediated relaxations (data not shown).

Endothelium-independent relaxation to the nitric oxide donor, sodium nitroprusside, following phenylephrine precontraction, approached 100% in all groups (Table 2). In addition, sensitivity remained unaltered by diabetes or

Table 2
Effects of 10 μM N^G-nitro-L-arginine (L-NNA) administration on relaxation responses of aorta to sodium nitroprusside

Group	*n*	Maximum relaxation (%)	+ L-NNA	$(-\log)EC_{50}$ (mol l^{-1})	+ L-NNA
Nondiabetic	8	98.7 ± 1.0	99.0 ± 0.6	8.02 ± 0.11	8.40 ± 0.12[a]
Diabetic	10	99.5 ± 0.3	98.0 ± 0.7	7.94 ± 0.08	8.02 ± 0.08
Diabetic + LY333531	9	99.6 ± 0.4	99.0 ± 1.1	8.11 ± 0.08	8.27 ± 0.10[b]

Data are mean ± S.E.M.
[a] $P<0.01$ vs. response prior to L-NNA administration.
[b] $P<0.05$ vs. response prior to L-NNA administration.

LY333531 treatment ((−log)EC_{50} approximately 7.9–8.1 for all groups). However, in the presence of 10 μM L-NNA, while there was no effect on diabetic control tissue responses to sodium nitroprusside, aortas from nondiabetic control and LY333531-treated diabetic mice had increased (−log)EC_{50} values of approximately 0.38 ($P<0.01$) and 0.16 ($P<0.05$) log units, respectively.

Maximum contraction to phenylephrine of diabetic aortas was increased by approximately 1.5-fold ($P<0.01$) compared to nondiabetic controls (Table 3). LY333531 treatment prevented this diabetic increase by approximately 48%, such that maximum tensions did not significantly differ from either diabetic or nondiabetic tissues. Similarly, sensitivity ((−log)EC_{50}) to phenylephrine of diabetic aortas was markedly increased by approximately 0.85 log units ($P<0.001$, Fig. 2); this was completely prevented by LY333531 treatment ($P<0.001$). Following preincubation with 10 μM L-NNA, there were increases in maximum contraction developed to phenylephrine across all groups. Maximum tensions were increased approximately 1.4-fold ($P<0.001$) for nondiabetic, 1.1-fold ($P<0.05$) for diabetic and 1.2-fold ($P<0.01$) for LY333531-treated diabetic aortas. Thus, while diabetes reduced the degree of L-NNA-dependent tension augmentation, LY333531 treatment resulted in a more control-like response. Sensitivity to phenylephrine was not significantly altered by L-NNA incubation in any group.

3.3. Corpus cavernosum study

Tissue weights did not significantly differ between groups (7.2 ± 0.3 mg, $n=13$, and 7.5 ± 0.3 mg, $n=12$, for nondiabetic and diabetic control, and 6.7 ± 0.4 mg, $n=10$, for LY333531 treated diabetic cavernosum, respectively).

Electrical stimulation of corpus cavernosum elicited frequency-dependent contractions (Fig. 3) that were abolished by incubation with 4 μM guanethidine (data not shown). Expressed relative to tissue weight, maximum contractions at 30 Hz did not significantly differ between groups (0.0636 ± 0.007 and 0.0590 ± 0.009 mN mg^{-1} for nondiabetic and diabetic control groups, and 0.0551 ± 0.009 mN mg^{-1} for treated tissue, respectively).

Table 3
Effects of 10 μM N^G-nitro-L-arginine (L-NNA) administration on contractile responses of aorta to phenylephrine

Group	n	Maximum contraction (mN)	+L-NNA	(−log)EC_{50} (mol l^{-1})	+L-NNA
Nondiabetic	10	8.82 ± 0.95	12.23 ± 0.83[a]	6.93 ± 0.13	7.14 ± 0.11
Diabetic	10	12.79 ± 0.69[b]	14.26 ± 0.68[c]	7.78 ± 0.11[d]	7.84 ± 0.12
Diabetic + LY333531	10	10.88 ± 0.70	13.11 ± 0.83[e]	6.97 ± 0.21	7.06 ± 0.17

Data presented as mean ± S.E.M.
[a] $P<0.001$ vs. response prior to L-NNA administration.
[b] $P<0.01$ vs. nondiabetic control.
[c] $P<0.05$ vs. response prior to L-NNA administration.
[d] $P<0.001$ vs. nondiabetic control.
[e] $P<0.01$ vs. response prior to L-NNA administration.

