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The calpain inhibitor, A-705253, corrects penile nitrergic nerve dysfunction in diabetic mice

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Abstract

Calpains, a superfamily of Ca^{2+}-activated proteases, are associated with an array of physiological and pathological events, including susceptibility to diabetes. Recently, increased calpain activity has been linked to reduced endothelium-derived nitric oxide-mediated vasodilatation in diabetes. However, a similar mechanism for neuronal-derived nitric oxide has not been examined. Thus, the aim was to investigate effects of the calpain inhibitor A-705253, *N*-(1-benzyl-2-carbamoyl-2-oxoethyl)-2-[E-2-(4-diethyl-aminomethylphenyl)ethen-1-yl]benzamide, on nitrergic neurovascular function in diabetic mice. Diabetes was induced by streptozotocin; duration was 6 weeks. Intervention A-705253 treatment (30 mg/kg/day) was given for 2 weeks following 4 weeks of untreated diabetes. After 6 weeks of diabetes, corpus cavernosa were isolated in organ baths for measurement of agonist- and electrical stimulation-evoked smooth muscle tensions. Adrenergic nerve- and phenylephrine-mediated contractions were not altered by diabetes or calpain inhibition. In contrast, maximum nitrergic nerve-mediated relaxation of phenylephrine-precontracted cavernosum was approximately 29% reduced by diabetes ($P<0.001$). This neurological deficit was 66% corrected by A-705253 treatment ($P<0.05$). Maximum nitric oxide-mediated endothelium-dependent relaxation to acetylcholine was attenuated approximately 39% by diabetes ($P<0.01$). Similarly, maximum endothelium-independent relaxation to the nitric oxide donor, sodium nitroprusside, was blunted approximately 23% by diabetes ($P<0.001$). A-705253 treatment partially improved endothelium-dependent relaxation to acetylcholine but had no effect on the deficit in response to nitroprusside. The data suggest that calpain contributes to the aetiology of diabetic nitrergic autonomic neuropathy and endothelial dysfunction, which may provide a novel therapeutic target for neurovascular complications.

Keywords: Calpain; Corpus cavernosum; Diabetes; Endothelium; Erectile dysfunction; Neuropathy; Nitric oxide; Smooth muscle

1. Introduction

The physiological roles of calpains (Ca^{2+}-activated non-lysosomal cysteine proteases) have not been comprehensively elucidated; they cleave a number of proteins that have been linked to important cellular events including proliferation, differentiation and apoptosis, thereby irreversibly modifying their function (Branca, 2004). In diabetes, increased calpain activity within rat mesenteric vasculature exposed to high glucose has been linked via deleterious effects on nitric oxide synthase activity, to reduced endothelial production of the potent vasodilator, nitric oxide (Stalker et al., 2003). More recently, these findings have been confirmed and extended in genetically obese diabetic rats (Stalker et al., 2005). Reduced production of nitric oxide in concert with its increased oxidative stress-driven degradation contributes to endothelial dysfunction in diabetes, augmenting vascular tone (Bayraktutan, 2002).

The vascular component of the aetiology of diabetic neuropathy is well established; endoneurial blood flow deficits accompany reductions in peripheral nerve functions including conduction velocity (Cameron et al., 2001). Penile erection involves nerve-mediated relaxation of corpus cavernosum vasculature and smooth muscle concurrent with restriction of venous outflow (Giuliano and Rampin, 2004). During the erectile process, sacral parasympathetic nerves release nitric oxide and acetylcholine as co-transmitters; cholinergic innervation of the endothelium stimulates nitric oxide release, providing

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a secondary source. Nitric oxide-dependent smooth muscle relaxation deficits in response to nerve stimulation or pharmacological agonism are evident in cavernosum from a number of diabetic animal models (Azadzoi and de Tejada, 1992; Gocmen et al., 2000; Keegan et al., 1999); similar findings are observed with erectile tissue from impotent diabetic men (de Tejada et al., 1989).

