RESEARCH PAPER

Insulin resistance in penile arteries from a rat model of metabolic syndrome

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BACKGROUND AND PURPOSE

Metabolic and cardiovascular abnormalities accompanying metabolic syndrome, such as obesity, insulin resistance and hypertension, are all associated with endothelial dysfunction and are independent risk factors for erectile dysfunction. The purpose of the present study was to investigate the vascular effects of insulin in penile arteries and whether these effects are impaired in a rat model of insulin resistance and metabolic syndrome.

EXPERIMENTAL APPROACH

Penile arteries from obese Zucker rats (OZR) and their counterpart, lean Zucker rats (LZR), were mounted on microvascular myographs and the effects of insulin were assessed in the absence and presence of endothelium and of specific inhibitors of nitric oxide (NO) synthesis, phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK). Insulin-induced changes in intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ were also examined.

KEY RESULTS

OZR exhibited mild hyperglycaemia, hypercholesterolemia, hypertryglyceridemia and hyperinsulinemia. Insulin induced endothelium- and NO-dependent relaxations in LZR that were impaired in OZR. Inhibition of PI3K reduced relaxation induced by insulin and by the β-adrenoceptor agonist isoprenaline, mainly in arteries from LZR. Antagonism of endothelin 1 (ET-1) receptors did not alter insulin-induced relaxation in either LZR or OZR, but MAPK blockade increased the responses in OZR. Insulin decreased $[Ca^{2+}]_i$, a response impaired in OZR.

CONCLUSIONS AND IMPLICATIONS

Insulin-induced relaxation was impaired in penile arteries of OZR due to altered NO release through the PI3K pathway and unmasking of a MAPK-mediated vasoconstriction. This vascular insulin resistance is likely to contribute to the endothelial dysfunction and erectile dysfunction associated with insulin resistant states.

Abbreviations

ED, erectile dysfunction; ET-1, endothelin 1; KPSS, high K^+-physiological saline solution; L-NNA, N^ω-nitro-L-arginine; LZR, lean Zucker rat; MAPK, mitogen-activated protein kinase; NOS, nitric oxide synthase; OZR, obese Zucker rat; PI3K, phosphatidylinositol 3-kinase; PSS, physiological saline solution; VSMC, vascular smooth muscle cells

Introduction

Erectile dysfunction (ED) is currently considered as an early sign of subclinical vascular disease and is a highly prevalent condition in patients with cardiovascular risk factors such as hypertension, dyslipidemia, central obesity and insulin resistance (Montorsi *et al.*, 2003). The cluster of these metabolic and cardiovascular abnormalities, jointly referred to as metabolic syndrome, further increases the risk for the development of ED (Fonseca and Jawa, 2005; Jackson, 2006).

Insulin resistance and hyperinsulinemia play a major role in the pathogenesis of type 2 diabetes and have been associated with the development of cardiovascular disease and hypertension (De Fronzo and Ferrannini, 1991). Insulin resistance is typically defined as a decreased sensitivity to the metabolic actions of insulin such as insulin-mediated glucose disposal. However, in addition to its classical physiological actions, insulin has vascular actions that couple the metabolic and haemodynamic homeostasis under healthy conditions (Kim *et al.*, 2006). The association between insulin resistance and cardiovascular disease was initially ascribed to the actions of insulin on vascular smooth muscle growth and extracellular matrix production (Tamaroglio and Lo, 1994). However, experimental evidence shows that endothelial function is impaired in insulin resistance and diabetic patients and diminished sensitivity or resistance to the actions of insulin in the vascular endothelium also contribute to the phenotype of the insulin-resistant states (Baron *et al.*, 1991; Steinberg *et al.*, 1996; Kim *et al.*, 2006).

Insulin binding to its receptor at the endothelial cells activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway that in turns phosphorylates endothelial nitric oxide synthase (eNOS) at Ser 1177 and induces nitric oxide (NO) release and vasodilatation (Zeng *et al.*, 2000). Stimulation of endothelial insulin receptors can also lead to the activation of the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway that is associated with gene expression, cell growth and the production of vasoconstrictor endothelin 1 (ET-1) (Cardillo *et al.*, 1999). In obese insulin-resistant men and patients with type 2 diabetes, the insulin-mediated activation of the PI3K/Akt pathway underlying both the metabolic and the vascular actions of the hormone is impaired, and endothelial dysfunction and blunted vasodilatation have been reported (Steinberg *et al.*, 1996; Cusi *et al.*, 2000).