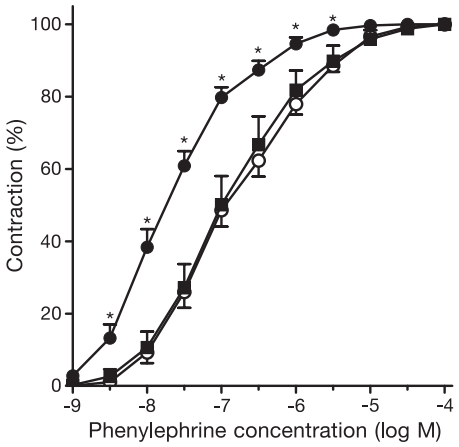

Fig. 2. Cumulative concentration–response curves for contraction to phenylephrine of aortas from nondiabetic and diabetic mice, and the effects of preventive LY333531 treatment. Groups: nondiabetic control (○, $n=10$); 4 week diabetic control (●, $n=10$); diabetic treated with 10 mg kg^{-1} day^{-1} LY333531 for 4 weeks from induction (■, $n=10$). Data are mean ± S.E.M. *$P<0.05$ vs. nondiabetic control and LY333531-treated diabetic groups. There were no significant differences between curves for nondiabetic control and LY333531-treated diabetic groups.

Electrical stimulation following phenylephrine precontraction in the presence of 1 μM atropine and 4 μM guanethidine produced frequency-dependent NANC nerve-mediated relaxation (Fig. 4); this was abolished by L-NNA preincubation (data not shown). Maximum NANC relaxation at 20 Hz was reduced by approximately 43% with diabetes compared to nondiabetic control tissues (42.5 ± 3.6% vs. 74.1 ± 4.7%, $P<0.001$). This deficit was 44% prevented by LY333531 treatment (56.3 ± 3.1%, $P<0.05$). However, NANC relaxa-

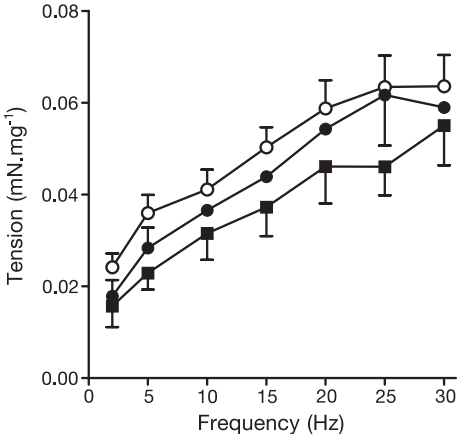

Fig. 3. Frequency–response curves for contraction to electrical field stimulation of corpus cavernosum from nondiabetic and diabetic mice, and the effects of preventive LY333531 treatment. Groups: nondiabetic control (○, $n=10$); 4 week diabetic control (●, $n=12$); diabetic treated with 10 mg kg^{-1} day^{-1} LY333531 for 4 weeks from induction (■, $n=7$). Data presented as mean ± S.E.M. There were no significant differences between curves for the three groups.

tion of treated cavernosum remained lower than the nondiabetic control value ($P<0.01$).

Maximum endothelium-dependent relaxation to acetylcholine (Fig. 5), following phenylephrine precontraction, was approximately 32% reduced by diabetes, compared to cavernosum from nondiabetic controls ($36.0 \pm 3.4\%$ vs. $53.1 \pm 3.0\%$, $P<0.01$). Treatment with LY333531 completely prevented the diabetic deficit ($56.9\% \pm 4.7$, $P<0.01$). Sensitivity to acetylcholine tended to decrease with diabetes; however, statistical significance was not attained. Thus, $(-\log)EC_{50}$ values were 7.07 ± 0.17 and 6.63 ± 0.11 for nondiabetic and diabetic control groups, and 6.93 ± 0.15 for treated cavernosum, respectively.

