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RESEARCH PAPER

Phosphatidylinositol 3-kinase-δ up-regulates L-type Ca^{2+} currents and increases vascular contractility in a mouse model of type 1 diabetes

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BACKGROUND AND PURPOSE

Vasculopathies represent the main cause of morbidity and mortality in diabetes. Vascular malfunctioning in diabetes is associated with abnormal vasoconstriction and Ca^{2+} handling by smooth muscle cells (SMC). Phosphatidylinositol 3-kinases (PI3K) are key mediators of insulin action and have been shown to modulate the function of voltage-dependent L-type Ca^{2+} channels ($Ca_V1.2$). In the present work, we investigated the involvement of PI3K signalling in regulating Ca^{2+} current through $Ca_V1.2$ ($I_{Ca,L}$) and vascular dysfunction in a mouse model of type I diabetes.

EXPERIMENTAL APPROACH

Changes in isometric tension were recorded on myograph. Ca^{2+} currents in freshly dissociated mice aortic SMCs were measured using the whole-cell patch-clamp technique. Antisense techniques were used to knock-down the PI3Kδ isoform.

KEY RESULTS

Contractile responses to phenylephrine and KCl were strongly enhanced in diabetic aorta independent of a functional endothelium. The magnitude of phenylephrine-induced $I_{Ca,L}$ was also greatly augmented. PI3Kδ expression, but not PI3Kα, PI3Kβ, PI3Kγ, was increased in diabetic aortas and treatment of vessels with a selective PI3Kδ inhibitor normalized $I_{Ca,L}$ and contractile response of diabetic vessels. Moreover, knock-down of PI3Kδ *in vivo* decreased PI3Kδ expression and normalized $I_{Ca,L}$ and contractile response of diabetic vessels *ex vivo*.

CONCLUSIONS AND IMPLICATIONS

Phosphatidylinositol 3-kinase δ was essential to the increased vascular contractile response in our model of type I diabetes. PI3Kδ signalling was up-regulated and most likely accounted for the increased $I_{Ca,L}$, leading to increased vascular contractility. Blockade of PI3Kδ may represent a novel therapeutic approach to treat vascular dysfunction in diabetic patients.

LINKED ARTICLE

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Abbreviations

$[Ca^{2+}]_i$, intracellular calcium concentration; AS-ODNs, antisense oligodeoxynucleotides; $Ca_V1.2$, voltage-dependent L-type Ca^{2+} channels; GPCR, G protein-coupled receptor; $I_{Ca,L}$, Ca^{2+} current through voltage-dependent L-type Ca^{2+} channel; MM-ODNs, mismatch oligodeoxynucleotides; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; SMCs, smooth muscle cells; VSMCs, vascular smooth muscle cells

Introduction

Abnormal vasoconstriction plays an important role in the pathophysiology of diabetes (Piercy and Taylor, 1998; Mayhan *et al.*, 1999; Lagaud *et al.*, 2001; Okon *et al.*, 2003) and greatly contributes to increased morbidity and mortality in patients with this disease. The contraction of vascular smooth muscle cells (VSMC) is tightly coupled to increases in the intracellular calcium concentration ($[Ca^{2+}]_i$). In smooth muscle, voltage-dependent L-type Ca^{2+} channels ($Ca_V1.2$; nomenclature follows Alexander *et al.*, 2009) represent the major pathway for Ca^{2+} entry and play a crucial role in excitation-contraction coupling (Jackson, 2000) and their activity is modulated by signal transduction mechanisms in VSMC (McDonald *et al.*, 1994). Phosphatidylinositol 3-kinases (PI3K) are a family of ubiquitously expressed enzymes, which possess both lipid and protein kinase activities (Anderson *et al.*, 1999). Class I PI3Ks are enzymes that selectively phosphorylate the 3′-OH position of the inositol in phosphatidylinositol 4,5-bisphosphate (PIP2). Class I PI3Ks have been subdivided further according to their structure and mode of activation by cell surface receptors. The Ia subgroup consists of p110α, p110β and p110δ catalytic subunits associated with a p85 adapter subunit to form a heterodimeric complex (Cantrell, 2000). All three isoforms of this class are activated by the binding of specific phospho-tyrosyl motifs to the two SH2 domains of the regulatory subunits. Class Ib PI3K is composed of the p110γ catalytic subunit associated with a p101 regulatory protein. PI3Kγ is stimulated by Gβγ heterodimers released after G protein-coupled receptor (GPCR) activation. The convenient classification of class I PI3K isoforms according to their mechanism of activation has been challenged by evidence that PI3Kβ can also be activated by GPCRs (Maier *et al.*, 1999; Murga *et al.*, 2000). PI3Ks have been implicated in the modulation of vascular smooth muscle contractility (Kawanabea *et al.*, 2004; Kim *et al.*, 2006; Morello *et al.*, 2009). However, there is still limited information on the role of individual PI3Ks in the control of vascular functions. It is known that class Ia and class Ib PI3Ks modulate $Ca_V1.2$ channnels and increase Ca^{2+} current (Macrez *et al.*, 2001; Le Blanc *et al.*, 2004). Angiotensin II activates Ca^{2+} entry by stimulating L-type Ca^{2+} channels through Gβγ-sensitive PI3Kγ in portal vein myocytes (Quignard *et al.*, 2001). Interestingly, PI3Kδ has been reported as being involved in the increased basal tonus in aorta and mesenteric arteries from hypertensive rats (Northcott *et al.*, 2002; 2004; 2005).

Because abnormal vasoconstriction is one of the many vascular complications of diabetes we hypothesized that PI3Kδ could be involved in the vascular dysfunction present in diabetes. In this study, we bring new insights on the role and signalling pathways involving PI3Kδ in vascular function and disease. Here we provide evidence for the first time that: (i) PI3Kδ participates in the control of vascular contractile response elicited by α_1-adrenoceptor stimulation; (ii) PI3Kδ levels are increased in a model of type I diabetes; and (iii) stimulation of a GPCR increases Ca^{2+} current through $Ca_V1.2$ channels ($I_{Ca,L}$) in a PI3Kδ-dependent manner, and accounts for the increased vascular contractility in type I diabetes.

Methods

Animals and induction of diabetes

All animal care and experimental protocols complied with guidelines for the humane use of laboratory animals and were approved by the animal ethics committee of the Federal University of Minas Gerais (protocol # 26/2007). We used male C57BL/6J mice. Diabetes was induced in 3-week-old mice by a single intraperitoneal injection of 120 $mg \cdot kg^{-1}$ streptozotocin, freshly dissolved in 10 $mmol \cdot L^{-1}$ citrate buffer, pH 4.5–5. Control mice were treated identically with vehicle alone. Mice that did not become diabetic within 3–4 days were excluded from the study. Experiments were started when mice had been hyperglycaemic for 9 weeks. The diabetic state was defined as a plasma glucose concentration exceeding 19.0 $mmol \cdot L^{-1}$ the day of the experiment. Average body weight was 20.94 ± 1.0 g and 26.97 ± 0.5 g for diabetic and control animals, respectively; and daily food intake was 5.7 ± 0.5 and 4.5 ± 0.08 (g per mouse), for diabetic and control animals respectively.

Organ chamber experiments

Rings from the thoracic aorta were obtained and set up as previously described (Rabelo *et al.*, 2003). Vessels that responded to 10 $\mu mol \cdot L^{-1}$ acetylcholine (ACh), with greater relaxation than 80% were considered as containing a functional endothelium. When necessary, the endothelium was removed by intraluminal perfusion with 0.5% CHAPS for 20 s followed by repeated washings. Phenylephrine or KCl was added in increasing cumulative concentrations. After 60 min washing, the vessels were pretreated with different drugs for 30 min and a second cumulative concentration–response curve for phenylephrine was constructed and compared with the

first one. Mechanical activity was recorded isometrically, as previously described (Lemos *et al.*, 2002).