The metabolic and cardiovascular abnormalities accompanying metabolic syndrome, that is, glucose intolerance, dyslipidemia, insulin resistance and hypertension, act as independent risk factors for ED and are all associated to some extent with endothelial dysfunction (Fonseca and Jawa, 2005), although the mechanisms for this dysfunction in the penile vasculature are not completely understood. Novel studies in an animal model of type 1 diabetes-associated ED have demonstrated an impairment of the PI3K/Akt/eNOS pathway in the erectile tissue associated with a decreased erectile capability (Musicki *et al.*, 2005). However, the effects of insulin on the penile vasculature of both healthy individuals and of type 2 diabetes and insulin resistance-

associated ED models are unknown. In the present study, we investigated the mechanisms underlying the vasoactive actions of insulin in penile arteries from the obese Zucker rat (OZR), a well-established genetic model of insulin resistance and metabolic syndrome caused by a dysfunctional gene of the leptin receptor. These animals progressively develop obesity, type 2 diabetes and hypertension (Guerre-Millo 1997). We have recently demonstrated profound alterations in vascular structure and endothelial dysfunction in dorsal penile arteries from the OZR (Villalba *et al.*, 2009), data consistent with the ED reported in these animals (Wingard *et al.*, 2007). Here, we aimed to further investigate whether the vascular effects of insulin are impaired in penile arteries under conditions of insulin resistance in the OZR.

Methods

Animal model

All animal care and experimental protocols conform to European Union guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Complutense University of Madrid. Male OZR (n = 47) and lean Zucker rats (LZR, n = 46) were obtained from Charles River Laboratories (Barcelona, Spain) and used for study at 17–18 weeks of age.

Experimental procedures for the functional experiments

On the day of the experiment, blood samples were obtained from the tail vein before death and plasma was frozen for determination of non-fasting glucose, cholesterol, triglycerides and insulin plasma levels. Glucose, total triglyceride and total cholesterol levels were determined by using commercially available kits. Plasma insulin levels were measured by specific enzyme-linked immunosorbent assay.

Immediately after killing of the animal (cervical dislocation and exsanguination), the penis was removed and placed in cold physiological saline solution (PSS) of the following composition (mM): NaCl 119, NaHCO$_3$ 25, KCl 4.7, K$_2$H$_2$PO$_4$ 1.17, MgSO$_4$ 1.18, CaCl$_2$ 1.5, EDTA 0.027 and glucose 11. The penile arteries, first- or second-order branches of the dorsal penile artery from LZR and OZR were carefully dissected by removing the connective and fat tissue, as described previously (Villalba *et al.*, 2009; Sánchez *et al.*, 2010). These arteries were used in order to assess erectile tissue function as they can supply blood to the cavernous tissue and are of a size in the rat that allows handling without damaging

the vascular endothelium (Villalba *et al.*, 2009). One arterial segment from a LZR and another from an OZR were mounted in parallel on double microvascular myographs (DMT, Denmark) and stretched to their optimal internal lumen diameter l_1 corresponding to 90% of the diameter the arteries would have *in situ* under an internal transmural pressure of 100 mmHg (Villalba *et al.*, 2009). Aortic rings from LZR and OZR were also used in order to compare the vascular effects of insulin with those on penile arteries. Thoracic aortas were isolated and cleaned from the fatty and connective tissue, and arterial rings were mounted in vascular myographs and a passive initial tension of 10 mN applied.