Endothelium-independent relaxation responses of cavernosum to sodium nitroprusside were not altered by diabetes or treatment. Thus, maximum relaxations were $50.1 \pm 3.9\%$, $n=10$, and $47.7 \pm 4.9\%$, $n=9$, for nondiabetic and diabetic controls, and $47.1 \pm 5.0\%$, $n=8$, for LY333531-treated tissues. $(-\log)EC_{50}$ values were 6.19 ± 0.14 and 5.97 ± 0.07 for nondiabetic and diabetic control groups, respectively, and 6.14 ± 0.12 for LY333531-treated cavernosum. L-NNA preincubation did not significantly alter sodium nitroprusside relaxation of either nondiabetic or diabetic control cavernosum (data not shown).

Contractile responses of corpus cavernosum to phenylephrine were not altered by diabetes or treatment. Thus, maximum tensions (mN mg^{-1}) were 0.086 ± 0.008, $n=11$, and 0.105 ± 0.011, $n=10$, for nondiabetic and diabetic control groups, and 0.084 ± 0.009, $n=6$, for LY333531-treated tissue, respectively. $(-\log)EC_{50}$ values were 6.33 ± 0.08 and 6.28 ± 0.06 for nondiabetic and diabetic control groups, and 6.18 ± 0.12 for LY333531-treated cavernosum. L-NNA preincubation did not significantly alter cavernosum contraction to phenylephrine (data not shown).

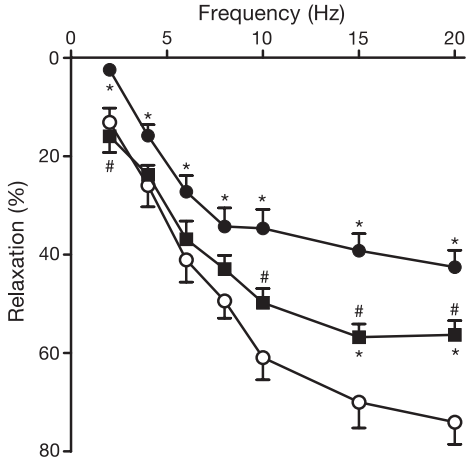

Fig. 4. Frequency–response curves for relaxation to electrical field stimulation in the presence of atropine and guanethidine, of corporus cavernosum from nondiabetic and diabetic mice, and the effects of preventive LY333531 treatment. Groups: nondiabetic control (○, $n=10$); 4 week diabetic control (●, $n=11$); diabetic treated with 10 mg kg^{-1} day^{-1} LY333531 for 4 weeks from induction (■, $n=8$). Data are mean ± S.E.M. *$P<0.05$ vs. nondiabetic control group; #$P<0.05$, effects of LY333531 treatment vs. the diabetic control group.

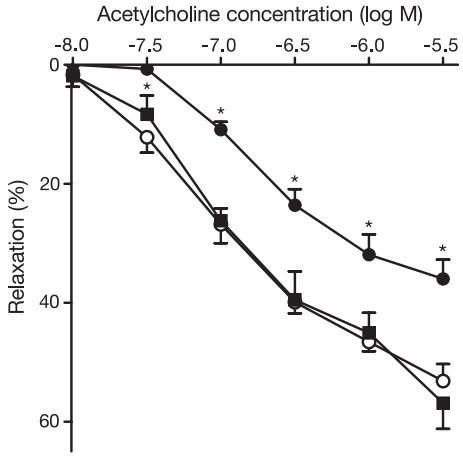

Fig. 5. Noncumulative concentration response curves for relaxation to acetylcholine of corpus cavernosum from nondiabetic and diabetic mice, and the effects of preventive LY333531 treatment. Groups: nondiabetic control (○, $n=10$); 4 week diabetic control (●, $n=9$); diabetic mice treated with 10 mg kg^{-1} day^{-1} LY333531 for 4 weeks from diabetes induction (■, $n=7$). Data are mean ± S.E.M. *$P<0.05$ vs. nondiabetic control and LY333531-treated diabetic groups. There were no significant differences between curves for nondiabetic control and LY333531-treated diabetic groups.