Activities of the endothelial and neuronal nitric oxide synthases (eNOS and nNOS) are positively regulated by the multiprotein heat shock protein 90 (hsp90) chaperone system (Bender et al., 1999; Garcia-Cardena et al., 1998). Recent findings suggest that increased calpain activity in diabetes augments dissociation of the hsp90-eNOS complex, reducing eNOS activity, hence vascular nitric oxide levels (Stalker et al., 2005). Moreover, increased hsp90-eNOS association can prevent high glucose-induced apoptosis in cultured human endothelial cells (Lin et al., 2005). However, effects of hyperglycaemia-induced calpain activity on a putative hsp90-nNOS linkage, and possible downstream effects on neuronal-derived nitric oxide-dependent function, have not been determined. Therefore, the aim was to examine the effects of a novel calpain inhibitor, A-705253, on neurovascular functions in streptozotocin-induced diabetic mouse corpus cavernosum.

2. Materials and methods

2.1. Animals

Male MF1 mice were purchased from Harlan (Bicester, Oxford, UK) at 3 months and were aged between 6 and 8 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 UK 'Animal (Scientific Procedures) Act, 1986'. Unless otherwise stated, chemicals were obtained from Sigma (Poole, Dorset, UK).

2.2. Diabetes induction, treatment and anaesthesia

Diabetes was induced by 125 mg/kg i.p. streptozotocin dissolved in sterile saline solution; duration was 6 weeks. Diabetic mice were weighed daily and were rejected if non-fasting blood glucose levels (Ascensia Esprit2 glucose meter, Bayer Diagnostics, Dublin, Ireland) of samples taken from the heart were less than 20 mmol/l on the day of experimentation. In addition to untreated nondiabetic and diabetic control groups, one group of diabetic mice received intervention treatment with A-705253 (*N*-(1-benzyl-2-carbamoyl-2-oxoethyl)-2-[E-2-(4-diethyl-aminomethylphenyl)ethen-1-yl]benzamide; Abbott GmbH, Ludwigshafen, Germany) at a dose of 30 mg/kg/day dissolved in water p.o. for 2 weeks, following 4 weeks of untreated diabetes. Similar dose levels of this drug have been shown to be neuro- and cardio-protective in ischaemic rodent models (Sandmann et al., 2002; Lubisch et al., 2003; Neuhof et al., 2004). Mice were anaesthetised by 5% isoflurane in air and 0.1 ml 10% urethane in 154 mmol/l saline per 10 g body weight i.p., and were exsanguinated following removal of the penis.

Table 1
Body weights and plasma glucose concentrations of nondiabetic and 6-week diabetic mice

Group	*n*	Body weight (g)		Plasma glucose (mmol l^{-1})
		Start	End	
Nondiabetic	13	47.5±1.4	–	11.9±0.7
Diabetic	14	47.5±0.8	42.3±1.0[a]	33.1±1.5[b]
Diabetic+A-705253	9	50.3±1.1	42.2±1.2[a]	30.6±1.9[b]

Data presented as mean±SEM.
[a] $P<0.001$ vs. start weight.
[b] $P<0.001$ vs. nondiabetic control.

2.3. Corpus cavernosum experiments

The penis was excised at its base and the glans penis and connective tissues surrounding the shaft were removed. The corpora cavernosum were then separated and each strip of tissue was mounted in a 10ml organ bath containing 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 mmol/l), maintained at 37 °C (pH 7.35) and gassed with a 95% O_2:5% CO_2 mixture. Resting tension, monitored by isometric transducers, was set at 0.5 g and tissues were equilibrated for 45 min with frequent changing of the bathing media. Cumulative concentration–response curves to the α_1-adrenoceptor agonist phenylephrine and endothelium-independent nitric oxide donor sodium nitroprusside (following an approximate 80% maximal phenylephrine precontraction) were established. Non-cumulative response-curves for endothelium-dependent relaxation to acetylcholine were also established. Transmural electrical field stimulation was delivered via platinum wire electrodes (duration 30s; 2–30 Hz; 5 ms pulses; 20 V). In the presence of the muscarinic antagonist atropine (1 μmol/l) and adrenergic neurone blocker guanethidine (3 μmol/l), non-adrenergic, non-cholinergic (NANC) transmitter mediated relaxations were achieved in response to electrical stimulation against phenylephrine-precontraction. In some experiments, tissues were preincubated with 10 μmol/l of the nitric oxide synthase inhibitor N^G-nitro-L-arginine (L-NNA). At the end of experiments, tissues were dabbed of excess solution and weighed.