At the beginning of each experiment, vasoconstrictor responses to 120 mM K^+ (high K^+-physiological saline solution, KPSS) confirmed vessel viability. The vasoactive effects of insulin were evaluated by adding cumulative concentrations of the hormone to arteries precontracted with phenylephrine (1 μM), in the absence or presence of specific inhibitors of NOS (N^ω-nitro-L-arginine, L-NNA, 100 μM), PI3K (LY-294002, 3 μM), cyclooxygenase (indomethacin, 1 μM), MAPK (PD-98059, 5 μM) and the non-selective antagonist of ET-1 receptors (bosentan, 10 μM). Because insulin effects were not reproducible in a second stimulation, two consecutive arterial segments of the same animal were used, one as a control and in the second the treatment was applied. The role of the vascular endothelium was assessed in arteries where the endothelium was mechanically removed by passing a human hair through the vessel lumen. The absence of functional endothelium was confirmed by the lack of relaxation to acetylcholine (ACh, 10 μM). The effects of the β-adrenoceptor agonist isoprenaline were also evaluated in the absence and presence of LY-294002. In order to assess the cAMP-dependent relaxations in penile arteries, concentration–response curves for the adenylate cyclase activator forskolin were also performed.

Measurements of intracellular Ca^{2+} $([Ca^{2+}]_i)$

Simultaneous measurements of $[Ca^{2+}]_i$ and force were performed in intact penile arteries as previously described (Villalba *et al.*, 2007). The myograph was mounted on an inverted microscope (Axiovert S100 TV) equipped for dual excitation wavelength microfluorimetry. Penile arteries were incubated in the dark in PSS with 8 μM Fura 2-acetoxymethyl ester for 3 h at 37°C, changing the solution after 1.5 h. Then arteries were washed for 45 min to remove remaining Fura 2-acetoxymethyl ester. After loading, the arteries were illuminated with alternating 340 and 380 nm light using a monochromator-based system (Deltascan, PTI). The fluorescence

emission was detected at a wavelength of 510 nm. At the end of each experiment, Ca^{2+}-insensitive signals were determined after quenching with 25 mM Mn^{2+}, and the values obtained were substracted from those obtained during the experiment. The ratio (R) F_{340}/F_{380} was taken as a measure of $[Ca^{2+}]_i$.

Arteries were initially stimulated with KPSS in order to test vessel viability. Then, the effect of a single dose of insulin (0. 1 μM) was evaluated in arteries pre-contracted with phenylephrine (1 μM). Finally, the effect of ACh (10 μM) was also tested.

Data analysis

Values are expressed as means $\pm$ SEM of n (number of arteries, 1–2 per animal). Relaxants responses to insulin are given as a percentage of precontraction induced by phenylephrine. The sensitivity of the arteries to the vasoactive agonists is expressed in terms of pEC_{50} values, where pEC_{50} is $-logEC_{50}$. EC_{50} is the concentration of the agonist required to produce 50% of the response and was calculated by using standard computer software (Prism 5.0, Graphpad, San Diego, CA, USA). The statistical differences between means were analysed by either unpaired Student's t-test or by one-way ANOVA. Probability levels lower than 5% were considered significant.

Materials

The sources of the compounds used in this MS were as follows: L-NNA, indomethacin, insulin, PD98059, isoprenaline, acetylcholine, forskolin and phenylephrine were from Sigma Chemicals (Madrid, Spain). LY 294002 and endothelin1 (ET-1) were from Tocris, Cookson, (Bristol, UK); Fura 2-acetoxymethyl ester (Fura-2-AM) was from Molecular Probes (OR, USA). Bosentan was a generous gift of F. Hoffman La Roche Laboratories (Basel, Switzerland). All drug and molecular target nomenclature follows Alexander et al., (2009).

Results

General parameters

At the time of the experiment, the OZR were significantly heavier than the LZR and exhibited mild hyperglycaemia, hyperinsulinemia and dyslipidemia with elevated total cholesterol and triglyceride levels (Table 1). In penile arteries from the OZR group, contractions to KPSS were reduced (2.11 $\pm$ 0.14 Nm^{-1} in LZR, $n = 46$; and 1.62 $\pm$ 0.10 Nm^{-1} in OZR, $n = 47$, $P < 0.01$), and the ACh-induced relaxations were impaired (58 $\pm$ 4% in LZR, $n = 27$; and