Freshly dissociated smooth muscle cells preparation

Smooth muscle cells (SMCs) from mice aorta were enzymically dissociated (Murakami *et al.*, 2003). Thereafter, the tissue was mechanically dispersed; three to five drops were plated onto glass coverslips containing PSS and the SMCs left for at least 20 min to adhere before the measurements of Ca^{2+} currents.

Whole-cell patch-clamp recording

$Ca_V1.2$ currents were isolated by eliminating K^+ currents by inclusion of Cs^+ and tetra-ethyl ammonium (TEA) in the patch pipette and Cs^+ in the bath solution. Patch pipettes were filled with (in mmol·L^{-1}): 130 CsCl, 10 TEA-Cl, 10 EGTA, 4 $MgCl_2$, 4 ATPMg and 10 HEPES, pH 7.2 with CsOH. The external solution contained (in mmol·L^{-1}): 130 CsCl, 20 $BaCl_2$, 0.5 $MgCl_2$, 10 HEPES and 5 glucose, pH 7.4 with CsOH, and osmolarity of 270–300 mOsmol·L^{-1}. Currents through $Ca_V1.2$ channels were evaluated using Ba^{2+} as charge carrier and further stimulated with Bay-K8644 (1 μmol·L^{-1}) or phenylephrine (1 μmol·L^{-1}) to increase signal to noise ratio. The currents were generated by 100 ms steps to 10 mV from a holding potential of –80 mV every 15 s. All inhibitors were applied acutely during current recordings for 2.5 min, the time necessary to reach steady state conditions. Data were collected after the whole-cell configuration was obtained and current amplitude had stabilized.

Western blot analysis

Western blot was performed as previously described (Capettini *et al.*, 2008). We used the following antibodies: mouse monoclonal IgG1 anti-PI3K p110δ (1:1000), goat polyclonal anti-p110α (1:200), rabbit polyclonal anti-p110β (1:200), rabbit polyclonal anti-p110γ (1:200) or rabbit polyclonal anti-actin (1:3000) at room temperature. All antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

In vivo *antisense oligonucleotides*

To silence the PI3Kδ isoform we used *in vivo* antisense oligodeoxynucleotides (AS-ODNs) (Capettini *et al.*, 2008). The 21-base phosphorothioated AS-ODNs were constructed based on the PI3K p110δ mouse sequence (5′-TAG.GCA.CCT.GCA.Gat.GTA.CTG-3′; GenBank access number NM-008840.2 purchased from Eurogentech North America Inc. (Fremont, CA, USA). The phosphorothioated mismatch oligodeoxynucleotides (MM-ODNs) sequence with the composition 5′-CCT.TCG.TAC.CCT.TTT.TCC-3′, was used as control ODNs. Male C57BL/6J mice received 2 nmol·L^{-1} AS-ODNs or MM-ODNs in the penile vein 12 h before the experiments (Capettini *et al.*, 2008).

Histological procedure and staining

Aorta rings from control and diabetic animals were fixed in formalin (10% w/v) in isotonic saline. Sections (5 μm) were stained and processed for light microscopic studies and morphometric analysis. Haematoxylin and eosin was used for determining the tissue area. In order to quantify the vessel wall thickness, four fields of each aorta were measured and images obtained with a panapochromatic objective ×40 in light microscopy (final magnification = ×100). The images were digitized through a JVC TK-1270/JGB microcamera and transferred to an analyser (Carl Zeiss- KS300 version 2, Kontron Electronics GmbH, Eiching bei Munchen, Germany).

Statistical analysis

Data were analysed by the use of GraphPad Prism© software (GraphPad Software Inc., USA) or SigmaPlot (SPSS Inc., USA) and are expressed as mean ± SEM. Two-way ANOVA with Bonferroni multiple comparisons post-test was used to compare concentration–response curves obtained in aortic rings and in Western blot experiments. Student's *t*-test was used on other experiments. The Δ area under the curves (AUC) was calculated as the difference between the contractile responses in the presence or in the absence of different drugs in control or diabetic mouse aortic rings. The relative % Δ AUC is the percentage of the Δ AUC correlated to the total area (in the absence of the drug). All statistical analyses were considered significant when $P < 0.05$.

Materials

Acetylcholine, Bay-K8644, bosentan, calphostin C, ibuprofen, L-NAME, LY294002, phenylephrine, streptozotocin, TEA and wortmannin were supplied by Sigma Aldrich (St Louis, MO, USA); IC87114 was from ICOS corporation (Bothell, WA, USA) and indomethacin from Calbiochem (San Diego, CA, USA).

Results

Vasoconstrictor response is increased in aorta of diabetic mice

Relaxation induced by ACh was not different between groups (Figure 1A) suggesting no endothelial dysfunction. Conversely, contractile responses to phenylephrine in diabetic mice were dramatically higher in either endothelium-containing or denuded