2.4. Statistical analysis

Data are expressed as means±SEM. They were subjected to Bartlett's test for homogeneity of variances before one-way analysis of variance (ANOVA). Where significance was reached ($P<0.05$), between groups differences were established using the Newman-Keuls multiple comparison test. Otherwise, data were analysed by Kruskal-Wallis non-parametric one-way ANOVA and Dunn's multiple comparison test. Whole curve analyses of concentration–response data were carried out using 2-way ANOVA. Concentration–response curves were fitted by sigmoid curves using the least squares method to calculate EC_{50}. All calculations were determined by a standard statistical software package (Prism3, Graphpad, San Diego, CA, USA).

3. Results

3.1. Plasma glucose concentrations and body and corpus cavernosum weights

Six weeks of diabetes caused an approximate 3-fold increase in plasma glucose concentrations and reduced body weights by roughly 15% (Table 1); these changes were unaffected by intervention A-705253 treatment. However, corpus cavernosum tissue weights did not significantly differ between groups; thus, weights in mg were 10.2±0.4, $n=13$, and 10.2±0.2, $n=14$, for nondiabetic and diabetic control, and 10.3±0.3, $n=9$, for A-705253 treated diabetic cavernosum, respectively.

3.2. Corpus cavernosum responses

Electrical stimulation of corpus cavernosum elicited frequency-dependent guanethidine- and tetrodotoxin-sensitive (data not shown) contractions mediated by noradrenergic nerves. There were no significant differences between groups at frequencies ranging from 2 through 30 Hz; thus, maximum tensions (at 30 Hz) were 0.121±0.016 g, $n=12$, and 0.099±0.011 g, $n=14$, for nondiabetic and diabetic controls, and 0.144±0.016 g, $n=9$, for A-705253 treated diabetic tissue, respectively.

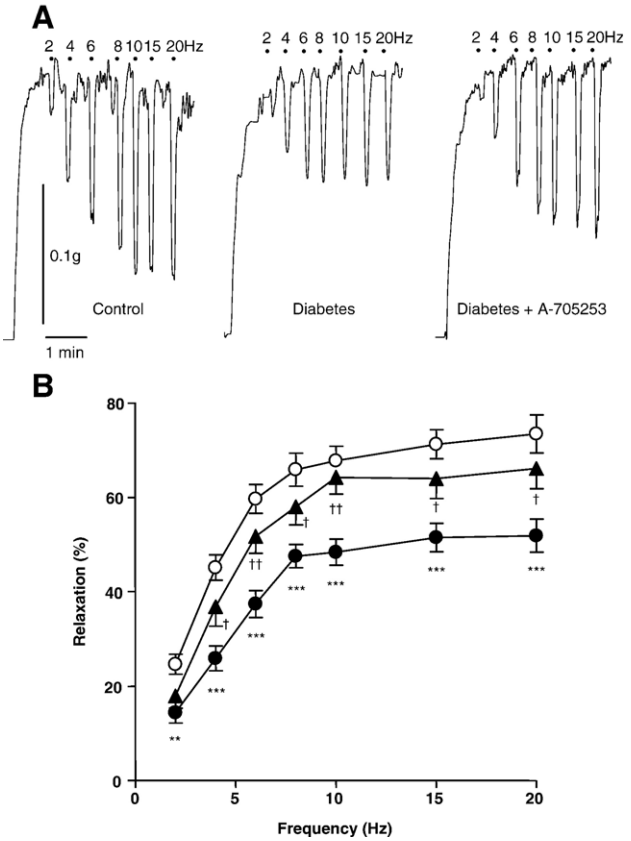

Fig. 1. (A, B) Frequency–response relations for relaxation, following phenylephrine precontraction in the presence of atropine and guanethidine, to electrical field stimulation of corpus cavernosum from nondiabetic and diabetic mice, and the effects of intervention A-705253 treatment. Typical traces (A), from nondiabetic, diabetic and A-705253-treated diabetic mice, for stimulation frequencies of 2–20 Hz; PE, phenylephrine. Frequency–response curves (B) for all groups: nondiabetic control (*open circles*, $n=12$); 6 week diabetic control (*closed circles*, $n=14$); diabetic treated with 30 mg/kg/day A-705253 for 2 weeks following 4 weeks of untreated diabetes (*closed triangles*, $n=9$). Data presented as mean±SEM. **, ***, $P<0.01$, $P<0.001$ vs. nondiabetic control group; †$P<0.05$, ††$P<0.01$, effects of A-705253 treatment vs. diabetic control group.