4. Discussion

In agreement with observations in nonmurine species (Durante et al., 1988; Azadzoi and Saenz de Tejada, 1992; Cameron and Cotter, 1992; Keegan et al., 1999), streptozotocin-diabetes attenuated nitric oxide-mediated endothelium-dependent relaxation to acetylcholine in mouse aorta and corpus cavernosum. Relaxations to the nitric oxide donor sodium nitroprusside were unaltered by diabetes in either tissue. This suggests that the dysfunction was at the level of the endothelium, as the ability of the smooth muscle to relax to nitric oxide was not compromised. In diabetic patients, endothelium-independent relaxation to nitric oxide donors is either unaltered (Johnstone et al., 1993) or depressed (McVeigh et al., 1992). The reason for the latter discrepancy with animal studies is not known, but likely reflects the progression and severity of vasculopathy.

In contrast to the present findings, a modest residual acetylcholine-mediated relaxation was demonstrated in the presence of nitric oxide synthase inhibition for mouse cavernosum (Gocmen et al., 1997). This may be due to an as yet unidentified agent such as endothelium-derived hyperpolarising factor. In the rat mesenteric vascular bed, diabetes suppresses both nitric oxide and endothelium-derived hyperpolarising factor-mediated vasodilatation (Cotter et al., 2002). In rabbit aorta, hyperglycaemia increases acetylcholine-stimulated cyclooxygenase-derived vasoconstrictor prostanoid production (Tesfamariam et al.,

1990). However, as nitric oxide synthase inhibition abolished aorta acetylcholine-mediated vasodilatation in this study, constrictor prostanoids did not contribute to the attenuated endothelium-dependent relaxation of diabetic mouse aorta.

This is the first study showing that chronic LY333531 treatment attenuates the development of nitric oxide-dependent endothelial dysfunction in cavernosum and aorta from diabetic mice. Similar effects of LY333531 were noted for both nitric oxide and endothelium-derived hyperpolarising factor deficits in diabetic rat mesenteric resistance vessels (Cotter et al., 2002). The amelioration of endothelium-dependent relaxation deficits in diabetic mice was greater for cavernosum than aorta, which could suggest that PKCβ activation is particularly relevant for microvascular complications.

Elevated PKC activity in the diabetic milieu occurs by several mechanisms; de novo synthesis of the activator, diacylglycerol, from glucose is increased (Koya and King, 1998). Increased oxidative stress in diabetes also stimulates PKC independent of hyperglycaemia, by liberating diacylglycerol from phospholipids (Numaguchi et al., 1996). Oxidative stress augments neurovascular endothelin-1 and angiotensin II systems and PKC activation is involved in their signalling pathways (Cameron et al., 2001; Touyz and Berry, 2002). Thus, these changes could also contribute to elevated vascular PKC activity in diabetes. Indeed, the balance of endothelin-1, angiotensin II and nitric oxide action has important regulatory roles in vascular function through complimentary interacting pathways. For example, endothelin-1 and angiotensin II act on endothelial cells to suppress nitric oxide-dependent vasodilation (Oriji and Keiser, 1996; Mollnau et al., 2002). Conversely, nitric oxide inhibits endothelin-1 induced PKC activation (Lang and Lewis, 1991). It is plausible, therefore, that the benefits of LY333531 on vasorelaxation could at least in part be due to inhibition of the signalling pathways for endothelin-1 and angiotensin II.

In aorta, diabetes caused a marked increase in contractile responses to the α_1-adrenoceptor agonist, phenylephrine. As LY333531 prevented the development of diabetes-like contractility in aorta, this suggests that the PKCβ isoform has a role in modulating smooth muscle contractility. Smooth muscle PKC activity is increased by diabetes and may cause phosphorylation of contractile proteins, which would augment phenylephrine responses (Inoguchi et al., 1992; Shimamoto et al., 1993). This may account for the actions of LY333531 on aorta. However, increased contractile responses were not observed in diabetic cavernosum, so such a mechanism may not be of universal vascular importance. In the rat mesenteric bed, diabetes did not enhance phenylephrine-induced contractions, and LY333531 was without effect on contractility (Cotter et al., 2002). Treatment with the lipid-lowering drug, rosuvastatin, prevented nitric oxide-dependent reductions of endothelium-dependent relaxation in aortas from diabetic mice, but was did not alter the enhanced contractile response to phenylephrine (Nangle, 2002). Therefore, the actions of LY333531 on aortic contractility in diabetic mice are unlikely to be caused by antagonism of phenylephrine-mediated contraction because of improved endothelial nitric oxide bioavailability. Rather, they likely reflect modulation of contractile stimulus–response signalling mechanisms.