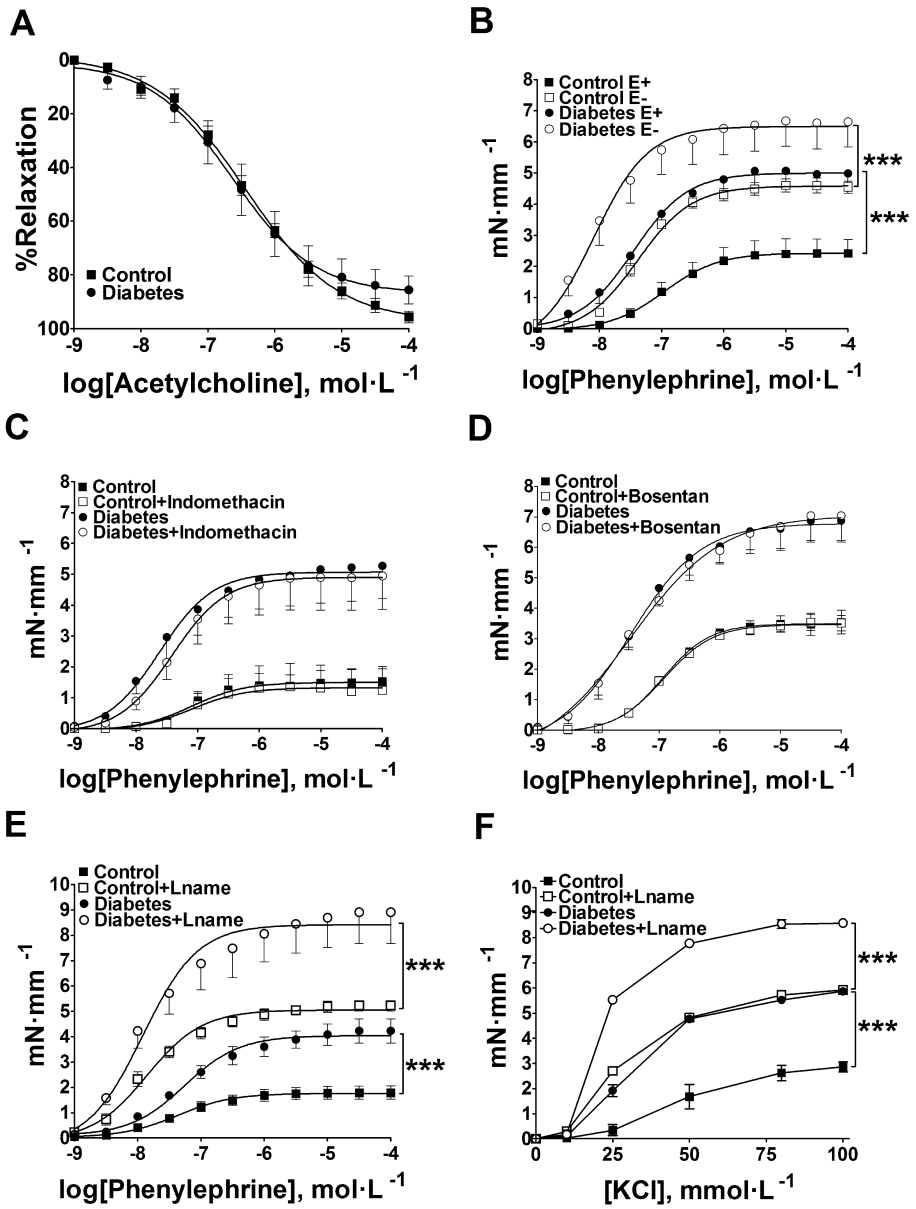

Figure 1

Concentration–response curves to acetylcholine (ACh), phenylephrine and KCl obtained in aortic rings from normoglycaemic (control) or diabetic mice. The data are represented as mean ± SEM. (A) ACh-induced relaxation in diabetic and control vessels with functional endothelium ($n = 8$). (B) Contractile responses to phenylephrine in endothelium-containing (E+) or denuded (E–) aortic rings ($n = 5$); ***$P < 0.001$. (C) Concentration–response curves to phenylephrine in endothelium-containing vessels with or without indomethacin ($n = 5$) or (D) bosentan ($n = 4$). Concentration–response curves to phenylephrine (E) and KCl (F) in endothelium-containing vessels L-NAME ($n = 4$). ***$P < 0.001$.

vessels (Figure 1B), making the participation of endothelium-derived contractile factors in this increased vasoconstriction unlikely. Consistent with these results, two cyclooxygenase inhibitors, indomethacin (10 μmol·L^{-1}; Figure 1C) and Ibuprofen (10 μmol·L^{-1}; data not shown), and the antagonist of endothelin receptors, bosentan (10 μmol·L^{-1}; Figure 1D), did not restore contractile responses in diabetic vessels to control levels. In an attempt to evaluate whether contractile dysfunction could be linked to an alteration in sensitivity or increased expression of α_1-adrenoceptors, we used KCl as vasoconstrictor. Our results show that contractile response to KCl was also equally enhanced in vessels from diabetic mice (Figure 1E). In the presence of L-NAME (300 μmol·L^{-1}), a nitric oxide synthase inhibitor, contractile responses to KCl and phenylephrine (Figure 1E,F) remained bigger in diabetic arteries compared with controls. Together, the above results point to a probable dysfunction in the aortic SMCs.

Morphological analysis

At this point it was interesting to analyse the possibility of morphological modifications in the structure of the vessel wall. Morphometrical analysis of sections showed decrease in the thickness and number of cells in the aorta of diabetic mice compared with the control group (Figure 2). Thus, these morphological alterations are not compatible with the greater vasoconstrictor response observed in aortas from diabetic mice.

Enhanced Ca^{2+} current through L-type voltage-dependent Ca^{2+} channels in diabetic smooth muscle cells

KCl-induced contraction has been shown to result in Ca^{2+} influx through $Ca_V1.2$ channels. To determine whether diabetes was associated with changes in L-type Ca^{2+} channels, we assessed whole-cell $I_{Ca,L}$ measured in freshly dissociated aortic vascular myocytes. Consistent with the enhanced aortic contraction induced by KCl depolarization, diabetes resulted in higher density of Ca^{2+} channel currents (by 96%, at 10 mV, in the presence of Bay K8644). Figure 3A shows the current density–voltage relationship. The voltage at which 50% of channel population was activated (V_{50}) was −14.24 ± 1.8 mV for diabetic and −11.20 ± 3.9 mV for control cells respectively ($P = 0.47$; $n = 9$). The slope factor was not different for diabetic (6.33 ± 0.91) and control (6.21 ± 1.59) cells ($n = 9$). Moreover, phenylephrine induced a large increase in current density (Figure 3A–C). The Ca^{2+} currents were abolished by nifedipine, confirming that Ca^{2+} entry was mainly through $Ca_V1.2$ channels (data not shown). No obvious differences in the kinetics of activation or inactivation were detected between control and diabetic SMCs. The cell capacitance measured in control and diabetic myocytes were 6.6 ± 0.3 pF ($n = 37$) and 6.0 ± 0.3 pF ($n = 32$), respectively, suggesting that there was no change in cell size induced by diabetes.

Mechanism involved in the augmentation of L-type Ca^{2+} current and contractile response

PI3K has been reported to modulate vascular contractility and $Ca_V1.2$ channels in SMCs (Le Blanc *et al.*, 2004). LY294002 (20 μmol·L^{-1}), an inhibitor

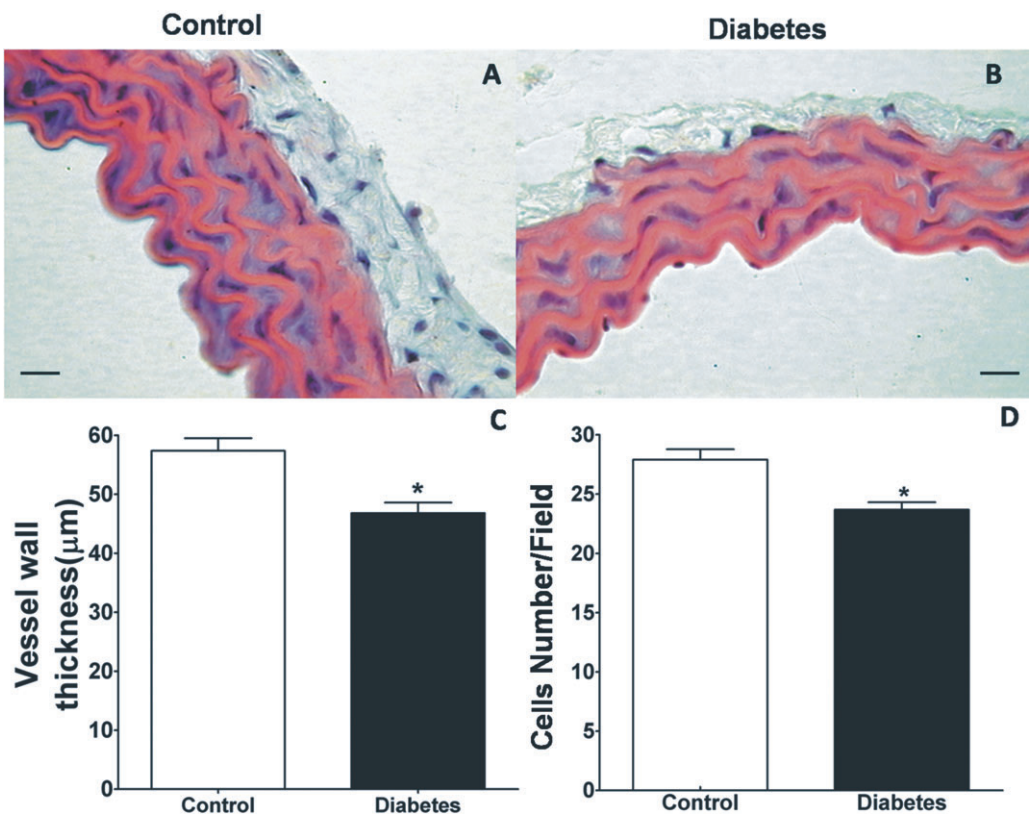

Figure 2

Morphometrical analyses in the structure of the vessel wall from normoglycaemic (control) or diabetic mice. Representative H&E-stained sections from (A) control and (B) diabetic mice aorta. (C) Average differences in vessel wall thickness ($n = 7$) and (D) cell numbers per field ($n = 8$) between control and diabetic mice. The data are represented as mean ± SEM; *$P < 0.05$. Scale bar: 10 μm.