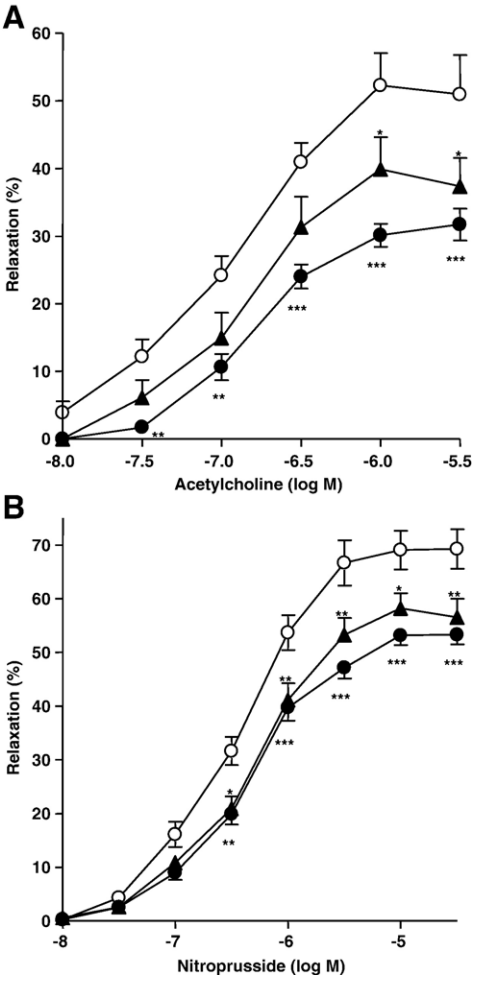

Fig. 2. (A, B) Concentration–response curves for relaxation, following phenylephrine precontraction, to acetylcholine (A) and sodium nitroprusside (B) of corpus cavernosum from nondiabetic and diabetic mice, and the effects of intervention A-705253 treatment. Groups: nondiabetic control (*open circles*: A, $n=13$; B, $n=12$); 6 week diabetic control (*closed circles*: A and B, $n=11$); diabetic treated with 30 mg/kg/day A-705253 for 2 weeks following 4 weeks untreated diabetes (*closed triangles*: A and B, $n=9$). Data presented as mean± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. nondiabetic control group.

Similarly, contractile responses to exogenous phenylephrine were not significantly altered by diabetes or A-705253 treatment. Thus, maximum tensions were 0.148±0.017 g, $n=12$, and 0.134±0.012 g, $n=10$, for nondiabetic and diabetic control groups, and 0.172±0.018 g, $n=9$, for A-705253 treated cavernosum, respectively. Sensitivity values [$(-\log)EC_{50}$] were 6.26±0.07 and 6.30±0.09 mol/l for nondiabetic and diabetic control groups, and 6.38±0.05 mol/l for treated diabetic cavernosum, respectively.

Electrical stimulation, following phenylephrine precontraction in the presence of 1 μmol/l atropine and 3 μmol/l guanethidine, produced L-NNA-sensitive (data not shown) frequency-

dependent relaxations mediated by nitrergic nerves (Fig. 1). Maximum relaxation (at 20 Hz) was reduced approximately 29% by untreated diabetes compared to nondiabetic control (51.9±3.5% vs. 73.5±4.0%, $P<0.001$). This deficit was 66% corrected by A-705253 treatment (66.1±4.3%, $P<0.05$), to the extent that responses did not significantly differ from nondiabetic controls.

Maximum endothelium-dependent relaxation to acetylcholine (Fig. 2A), following phenylephrine contraction, was reduced approximately 39% by diabetes compared to nondiabetic control (31.7±2.3% vs. 52.3±4.8%, $P<0.01$). A-705253 treatment failed to reverse the diabetic deficit; hence, the maximum response remaining depressed compared to nondiabetic control (39.9±4.7%, $P<0.05$). However, a trend towards improvement with A-705253 treatment compared to the diabetic control data, while not statistically significant for maximal relaxation or other individual data points, was apparent for whole curve analysis (two-way ANOVA; $P<0.001$). Sensitivity to acetylcholine was not significantly altered by diabetes or treatment; thus, (−log) EC_{50} values were 6.96±0.07 and 6.81±0.11 mol/l for nondiabetic and diabetic controls, and 6.93±0.11 mol/l for A-705253 treated diabetic cavernosum, respectively.