Nitric oxide synthase inhibition with L-NNA augmented phenylephrine-mediated contraction of aorta to a greater extent in nondiabetic and LY333531-treated diabetic than in untreated diabetic mice. In addition, L-NNA increased sensitivity to sodium nitroprusside in aorta from nondiabetic and LY333531-treated diabetic mice, but not untreated diabetic controls. Thus, diabetes markedly diminished spontaneous as well as agonist-induced production or bioavailability of nitric oxide. The preventative effect of PKCβ inhibition may be via increased nitric oxide synthase expression and/or activation (Hirata et al., 1995; Ohara et al., 1995). Comparable effects were seen following rosuvastatin therapy (Nangle, 2002); statins can also increase nitric oxide synthase expression and activity (Laufs et al., 1998). However, similar findings following L-NNA incubation were not observed for cavernosum, suggesting that at least under the in vitro experimental conditions, basal nitric oxide release is minimal compared to aorta.

This study is the first to demonstrate that diabetic autonomic nitrergic nerve function is protected by PKCβ inhibition. Diabetic peripheral nerve blood flow and somatic motor and sensory conduction velocity deficits were largely corrected by LY333531 and nonspecific PKC inhibitor treatment in rats (Cameron et al., 1999; Cameron and Cotter, 2002; Cotter et al., 2002). In the latter case, these effects were blocked by co-treatment with a nitric oxide synthase inhibitor, emphasising the importance of a vascular action as opposed to a direct neuronal effect.

It is plausible that in addition to nitric oxide synthase in vessel endothelium, the neuronal isoform of nitric oxide synthase is upregulated in nerve by LY333531. However, unlike the case for vessels, peripheral nerve diacylglycerol and PKC activity are not elevated in diabetic rats or mice (Cameron et al., 1999; Yagihashi et al., 2001). Alternatively, improved nerve blood flow via inhibition of vascular PKCβ and prevention of nitric oxide-mediated endothelial dysfunction, rather than changes in nerve PKC activity, may account for the protection of cavernosum NANC nerve function in mice. As noted for nerve trunks (Cameron et al., 1991), autonomic ganglion blood flow shows an early and marked reduction with diabetes in rats (Cameron and Cotter, 2001). Thus, LY333531-induced elevation of major pelvic ganglion and pelvic nerve blood flow could indirectly protect NANC neuronal cell bodies and nerve fibres, hence function.

In summary, PKCβ inhibition has marked beneficial effects on aorta and corpus cavernosum agonist- and spontaneously induced nitric oxide-dependent endothelial function in diabetes. Furthermore, LY333531 protected against

nitric oxide-mediated NANC nerve dysfunction, which could have implications for the treatment of diabetic impotence. Treatment also prevented altered adrenergic contractility in diabetic mouse aorta. Improving vascular and neural and nitric oxide pathway function by PKCβ inhibition may prove a useful therapeutic approach for diabetic impotence and vasculopathy.

Acknowledgements

This work was supported by Juvenile Diabetes Research Foundation International grant no. 1-2000-45.

References

Andersson, K.E., Wagner, G., 1995. Physiology of penile erection. Physiol. Rev. 75, 191–236.

Azadzoi, K.M., Saenz de Tejada, I., 1992. Diabetes mellitus impairs neurogenic and endothelium-dependent relaxation of rabbit corpus cavernosum smooth muscle. J. Urol. 148, 1587–1591.

Bivalacqua, T.J., Champion, H.C., Hellstrom, W.J.G., Kadowitz, P.J., 2000. Pharmacotherapy for erectile dysfunction. Trends Pharmacol. Sci. 21, 484–489.

Cai, H., Harrison, D.G., 2000. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ. Res. 87, 840–844.

Cameron, N.E., Cotter, M.A., 1992. Impaired contraction and relaxation in aorta from streptozotocin-diabetic rats: role of the polyol pathway. Diabetologia 35, 1011–1019.

Cameron, N.E., Cotter, M.A., 2001. Diabetes causes an early reduction in autonomic ganglion blood flow in rats. J. Diabetes Its Complicat. 15, 198–202.

Cameron, N.E., Cotter, M.A., 2002. Effects of protein kinase Cβ inhibition on neurovascular dysfunction in diabetic rats: interaction with oxidative stress and essential fatty acid dysmetabolism. Diabetes Metab. Res. Rev. 18, 315–323.