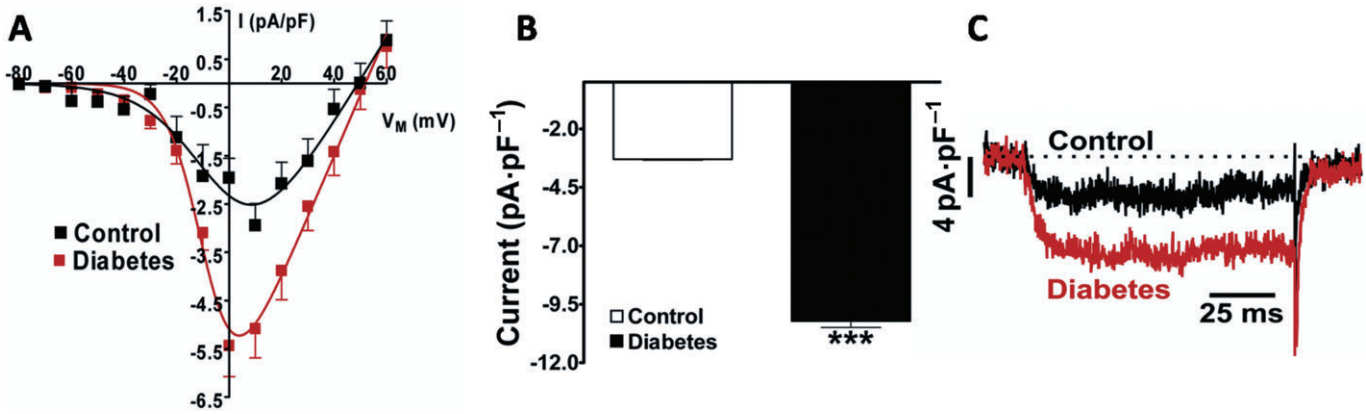

Figure 3

Vascular smooth muscle cells' L-type calcium current from normoglycaemic or diabetic mice. Panel (A) shows current density–voltage relationships in the presence of Bay-K8644 (control, $n = 9$ and diabetic, $n = 9$). (B) Average peak Ca^{2+} current density ($I_{Ca,L}$) ± SEM for control ($n = 20$) and diabetic ($n = 10$) mice. Here the vascular myocytes were stimulated with phenylephrine at 1 μM. (C) Representative original records of $I_{Ca,L}$ in the presence of phenylephrine. ***$P < 0.001$. $I_{Ca,L}$, Ca^{2+} current through voltage-dependent L-type Ca^{2+} channel.

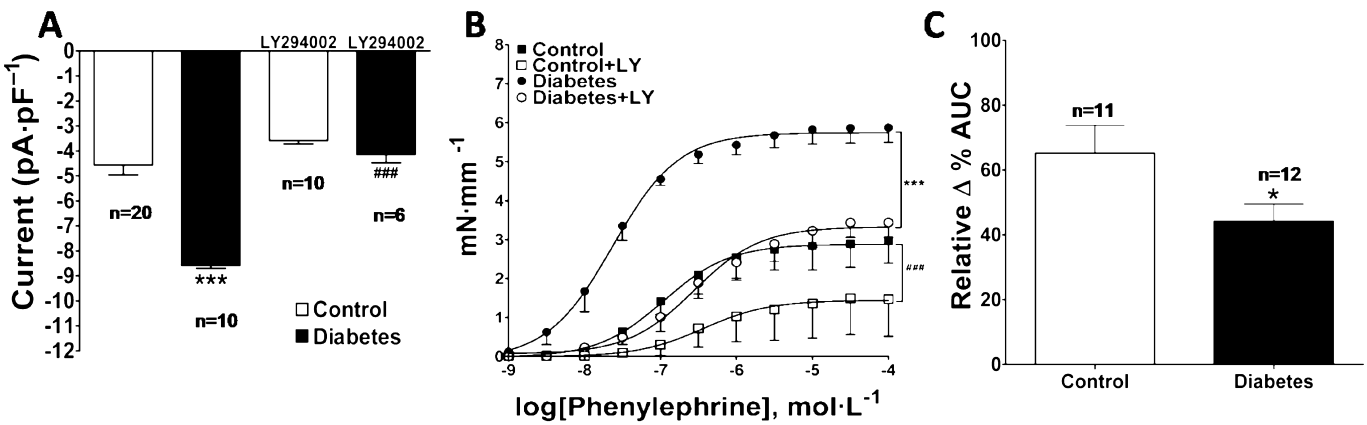

Figure 4

Effect of LY294002 on phenylephrine-induced $I_{Ca,L}$ and contractile responses in normoglycaemic (control) and diabetic mice. The data are represented as mean ± SEM. Effect of LY294002 (20 μmol·L^{-1}) on (A) $I_{Ca,L}$ induced by phenylephrine (1 μmol·L^{-1}) in aortic SMC; control versus diabetic ***$P < 0.001$, diabetic versus diabetic+LY294002 $^{###}P < 0.001$ and on (B) phenylephrine-induced contractile responses in aortic rings. (C) Inhibitory effect of LY294002 represented by the relative Δ % area under the curves shown in (B); *$P < 0.05$. $I_{Ca,L}$, Ca^{2+} current through voltage-dependent L-type Ca^{2+} channel.

that acts on all PI3K isoforms, prevented the phenylephrine-induced enhancement of $I_{Ca,L}$ in diabetic aorta SMCs (Figure 4A). LY294002 (20 μmol·L^{-1}) also decreased phenylephrine-induced contraction in diabetic animals. However, the decrease in contraction was greater in controls, as shown by the relative Δ % AUC (Figure 4B,C). We repeated the same experiment but using wortmannin (0.3 μmol·L^{-1}), another inhibitor that acts on all PI3K isoforms, with similar results (Figure 5).

Western blot analyses of aortic homogenates revealed the presence of PI3Kδ in control animals and increased expression of PI3Kδ was found in diabetic mice aorta (Figure 6A). PI3Kα was also expressed but in very low levels and no increase in expression was seen in diabetic animals (Figure 6B). Traces of PI3Kβ and PI3Kγ were also detected, and their expression was significantly decreased in diabetic aortas (Figure 6C,D).