Following phenylephrine contraction, maximum endothelium-independent relaxation to sodium nitroprusside (Fig. 2B) was approximately 23% reduced by diabetes, compared to nondiabetic control (53.3±1.8% vs. 69.3±3.7%, $P<0.001$). A-705253 treatment failed to correct the diabetic deficit; hence, relaxation remained significantly attenuated compared to nondiabetic control (58.2±2.9%, $P<0.05$). Sensitivity was not significantly altered by diabetes or treatment; thus, (−log) EC_{50} values were 6.47±0.06 and 6.34±0.05 mol/l for nondiabetic and diabetic controls, and 6.31±0.06 mol/l for A-705253 treated diabetic cavernosum, respectively.

4. Discussion

As previously reported, streptozotocin-diabetes attenuated nitric oxide-mediated mouse corpus cavernosum smooth muscle relaxation in response to NANC-neurostimulation (Gocmen et al., 2000; Nangle et al., 2003). This deficit is present after 4 weeks of diabetes to a similar extent to that noted after 6 weeks (Nangle et al., 2003), therefore, the present study demonstrates that calpain inhibition can substantially reverse the diabetic deficit, restoring neuronal nitric oxide production sufficiently to preserve nitrergic nerve function. This could implicate hyperglycaemia-induced calpain activity in the dissociation of the hsp90-nNOS complex, as demonstrated for hsp90-eNOS binding (Stalker et al., 2003, 2005).

In penile tissue from streptozotocin-diabetic rats, nNOS expression within nitrergic nerves is selectively and gradually lost until eventual apoptosis of cell bodies within the pelvic ganglia nullifies the effects of restoration of glycaemic control by insulin treatment (Cellek et al., 2003). The process begins distally, suggesting possible reduction in axonal transport of the nNOS protein to the nerve terminal, with nNOS levels halved as early as 4 weeks. If a similar mechanism occurs in diabetic mice, the beneficial effects of A-705253 treatment started at 4-weeks might be explained by a compensatory increase in hsp90-nNOS association, and therefore, nitric oxide production, particularly as glucose levels remained unaltered by treatment. Calpain-mediated proteolysis of nNOS has been observed in cultured hippocampal neurons exposed to neurotoxic stimuli, and possibly contributes to the reduced survival noted in these cells (Araujo et al., 2004). Furthermore, calpain inhibition can improve motoneuron survival under similar conditions, and partially prevents loss of muscle function following a sciatic nerve crush injury (Kieran and Greensmith, 2004).

Vascular integrity contributes to the preservation of nerve function in diabetes, including conduction velocity (Cameron et al., 2001). In addition, early and pronounced reductions in autonomic ganglion blood flow are observed in diabetic rats (Cameron and Cotter, 2001). Therefore, it is not inconceivable that A-705253 treatment might have promoted nitrergic neuronal cell body survival and possibly axonal transport of nNOS in the major pelvic ganglia, by beneficial effects on nutritive vascular supply. Increased bioavailability of eNOS-derived nitric oxide in vascular tissue can augment vasodilator function in diabetes (Bayraktutan, 2002). However, despite benefits of calpain inhibition in prevention of myocardial infarction in rabbits and on nitric oxide-dependent function in diabetic mesenteric vasculature (Neuhof et al., 2004; Stalker et al., 2005), chronic A-705253 treatment only partially reverse vascular dysfunction in the present study. However, this does not necessarily preclude positive effects of A-705253 on eNOS activity in the diabetic state. Thus, improved responsiveness to cholinergic agonism may have been masked by reduced responsiveness of smooth muscle to nitric oxide, evidenced by reduced endothelium-independent relaxation to the nitric oxide donor, sodium nitroprusside, in this experimental model. Calpain inhibition did not improve responses to nitroprusside. Taken together, the results suggest that the endothelium is the target for eNOS changes in diabetes, and that calpain changes do not have functional proteolytic relevance for the nitric oxide-guanylyl cyclase-cyclic guanosine monophosphate smooth muscle dilator pathway.