Cameron, N.E., Cotter, M.A., Low, P.A., 1991. Nerve blood flow in early experimental diabetes in rats: relation to conduction deficits. Am. J. Physiol. 261, E1–E8.

Cameron, N.E., Cotter, M.A., Jack, A.M., Basso, M.D., Hohman, T.C., 1999. Protein kinase C effects on nerve function, perfusion and Na^+, K^+-ATPase activity and glutathione content in diabetic rats. Diabetologia 42, 1120–1130.

Cameron, N.E., Eaton, S.E.M., Cotter, M.A., Tesfaye, S., 2001. Vascular factors and metabolic interactions in the pathogenesis of diabetic neuropathy. Diabetologia 44, 1973–1988.

Cotter, M.A., Jack, A.M., Cameron, N.E., 2002. Effects of the protein kinase Cβ inhibitor LY333531 on neural and vascular function in rats with streptozotocin-induced diabetes. Clin. Sci. 103, 311–321.

Durante, W., Sen, A.K., Sunahara, F.A., 1988. Impairment of endothelium-dependent relaxation in aortae from spontaneously diabetic rats. Br. J. Pharmacol. 94, 463–468.

Gocmen, C., Ucar, P., Singirik, E., Dikmen, A., Baysal, F., 1997. An in vitro study of nonadrenergic–noncholinergic activity on the cavernous tissue of mouse. Urol. Res. 25, 269–275.

Gocmen, C., Secilmis, A., Kumcu, E.K., Ertug, P.U., Onder, S., Dikmen, A., Baysal, F., 2000. Effects of vitamin E and sodium selenate on neurogenic and endothelial relaxation of corpus cavernosum in the diabetic mouse. Eur. J. Pharmacol. 398, 93–98.

Hirata, K., Kuroda, R., Sakoda, T., Katayama, M., Inoue, N., Suematsu, M., Kawashima, S., Yokoyama, M., 1995. Inhibition of endothelial nitric oxide synthase activity by protein kinase C. Hypertension 25, 180–185.

Inoguchi, T., Battan, R., Handler, E., Sportsman, J.R., Heath, W., King, G.L., 1992. Preferential elevation of protein kinase C isoform βII and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycaemic control by islet cell transplantation. Proc. Natl. Acad. Sci. U. S. A. 89, 11059–11063.

Inoguchi, T., Pu, X., Kunisaki, M., Higashi, S., Feener, E.P., King, G.L., 1994. Insulin's effect on protein kinase C and diacylglycerol induced by diabetes and glucose in vascular tissues. Am. J. Physiol. 267, E369–E379.

Ishii, H., Jirousek, M.R., Koya, D., Takagi, C., Xia, P., Clermont, A., Bursell, S-E., Kern, T.S., Ballas, L.M., Heath, W.F., Stramm, L.E., Feener, E.P., King, G.L., 1996. Amelioration of vascular dysfunctions in diabetic rats by an oral PKC β inhibitor. Science 272, 728–731.

Jack, A., Cameron, N.E., Cotter, M.A., 1999. Effects of the diacylglycerol complexing agent, cremophor, on nerve conduction velocity and perfusion in diabetic rats. J. Diabetes Its Complicat. 13, 2–9.

Johnstone, M.T., Creager, B.S.N., Scales, K.M., Cusco, J.A., Lee, B.K., Creager, M.A., 1993. Impaired endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. Circulation 88, 2510–2516.

Keegan, A., Cotter, M.A., Cameron, N.E., 1999. Effects of diabetes and treatment with the antioxidant α-lipoic acid on endothelial and neurogenic responses of corpus cavernosum in rats. Diabetologia 42, 343–350.

Koya, D., King, G.L., 1998. Protein kinase C activation and the development of diabetic complications. Diabetes 47, 859–866.

Lang, D., Lewis, M.J., 1991. Endothelium-derived relaxing factor inhibits the endothelin-1-induced increase in protein kinase C activity in rat aorta. Br. J. Pharmacol. 104, 139–144.

Laufs, U., La Fata, V., Plutzky, J., Liao, J.K., 1998. Upregulation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors. Circulation 97, 1129–1135.