Table 1

Metabolic parameters of LZR and OZR

	LZR	N	OZR	n
Body weight (g)	384 ± 7	46	$474 \pm 7^{***}$	47
Weeks	17–18		17–18	
$[Glucose]_{blood}$ (mg/ml)	1.16 ± 0.13	12	$1.75 \pm 0.20^*$	12
$[Insulin]_{plasma}$ (ng/ml)	1.10 ± 0.20	6	$4.30 \pm 0.50^{***}$	6
$[Cholesterol]_{plasma}$ (mg/ml)	0.92 ± 0.04	12	$1.83 \pm 0.13^{***}$	12
$[Triglycerides]_{plasma}$ (mg/ml)	0.99 ± 0.13	12	$3.34 \pm 0.33^{***}$	12

Data are means $\pm$ SEM of the number n of animals. Significant differences were analyzed by an unpaired Student t-test. $^*P < 0.05$; $^{**}p < 0.001$; $^{***}P < 0.0001$ vs LZR. LZR, lean Zucker rat; OZR, obese Zucker rat.

$43 \pm 5\%$ in OZR, $n = 38$, $P < 0.05$), indicating reduced contractility and endothelial dysfunction respectively.

Structure of penile arteries

Normalized internal lumen diameters (l_1) of penile arteries were significantly smaller in OZR (129 ± 4 μm, $n = 36$) than in LZR (155 ± 6 μm, $n = 38$, $P < 0.001$), indicating inward vascular remodelling in insulin-resistant animals. Penile weights were significantly lower in OZR than LZR (0.5 ± 0.1 g and 0.7 ± 0.1 g, $n = 7$, $P < 0.01$, respectively), also suggesting structural remodelling of penile erectile tissue. Masson's trichrome staining confirmed wall thickening and smaller internal diameters in arteries from OZR (not shown).

Effect of insulin on penile arteries from LZR and OZR

Exogenously applied insulin (0.1–100 nM) induced concentration-dependent relaxations in penile arteries of LZR pre-contracted with phenylephrine (Figure 1). The pEC_{50} and response to the highest concentration of insulin used (100 nM) was 8.02 ± 0.12 and $24.3 \pm 3.6\%$ ($n = 27$) of the phenylephrine-induced contraction respectively (Figure 1A,C). The relaxant responses to insulin were impaired in penile arteries from OZR (insulin-induced (100 nM) relaxation of $7.5 \pm 2.5\%$, $n = 31$, $P < 0.01$ vs. LZR) (Figure 1B,C). In aortas from OZR, responses to insulin were also blunted and, in some samples, turned into contractions, the response to 100 nM insulin being $-4.1 \pm 10.9\%$ ($n = 10$) of the phenylephrine-induced contraction. In LZR aortas, the corresponding response to insulin was greater ($24.0 \pm 7.2\%$, $n = 11$) of the phenylephrine-induced contraction ($P < 0.01$).

Effects of endothelial cell removal on insulin relaxant responses

Mechanical removal of the endothelium impaired insulin-induced relaxations in penile arteries from LZR but did not significantly alter responses in OZR (Figure 2A,B, Table 2). There was a significant linear correlation between the 100 nM insulin-induced relaxation and that elicited by the endothelial cell agonist ACh (10 μM) in both LZR and OZR (Figure 2C,D). The absence of functional endothelium was verified by the blunted vasodilator effect of ACh after endothelial cell removal (Figure 2E,F, Table 2).

Effects of eNOS and PI3K inhibitors on insulin relaxant responses

Treatment with the NOS blocker L-NNA (100 μM) produced a significant inhibition of the insulin-induced relaxation in penile arteries of LZR but not of OZR (Figure 3A,B, Table 2). The maximal ACh relaxant responses were significantly diminished in both LZR and OZR after NOS blockade (Figure 3C,D, Table 2).

Inhibition of PI3K with LY-294002 (1 μM) markedly reduced the relaxant effect of insulin in arteries of LZR, and to a lesser extent in OZR (Figure 4A,B, Table 2), without significantly altering the relaxant effects of ACh in either LZR or OZR (Figure 4C,D, Table 2). Interestingly, LY-294002 also inhibited the relaxant responses induced by the β-adrenoceptor agonist isoprenaline in LZR but not in OZR (Figure 5A,B). The maximal relaxant effect of isoprenaline was significantly impaired in OZR compared with controls (Figure 5C). However, the relaxant effect of the adenylate cyclase activator forskolin was not different in penile arteries from OZR compared with LZR (Figure 5D), pEC_{50} being 6.18 ± 0.21 and 6.30 ± 0.21 ($n = 15$, not significantly different) respectively.