To determine whether the PI3Kδ isoform is required for the unique response to phenylephrine in diabetic vascular myocytes, IC87114 (10 μmol·L^{-1}) was used to selectively inhibit PI3Kδ (Sadhu *et al.*, 2003a,b); Interestingly, PI3Kδ inhibition did prevent phenylephrine-induced enhancement of $I_{Ca,L}$ in diabetic vascular myocytes (Figure 7A) and normalized contractile response in the diabetic aorta (Figure 7B). These data suggest an

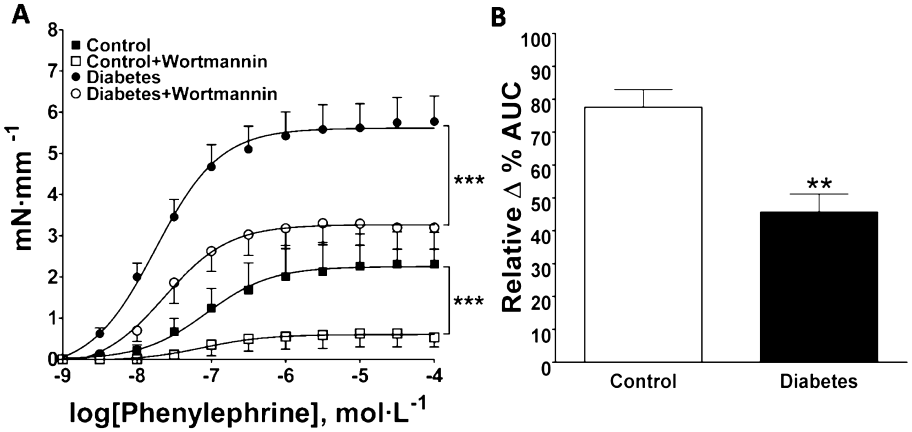

Figure 5

Effect of wortmannin on phenylephrine-induced contraction in aortic rings from normoglycaemic (control) and diabetic mice. The data are represented as mean ± SEM. (A) Concentration–response curves to phenylephrine with or without wortmannin (0.3 μmol·L^{-1}; $n = 6$). (B) Inhibitory effect of wortmannin represented by the relative Δ % area under the curves shown in (A); $^{**}P < 0.01$; $^{***}P < 0.001$.

important role of PI3Kδ in the pathophysiology of vascular disease in diabetes.

To further support the hypothesis above, we specifically knocked down PI3Kδ expression by using AS-ODNs *in vivo*. The efficiency of the AS-ODNs to block expression of PI3Kδ isoform was evaluated by functional experiments in the organ bath and patch-clamp and by Western blot analysis. In diabetic vessels, there was decrease in $I_{Ca,L}$ (Figure 7C) and contraction (Figure 7D) induced by α_1-adrenoceptor stimulation after PI3Kδ knock-down to levels similar to those observed with pharmacological inhibition with IC87114 (Figure 7A,B). MM-ODNs were used as controls and had no significant effect (Figure 7C,D). The lowest level of PI3Kδ expression in the mouse aorta (~86% reduction in control and 90% in diabetic vessels) was achieved 12 h after the AS-ODNs injection while MM-ODNs had no effect (Figure 7E). AS-ODNs knock-down of PI3Kδ did not affect expression of either PI3Kα, PI3Kβ and PI3Kγ (Figure 8). The increase in KCl-elicited vessel contraction (Figure 9A) and activation of voltage-dependent Ca^{2+} current (Figure 9B–D) in diabetic mice were also prevented by knocking down PI3Kδ. Taken together, our data provide compelling evidence that PI3Kδ signalling is up-regulated in diabetic aortic vessels and that its modulation clearly affects vascular function in our model of type 1 diabetes.

Protein kinase C (PKC) is well recognized to be involved on the control of SMCs contractility through modulation of the activity of $Ca_V1.2$ channels (Crozatier, 2006). Therefore, we also evaluated the involvement of PKC in the abnormal vasoconstriction of the diabetic mouse aorta. However, calphostin C (10 μmol·L^{-1}), a selective inhibitor of PKC, did not modify the contractile response to phenylephrine either in control or in aorta of diabetic mice (Figure 10), while it inhibited contractions induced by phorbol ester (data not shown). These results suggest that the conventional and novel isoforms of PKC are not involved in the increased contractility of the aorta of diabetic mice.

Discussion

The major finding of this work is that the δ isoform of PI3K is specifically involved in the signal transduction pathway leading to increased Ca^{2+} currents through $Ca_V1.2$ in VSMCs and accounts for the increased vascular contractility in our mouse model of type I diabetes, elicited by stimulation of α_1-adrenoceptors.

Macro- and microvascular disease conditions currently represent the principal causes of morbidity and mortality in patients with type I or type II diabetes mellitus (Nuzum and Pharm, 2009; Orasanu and Plutzky, 2009). Altered vascular contractility and impaired endothelium-dependent vasodilation have been demonstrated in various vascular beds in different animal models of diabetes and in humans with type I or type II diabetes (Kamata *et al.*, 1988; Matsumoto *et al.*, 2004). We show in this study that the responses of aortic rings from diabetic mice, to phenylephrine and KCl were about three times greater. Aortic pulse-wave velocity has been shown to be associated with cardiovascular risk (Blacher *et al.*, 1999). Moreover, aortic stiffness has been shown as an independent predictor of all-cause and cardiovascular mortality (Laurent *et al.*,

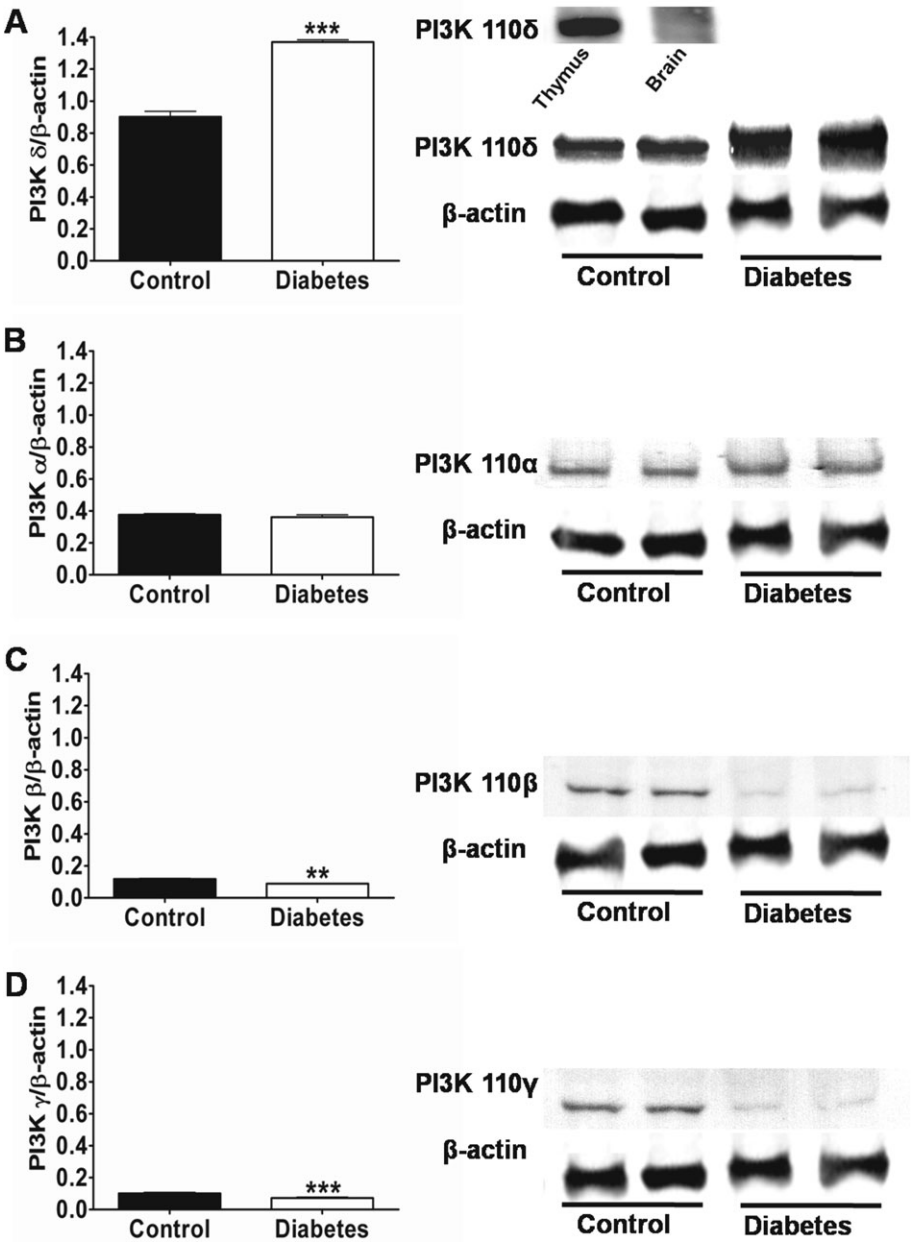

Figure 6

Western blot analysis of PI3Ks isoforms in aortas from normoglycaemic (control) and diabetic mice. On the left are shown the mean ± SEM values of three different experiments, and on the right representative gels. Results were normalized by β-actin content in samples. (A) PI3Kδ; (B) PI3Kα; (C) PI3Kβ and (D) PI3Kγ ***$P < 0.001$ and **$P < 0.01$. PI3K, phosphatidylinositol 3-kinase.