McVeigh, S.E., Brennan, G.M., Johnston, G.D., McDermott, B.J., McGrath, L.T., Henry, W.R., Andrews, J.W., Hayes, J.R., 1992. Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia 35, 771–776.

Mollnau, H., Wendt, M., Szocs, K., Lassegue, B., Schulz, E., Oelze, M., Li, H., Bodenschatz, M., August, M., Kleschyov, A.L., Tsilimingas, N., Walter, U., Forstermann, U., Meinertz, T., Griendling, K., Munzel, T., 2002. Effects of angiotensin II infusion on the expression and function of NAD(P)H oxidase and components of nitric oxide/cGMP signalling. Circ. Res. 90, E58–E65.

Mulvany, M.J., Halpern, W., 1976. Mechanical properties of smooth muscle cells in situ. Nature 260, 617–619.

Nakamura, J., Kato, K., Hamada, Y., Nakayama, M., Chaya, S., Nakashima, E., Naruse, K., Kasuya, Y., Mizubayashi, R., Miwa, K., Yasuda, Y., Kamiya, H., Ienaga, K., Sakakibara, F., Koh, N., Hotta, N., 1999. A protein kinase C-beta-selective inhibitor ameliorates neural dysfunction in streptozotocin-induced diabetic rats. Diabetes 48, 2090–2095.

Nangle, M.R., 2002. Aorta and corpus cavernosum dysfunction in diabetic rodents: effects of rosuvastatin and the role of nitric oxide. PhD thesis. University of Aberdeen, Aberdeen, Scotland, UK.

Numaguchi, K., Shimokawa, H., Nakaike, R., Egashira, K., Takashita, A., 1996. PKC inhibitors prevent endothelial dysfunction after myocardial ischemia–reperfusion. Am. J. Physiol. 270, H1634–H1639.

Ohara, Y., Sayegh, H.S., Yamin, J.J., Harrison, D.G., 1995. Regulation of endothelial constitutive nitric oxide synthase by protein kinase C. Hypertension 25, 415–420.

Oriji, G.K., Keiser, H.R., 1996. Action of protein kinase C in endothelin-induced contractions in rat aortic rings. Am. J. Physiol. 271, C398–C404.

Pieper, G.M., 1998. Review of alterations in endothelial nitric oxide production in diabetes: protective role of arginine on endothelial dysfunction. Hypertension 31, 1047–1060.

Piercy, V., Taylor, S.G., 1998. A comparison of spasmogenic and relaxant responses in aortae from C57/BL/KsJ diabetic mice with those from their non-diabetic litter mates. Pharmacology 56, 267–275.

Saenz de Tejada, I., Goldstein, I., Azadzoi, K.M., Krane, R.J., Cohen, R.A., 1989. Impaired neurogenic and endothelium-mediated relaxation of penile smooth muscle from diabetic men with impotence. N. Engl. J. Med. 320, 1025–1030.

Shimamoto, Y., Shimamoto, H., Kwan, C.Y., Daniel, E.E., 1993. Differential effects of putative protein kinase C inhibitors on contraction of rat aortic smooth muscle. Am. J. Physiol. 264, H1300–H1306.

Tesfamariam, B., Brown, M.L., Deykin, D., Cohen, R.A., 1990. Elevated glucose promotes generation of endothelium-derived vasoconstriction prostanoids in rabbit aorta. J. Clin. Invest. 85, 929–932.

Tesfamariam, B., Brown, M.L., Cohen, R.A., 1991. Elevated glucose impairs endothelium-dependent relaxation by activating protein kinase C. J. Clin. Invest. 87, 1643–1648.

Touyz, R.M., Berry, C., 2002. Recent advances in angiotensin II signalling. Braz. J. Med. Biol. Res. 35, 1001–1015.

Yagihashi, S., Yamagishi, S.I., Wada Ri, R., Baba, M., Hohman, T.C., Yabe-Nishimura, C., Kokai, Y., 2001. Neuropathy in diabetic mice overexpressing human aldose reductase and effects of aldose reductase inhibitor. Brain 124, 2448–2458.

Xia, P., Inoguchi, T., Kern, T.S., Engerman, R.L., Oates, P.J., King, G.L., 1994. Characterization of the mechanism for the chronic activation of diacylglycerol–protein kinase C pathway in diabetes and hypergalactosemia. Diabetes 43, 1122–1129.