Effect of ET-1 receptor antagonist and MAPK inhibitors on arterial responses to insulin

The antagonist of ET-1 receptors, bosentan, did not modify the relaxant responses to insulin
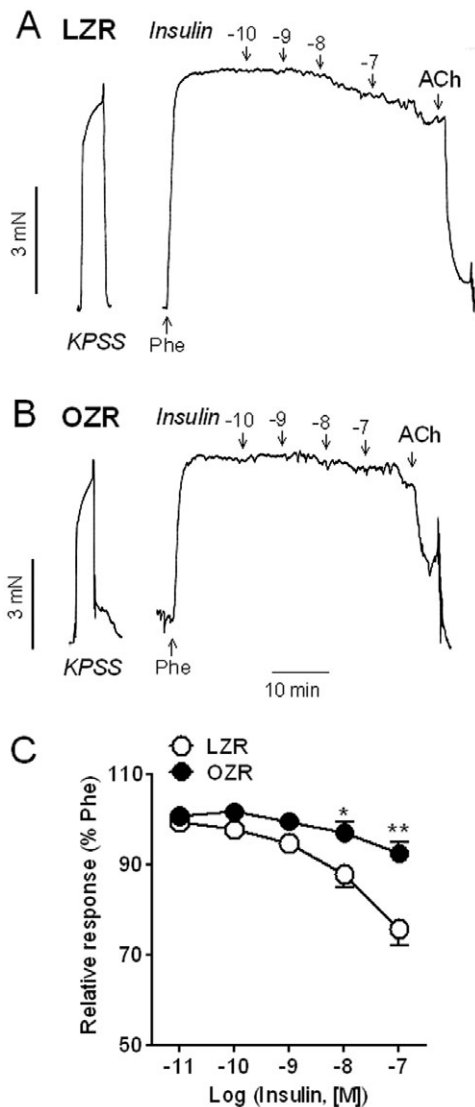
Figure 1

Relaxant responses to insulin were significantly impaired in penile arteries from OZR compared with LZR. (A, B) Representative traces showing the insulin-induced relaxations in penile arteries from (A) LZR and (B) OZR. (C) Average concentration–response curves for the relaxant effect of insulin in arteries from LZR and OZR. Data are shown as the mean ± SEM of 26 arteries (24 animals, LZR) and 28 arteries (25 animals, OZR). *$P < 0.05$, **$P < 0.01$ versus LZR. Vertical bars show force in mN and horizontal bars show time in min. ACh, acetylcholine; KPSS, high K$^+$-physiological saline solution; LZR, lean Zucker rat; OZR, obese Zucker rat; Phe, phenylephrine.

(Figure 6A,B) in penile arteries from either LZR or OZR. However, bosentan inhibited the contractions induced by ET-1 in both LZR (Figure 6C,D) (pEC$_{50}$ values: 8.94 ± 0.13, $n = 8$, vs. 7.23 ± 0.12, $n = 10$, $P < 0.0001$, in control and treated LZR arteries, respectively) and OZR (pEC$_{50}$ 9.20 ± 0.34, $n = 7$, vs. 7.23 ± 0.11, $n = 12$, $P < 0.01$, in control and treated arteries, respectively).

The MAPK inhibitor, PD-98059, did not alter the relaxant responses to insulin in LZR (Figure 7A), but significantly enhanced those in OZR (Figure 7B). The relaxant responses to insulin in the presence of PD-98059 were significantly inhibited by NOS blockade in LZR (Figure 7C), but blunted only after combined blockade of NOS and cyclooxygenase in OZR (Figure 7D).