2001). Therefore, the comprehension of the mechanism(s) involved in increased contractility of aorta is crucial.

Increased arterial contractility in diabetes may be secondary to an abnormal response of smooth muscle (Kamata *et al.*, 1988; Taylor *et al.*, 1994) or endothelial dysfunction (Pieper *et al.*, 1992; 1997). In this work we have evaluated the role of the endothelium in many ways and show that: (i) vasodilation in response to ACh was not impaired in diabetic aorta; and (ii) removal of the endothelial layer, inhibition of prostaglandin synthesis with indomethacin or ibuprofen, inhibition of nitric oxide synthase with L-NAME or blockade of endothelin-1 receptors with bosentan did not normalize contraction of diabetic vessel to the level found in controls. Altogether these results suggest that the endothelium did not play a major role in mediating increased vascular contractility in our model of diabetes. A considerable body of evidence

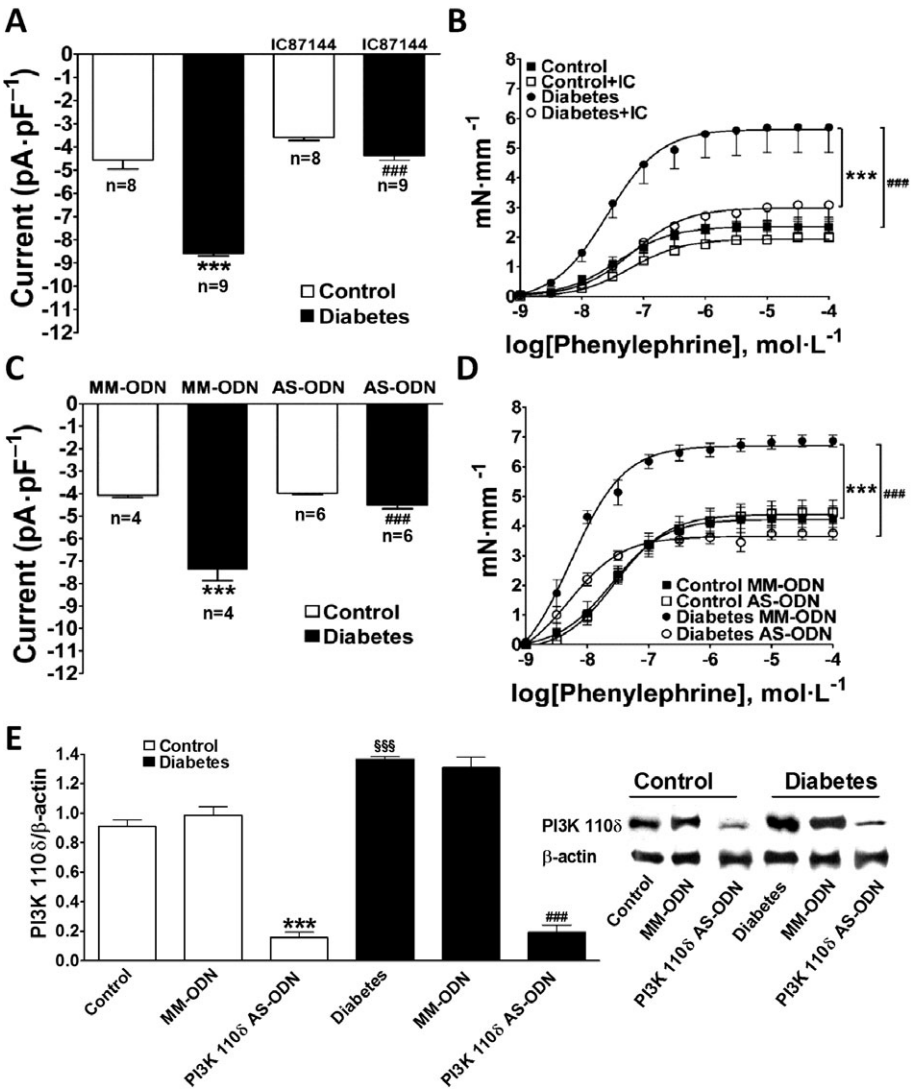

Figure 7

Effect of selective inhibition of the PI3Kδ isoform with IC87114 or antisense knock-down (AS-ODNs) on $I_{Ca,L}$ and contractile responses from normoglycaemic (control) and diabetic mice. The data represents mean ± SEM. (A) Effect of IC87114 on $I_{Ca,L}$ induced by phenylephrine in VSMC. Control versus diabetic $^{***}P < 0.001$, diabetic versus diabetic + IC87114 $^{###}P < 0.001$. (B) Effect of IC87114 on contractile responses to phenylephrine in endothelium-denuded aortic rings. Diabetes versus diabetes+IC87114 $^{***}P < 0.001$, control versus diabetes $^{###}P < 0.001$. (C) Effect of AS-ODN and its mismatch oligonucleotide (MM-ODN) on $I_{Ca,L}$ induced by phenylephrine in VSMC. Control MM-ODN versus diabetic MM-ODN $^{***}P < 0.001$, diabetic MM-ODN versus diabetic AS-ODN $^{###}P < 0.001$. (D) Effect of AS-ODN and MM-ODN on contractile responses to phenylephrine in aortic rings. Control MM-ODN versus diabetic MM-ODN $^{***}P < 0.001$, diabetic MM-ODN versus diabetic AS-ODN $^{###}P < 0.001$. (E) Western blot analysis of the efficiency of AS-ODN to block the expression of the PI3kδ isoform in aortas from control and diabetic mice. Control versus diabetic $^{§§§}P < 0.001$, control MM-ODN versus control AS-ODN $^{***}P < 0.001$ and diabetic MM-ODN versus diabetic AS-ODN $^{###}P < 0.001$. AS-ODNs, antisense oligodeoxynucleotides; $I_{Ca,L}$, Ca^{2+} current through voltage-dependent L-type Ca^{2+} channel; MM-ODNs, mismatch oligodeoxynucleotides; PI3K, phosphatidylinositol 3-kinase; VSMC, vascular smooth muscle cell.

indicates a decreased endothelium-dependent relaxation in diabetes (Hink *et al.*, 2001; Ding *et al.*, 2005). However, our results agree with other reports that have shown unaltered or even augmented endothelium-dependent relaxation in diabetes (Brands and Fitzgerald, 1998; Pieper, 1999; Kobayashi *et al.*, 2005). Moreover, some clinical data seem to be in agreement with these observations (Jaap and Tooke, 1995; Cipolla *et al.*, 1996). Possible explanations for these apparent discrepancies may be related to time-dependent changes in endothelial function (Kobayashi and Kamata, 1999; Kobayashi *et al.*, 2005), the vascular bed studied and model and severity of diabetes (De Vriese *et al.*, 2000; Kobayashi *et al.*, 2005).