In the majority of studies, penile smooth muscle relaxation to nitroprusside is unaltered by experimental diabetes (Gocmen et al., 2000; Keegan et al., 1999); however, decreased responsiveness has been reported (Thompson et al., 2001). Of interest, whilst 6 weeks of diabetes does not alter penile smooth muscle responsiveness to nitric oxide in the C57 mouse model (Nangle et al., 2003), streptozotocin-MF1 mice have previously been shown to develop reduced nitroprusside sensitivity over a similar time period (Nangle et al., 2004). If smooth muscle insensitivity is indeed masking increased eNOS functioning in diabetic mice treated with A-705253, then calpain inhibition in an experimental model such as the C57 mouse could reveal improved endothelial functioning under similar experimental conditions. Overall, the severity and progression of vascular damage in diabetes, possibly in association with elevated plasma lipids, may explain differences in responsiveness to nitric oxide donors in man (van Etten et al., 2002).

A further possible mechanism to explain the incomplete correction of endothelium-dependent vasodilation by calpain inhibition relates to the fact that nitric oxide can be rapidly scavenged by increased free radicals such as the superoxide anion

in diabetes (Bayraktutan, 2002). This process may continue unabated in mice treated with calpain inhibitor, disguising any benefits of increased eNOS activity. In association, levels of superoxide dismutase, the catabolic enzyme for superoxide, along with endogenous antioxidants such as vitamin E, glutathione and α-lipoic acid, are decreased in diabetes (Bayraktutan, 2002; Brownlee, 2001) and calpain inhibition may not be relevant to their restoration. The reversal of nitrergic nerve dysfunction by A-705253 treatment despite reduced smooth muscle sensitivity to nitric oxide is of particular interest. Nitric oxide-dependent roles of the endothelium may be more susceptible to, and thereby may even precede, hyperglycaemic and oxidative damage within nitrergic nerves. Indeed, endothelial dysfunction is thought be a causative factor for many nerve dysfunctions in diabetes (Cameron et al., 2001). Hsp90 augments constitutive NOS activity by promoting Ca^{2+}/calmodulin binding (Song et al., 2001). In diabetes, basal intracellular Ca^{2+} concentrations are, at least modestly, increased in most tissues (Levy et al., 1994). Therefore, as both calpains and constitutive NOS are Ca^{2+}-activated, a scenario of increased calpain activity-driven hsp90-NOS dissociation may, at least partially, account for the reduced eNOS and nNOS-mediated functions observed in diabetes.

Interestingly, mutations in the calpain 10 gene (*CAPN10*) have been linked to the development of type II (non-insulin-dependent) diabetes (Horikawa et al., 2000). Although this association has been observed in Mexican Americans, Finns and Germans, populations such as Samoans and Japanese do not seem to develop this genetic susceptibility (Cox et al., 2004; Harris et al., 2004); highlighting the complex interplay between genetics and environmental factors in diabetes. In Caucasians from the United Kingdom, *CAPN10* polymorphism confers an increased susceptibility to type II diabetes that has been linked to enhanced hyperaemia and decreased resistance of the microvasculature (Shore et al., 2002). However, the direct role of calpains, including calpain 10, in the development and progression of diabetic complications are less well characterised. Murine pancreatic islets and muscle strips exposed to calpain inhibitors increase insulin secretion in response to glucose and develop reduced insulin-mediated glucose transport, respectively, implicating calpain protease activity in the regulation of glucose metabolism (Sreenan et al., 2001). Despite this possibility, A-705253 treatment did not significantly alter glycaemic control in the present study.

In summary, diabetic mice treated with the novel calpain inhibitor, A-705253, recovered corpus cavernosum nitrergic nerve function and partially recovered endothelium-dependent relaxation despite reduced smooth muscle sensitivity to nitric oxide. These data implicate hyperglycaemia-driven calpain activity in the dysfunction of neuronal and endothelial nitric oxide synthase systems, and suggest that calpain inhibition may be a novel therapeutic target for nitric oxide mediated neurovascular function, at least for patients with diabetic impotence.

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