Effect of insulin on [Ca^{2+}]$_i$

Stimulation of penile arteries from LZR and OZR with a high K$^+$ solution (KPSS) induced simultaneous increases in both [Ca^{2+}]$_i$ [Δ(F340/F380) = 0.51 ± 0.05, $n = 7$, in LZR, and 0.35 ± 0.04, $n = 7$, in OZR, $P < 0.05$] and tension (1.17 ± 0.16 Nm^{-1}, $n = 7$ in LZR and 0.95 ± 0.18 Nm^{-1}, $n = 7$, in OZR, $P < 0.01$) (Figure 8A,C). The α$_1$-adrenoceptor agonist phenylephrine (1 μM) also evoked sustained increases in [Ca^{2+}]$_i$ [Δ(F340/F380) = 0.38 ± 0.06, $n = 7$, in LZR and 0.26 ± 0.04, $n = 7$, in OZR, $P < 0.05$] and tension (2.49 ± 0.26 Nm^{-1}, $n = 7$ in LZR and 1.31 ± 0.24 Nm^{-1}, $n = 7$, in OZR, $P < 0.01$) (Figure 8A,C).

The relaxations induced by a single dose of both insulin (100 nM) and ACh (10 μM) were accompanied by simultaneous decreases in [Ca^{2+}]$_i$ in penile arteries from LZR pre-contracted with phenylephrine (Figure 8A,B). These decreases in [Ca^{2+}]$_i$ were impaired, along with relaxation, in arteries from OZR (Figure 8C,D). In some arteries from OZR, stimulation with insulin was eventually accompanied by increases in [Ca^{2+}]$_i$ (Figure 8C).

Discussion

The present study demonstrates that insulin induced endothelium-dependent relaxations in penile arteries through the release of NO via the PI3K/eNOS pathway. This pathway was impaired in arteries from insulin-resistant OZR thus unmasking MAPK-dependent vasoconstrictor effects of insulin. Penile endothelial dysfunction in insulin resistant animals, measured here as a decreased insulin-stimulated, NO-mediated, relaxation of the penile arterial rings, correlates with abnormal structure and vascular remodelling (Villalba *et al.*, 2009), and probably contributes to the decreased erectile capability reported for this animal model (Wingard *et al.*, 2007) and to the ED associated to insulin-resistant states, such as diabetes, obesity and hypertension (Fonseca and Jawa, 2005).

The impaired relaxant effect of insulin in penile arteries of OZR was also found in the aorta of the same animals and is in agreement with the reported impairment of the insulin-mediated vasodilatation in obese subjects (Baron *et al.*, 1991; Steinberg *et al.*,
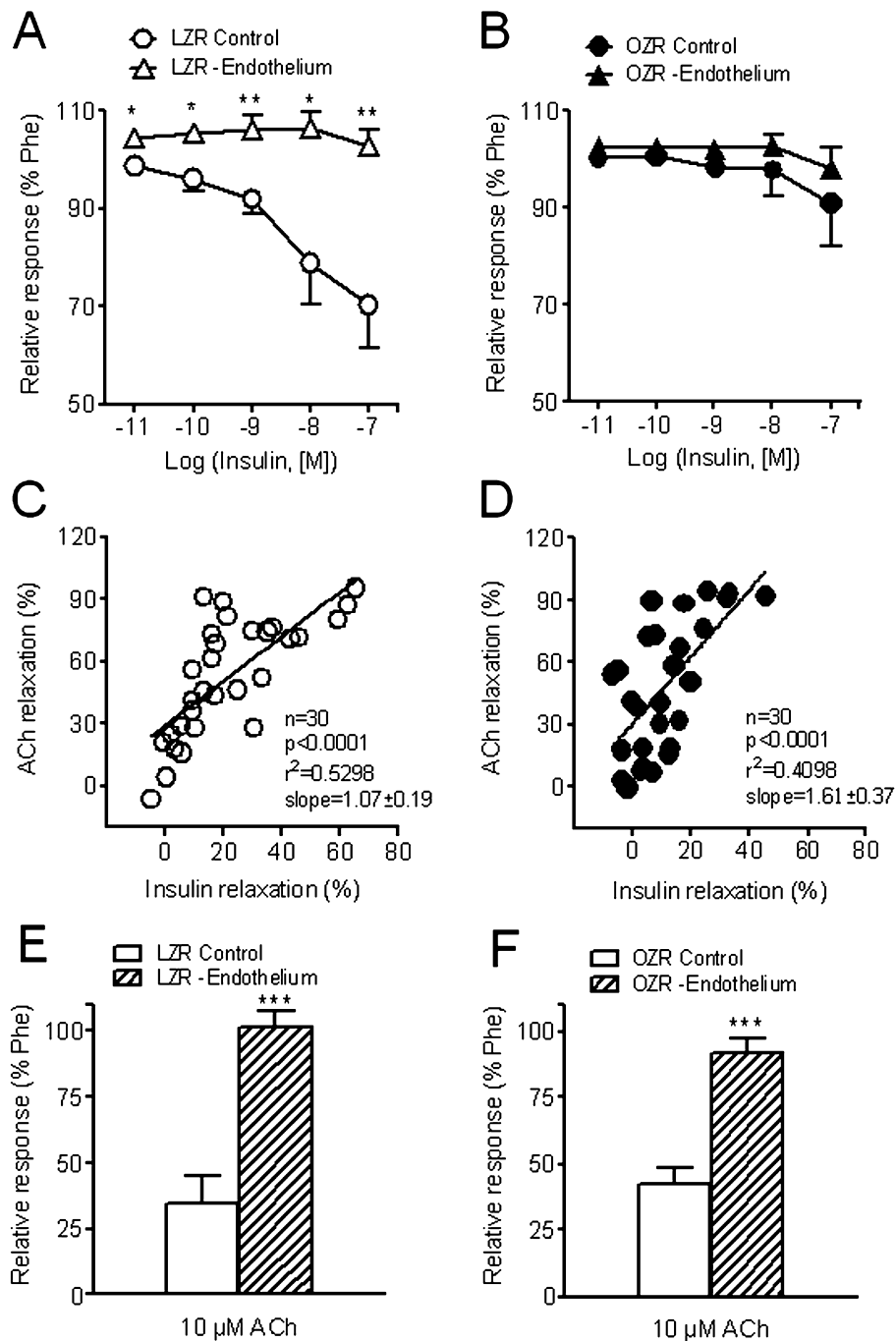
Figure 2