An alternative explanation for the increased contractile response lies on an enhanced function of VSMC. The possibility that the morphological

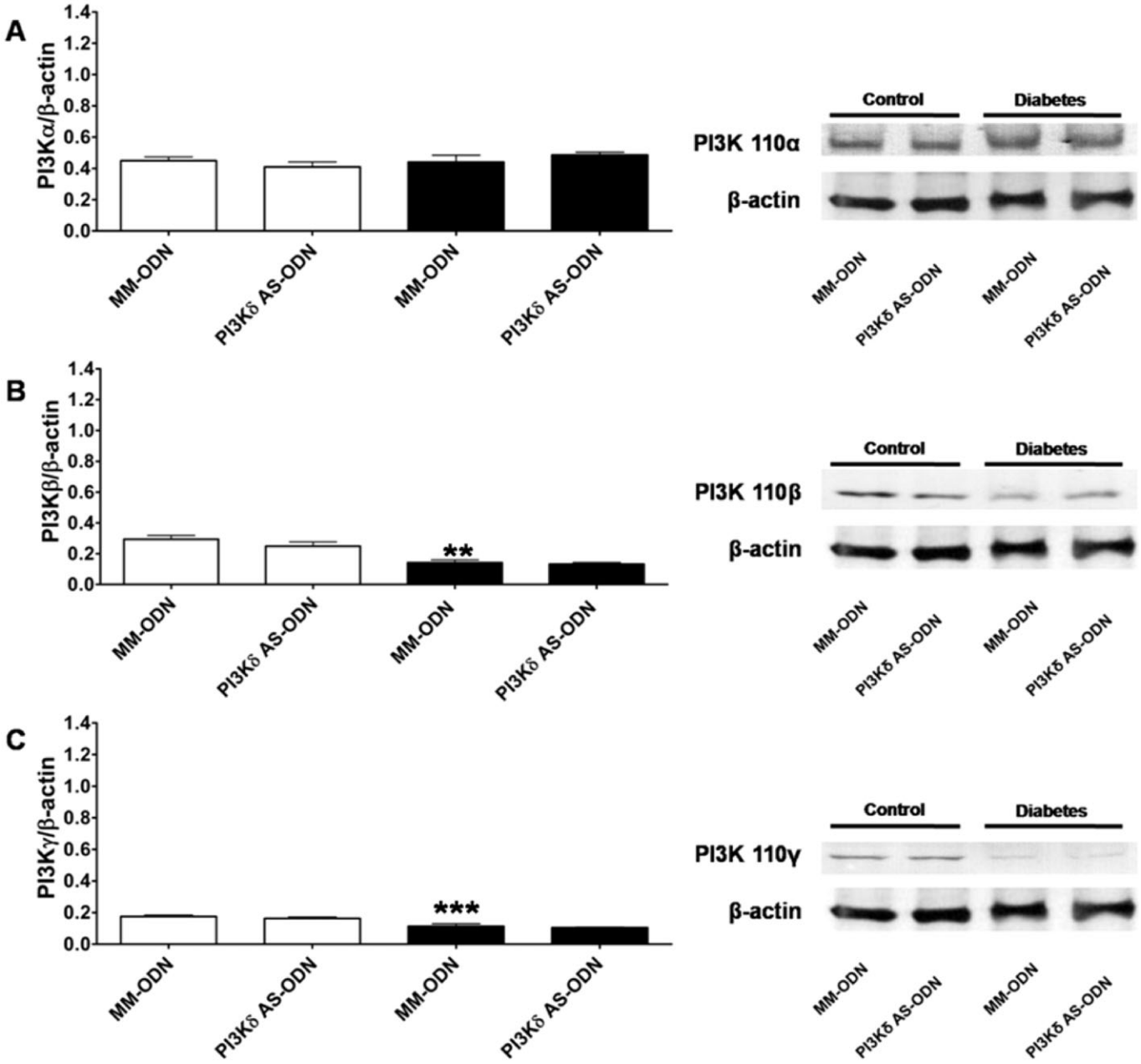

Figure 8

Western blot analysis of the effect of antisense knock-down (AS-ODNs) of PI3Kδ on the expression of PI3Kα (A), PI3Kβ (B) and PI3Kγ (C) isoforms in aortas from normoglycaemic (control) and diabetic mice. On the left are shown the mean ± SEM values of three different experiments, and on the right representative gels. Results were normalized by β-actin content in samples. Control MM-ODN versus diabetic MM-ODN ***$P < 0.001$, **$P < 0.01$. AS-ODNs, antisense oligodeoxynucleotides; MM-ODNs, mismatch oligodeoxynucleotides; PI3K, phosphatidylinositol 3-kinase.

alterations in diabetic vascular tissue could be responsible for its increased vasoconstriction is unlikely, as the decrease in the vessel wall thickness and in the cell number observed here did not support this hypothesis.

A growing body of evidence indicates that the PI3K signalling pathway mediates agonist-induced contraction (Vecchione *et al.*, 2005) and increase in calcium influx through $Ca_V1.2$ channels (Blair and Marshall, 1997; Viard *et al.*, 1999; 2004; Macrez *et al.*, 2001). Therefore, a series of experiments was undertaken to determine the role of PI3K and $Ca_V1.2$ channels on increased contractility in diabetes. Two inhibitors of PI3K, LY294002 and wortmannin produced a decrease in contraction in diabetic vessels and the selective inhibitor of the p110δ catalytic subunit of PI3K, IC87114, normalized contraction in aortic rings from diabetic mice suggesting an