Effect of endothelial cell removal on the relaxant effects of insulin in penile arteries from LZR and OZR. (A, B) Average relaxant responses to insulin in endothelium-intact and endothelium-denuded penile arteries from (A) LZR and (B) OZR. (C, D) There was a significant linear correlation between insulin-dependent and ACh-dependent relaxations in both (C) LZR and (D) OZR. (E, F) Average ACh (10 μM) relaxant responses in endothelium-intact and endothelium-denuded penile arteries from (E) LZR and (F) OZR. Data are expressed as the mean ± SEM of 6–7 arteries (A, B, E, F) and 30 arteries (C, D), 1–2 per animal. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$ versus endothelium-intact arteries. Unpaired Student's *t*-test. ACh, acetylcholine; LZR, lean Zucker rat; OZR, obese Zucker rat; Phe, phenylephrine.

1996) and in skeletal muscle arterioles (Eringa *et al.*, 2007) and coronary arteries (Katakam *et al.*, 2005) from OZR. The present experiments showed that insulin-induced relaxation in penile arteries was

endothelium-dependent and NO-mediated in healthy animals, as shown by the inhibitory effect of endothelium removal and NOS blockers, and also by the significant correlation between the relaxant

Table 2

Effects of endothelium removal (-Endo) and of the inhibitors of NOS, L-NNA (100 µM), and PI3K, LY-294002 (1 µM) on the relaxant responses to insulin and to acetylcholine in penile arteries from LZR and OZR

| | Insulin (100 nM) | | | | Acetylcholine (10 µM) | | | |
	LZR	N	OZR	n	LZR	n	OZR	n
Control	29.7 ± 8.9	7	9.1 ± 9.0	7	65.1 ± 9.9	7	57.4 ± 6.1	7
-Endo	−2.7 ± 3.3[b]	7	2.0 ± 4.2	6	4.5 ± 4.1[c]	7	7.8 ± 5.2[c]	6
Control	34.9 ± 6.8	8	16.0 ± 8.8	6	53.8 ± 9.9	8	43.1 ± 15.3	6
L-NNA	7.4 ± 8.4[a]	7	8.0 ± 5.3	7	29.3 ±8.5[a]	7	16.8 ± 6.1[a]	7
Control	20.2 ± 4.8	6	12.6 ± 3.4	7	58.9 ± 6.9	10	57.3 ± 10.9	10
LY-294002	2.0 ± 1.5[c]	7	3.7 ± 0.9[b]	8	44.7 ± 12.1	10	45.6 ± 12.1	11