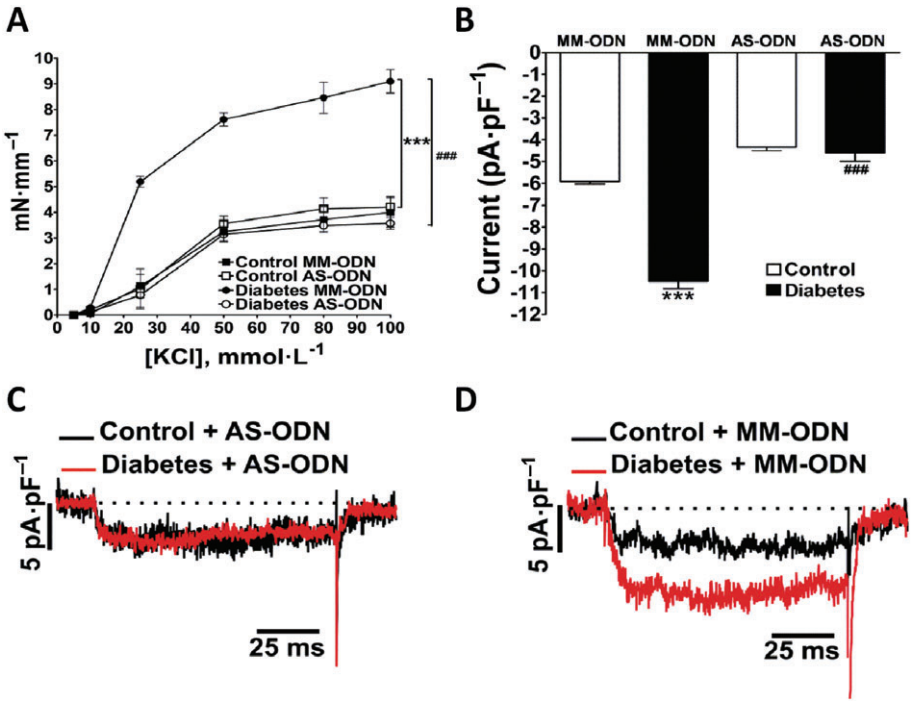

Figure 9

Effect of antisense oligonucleotides (AS-ODN) knock-down of PI3Kδ on KCl-induced contractions and on activation of $I_{Ca,L}$ in the presence of Bay-K8644. PI3Kδ AS-ODN knock-down normalized (A) contractile responses to KCl in diabetic aortic rings ($n = 7$) and (B, C) $I_{Ca,L}$ in smooth muscle cells in the presence of Bay-K8644 (1 μmol·L^{-1}; $n = 3$). (B) Represents the average peak Ca^{2+} current density ($n = 5$), and (C, D) representative traces. Control mismatch oligonucleotide (MM-ODN) did not have any effect (A, B, D). In (A) diabetic MM-ODN versus control MM-ODN ***$P < 0.001$ and diabetic MM-ODN versus diabetic AS-ODN $^{###}P < 0.001$. In (B) control MM-ODN versus diabetic MM-ODN ***$P < 0.001$ and diabetic MM-ODN versus diabetic AS-ODN $^{###}P < 0.001$. $I_{Ca,L}$, Ca^{2+} current through voltage-dependent L-type Ca^{2+} channel; PI3K, phosphatidylinositol 3-kinase.

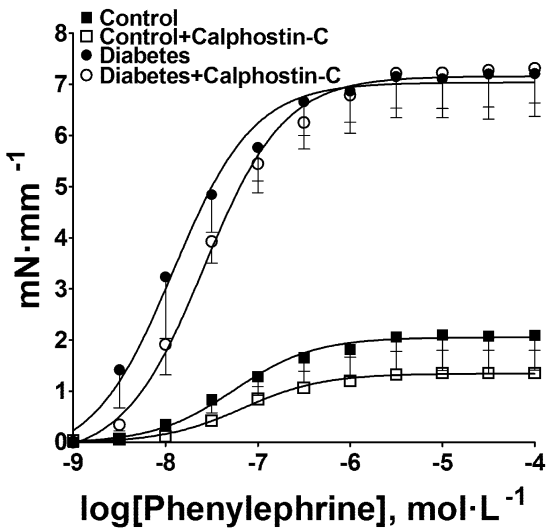

Figure 10

Effect of calphostin C (10 μmol·L^{-1}) on contractile response to phenylephrine in aortas from control and diabetic mice. The data are means (±SEM) of five experiments.

involvement of PI3Kδ in the increased contractility. These data were further corroborated by antisense specific knock-down of p110δ, which reduced the contractile responses in diabetic aortas following stimulation with phenylephrine, to the level of those observed in control vessels. Taken together, these data clearly show that the PI3Kδ isoform was implicated in the increased vasoconstriction found in diabetic aortic rings.

Knock-down of p110δ also normalized contraction to KCl in diabetic vessels. As KCl-induced contraction of aorta is mainly the result of Ca^{2+} influx through $Ca_V1.2$ channels we next evaluated the role of PI3K in regulating these channels. We used freshly dissociated mice aortic SMC to avoid adaptations of cells to culture conditions and modification in the pattern of PI3K isoforms expression, as reported in previous work (Macrez *et al.*, 2001; Vecchione *et al.*, 2005). Peak Ca^{2+} current density was higher in SMCs from diabetic animals. The increase in $I_{Ca,L}$ was seen in cells stimulated with phenylephrine or Bay-K8644, as well. The Ca^{2+} signal is the primary determinant of the contraction of the vascular smooth muscle (Akata, 2007) and is in good

agreement with the increased contractility found in diabetic vessels, in despite the small reduction (~15%) of the number of cells.

A previous report showed that increased PI3Kδ activity leads to increased L-type Ca^{2+} channel activity in rat portal vein myocytes (Macrez *et al.*, 2001). We fond here that LY294002, IC87114 and knockdown of p110δ normalized $I_{Ca,L}$ in SMC from diabetic aorta to the level found in control SMC. Altogether, these results clearly indicated that PI3Kδ was responsible for the increased Ca^{2+} channel current and the consequently increased contractility in our model of diabetes.

The increased Ca^{2+} influx through $Ca_V1.2$ channels showed in this work are in agreement with data from the literature showing higher Ca^{2+} influx in cerebral artery myocytes during acute hyperglycaemia and in the dB/dB mouse (Navedo *et al.*, 2010). However, our results are in disagreement with the reported decrease in Ca^{2+} influx observed in coronary smooth muscle from alloxan-induced diabetic Yucatan pigs (Witczak *et al.*, 2006). The discrepancy between our results and theirs may well be due to the different species and vascular beds studied.

One mechanism by which PI3Kδ activity may be increased in diabetes is by enhancing protein expression. Thus, we used Western blot analyses to measure the expression of the catalytic subunits of the class I PI3K family and found that the main isoform present was p110δ with very small amounts of p110α, p110β and p110γ in control animals. However, an increased expression of one particular isoform of the catalytic subunit of PI3K, p110δ, in diabetic animals has been reported. Northcott *et al.* (2002; 2004; 2005) showed p110δ expression to be increased in aortas from L-NNA, DOCA-salt and spontaneously hypertensive rats and to play a role in the increased spontaneous tone found in the aorta of these models of hypertension. The reasons for a decrease in expression of p110β and p110γ isoforms found in our study are not apparent and deserve further investigation. However, our data clearly indicate that these PI3K isoforms are not involved in the increased $I_{Ca,L}$ and vasoconstriction found in our model of type I diabetes.

Notably, non-selective inhibition of PI3K with LY294002 and wortmannin was more effective in inhibiting contraction in control than in diabetic arteries, while PI3Kδ inhibition was only effective in diabetic animals. These results are consistent with the increased expression of PI3Kδ and fall in β and γ isoforms found in diabetic animals and suggest that different PI3K isoforms regulate contraction in normal and diabetic arteries. Another important point to consider is that LY294002 and wortmannin were much more effective in inhibiting contraction than $Ca_V1.2$ channels in control animals. These results suggest that an additional mechanism independent of $Ca_V1.2$ channels and of PI3Kδ would be contributing to the inhibition of the contraction. It is already known that activation of the Rho/Rho kinase pathway by PI3K leads to an increased level of myosin light chain phosphorylation and consequent vasoconstriction (Miao *et al.*, 2002; Wang *et al.*, 2006). In view of these considerations, it interesting to speculate that in control vessels, another isoform of PI3K could be contributing to the inhibition of the contraction via Rho activation.

It should be noted that the metabolic state of our animals may not completely reflect the pathogenesis of diabetic vascular complications in humans, as type 1 diabetic humans receiving insulin, have either normal or elevated body weight (Chaturvedi *et al.*, 1995). However, it is also important to consider that often, symptoms of diabetes are not severe, or may be absent, and consequently hyperglycaemia of sufficient degree to cause pathological and functional changes may be present for a long time before the diagnosis is made (Alberti and Zimmet, 1998).

In summary, our data show that over-expression of the PI3Kδ isoform mediates the increased Ca^{2+} current through $Ca_V1.2$ channels that drives the increased contractility in diabetic vessels. Given that selective inhibition of the PI3Kδ isoform appears to be highly effective in blocking vasoconstriction in our model of type 1 diabetes, PI3Kδ could represent a novel target to expand the therapeutic strategy to treat vascular disease in diabetic patients.

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Conflict of interest

The authors state no conflict of interest.

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