Values represent mean ± SEM of the number n of individual arteries, 1–2 per animal. The relaxant responses to insulin and acetylcholine are expressed as percentage of the contraction induced by phenylephrine (1 µM), prior to the addition of the agonist in each artery. Significant differences from controls were assessed by an unpaired Student's t-test.
[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$.
L-NNA, N$^\omega$-nitro-L-arginine; LZR, lean Zucker rat; NOS, nitric oxide synthase; OZR, obese Zucker rat; PI3K, phosphatidylinositol 3-kinase.

effect of insulin and that of the endothelial agonist ACh. Furthermore, our data provide pharmacological evidence for the involvement of the PI3K/Akt pathway in the vasoactive effects of insulin in penile arteries. This pathway plays a key role in the physiology of erection, as it is responsible for the sustained production of NO and full erection upon stimulation of eNOS by shear stress, after the increased blood flow to the corpus cavernosum caused by nerve-derived NO released by sexual stimuli (Hurt et al., 2002; Prieto, 2008).

The functional significance of the NO-mediated vascular effects of insulin via the PI3K/Akt/eNOS pathway has earlier been reported in studies showing vascular protection by the hormone, in the coronary circulation. Thus, in vivo administration of insulin improved endothelium-dependent vasodilatation and reduced post-ischaemic myocardial apoptotic death (Gao et al., 2002; Ma et al., 2006). On the other hand, the endothelial dysfunction reported for insulin-resistant aging animals was associated with impaired arterial insulin-induced vasorelaxation and eNOS phosphorylation (Li et al., 2009). The activity of the PI3K/Akt pathway and phosphorylation of eNOS have been reported to be also impaired in models of type 1 diabetes-associated ED due to the O-linked glycosylation and inactivation of eNOS by hyperglycaemia (Federici et al., 2002; Musicki et al., 2005). In the present study, we further demonstrated that the PI3K/Akt pathway is impaired in penile tissue from an animal model of insulin resistance/metabolic syndrome, as shown by the blunted vasodilator effects elicited not only by insulin but also by the β-adrenoceptor agonist isoprenaline, and by the reduced effect of the selective PI3K inhibitor on these relaxations in

penile arteries from OZR. In contrast to that reported for type 1 diabetes models, impairment of the PI3K/Akt pathway is unlikely to be due to hyperglycaemia, because the insulin-resistant OZR animals used in our study were hyperinsulinemic but they only exhibited mild hyperglycaemia. On the other hand, decreased insulin-stimulated production of NO and impaired vasodilatation have been ascribed to decreased eNOS expression in skeletal muscle arterioles from OZR (Eringa et al., 2007), but in penile arteries we have recently shown that eNOS protein content is unaltered (Villalba et al., 2009). The functional data of the present study confirm earlier biochemical studies demonstrating that insulin-stimulated PI3K activity and serine phosphorylation of eNOS were markedly reduced in microvessels from OZR (Jiang et al., 1999). Moreover, reduced insulin-stimulated vasodilatation (Kobayashi et al., 2004) coupled to reduced phosphorylation and expression of Akt also occurs in arteries from type 2 diabetic patients (Okon et al., 2005) and rodent models (Kobayashi et al., 2004). The present data therefore suggest that penile endothelial dysfunction and impaired NO-mediated vasodilatation was in part due to the impaired PI3K/eNOS pathway in penile arteries from insulin-resistant OZR. In a recent study in which the contribution from vascular insulin signalling to eNOS in regulating endothelial function and blood pressure was assessed, high levels of free fatty acids rather that defective upstream signalling via Akt were demonstrated to be responsible for the impaired insulin-stimulated eNOS phosphorylation, abnormal endothelial function and hypertension in mice with diet-induced obesity (Symons et al., 2009). The 17–18-week-old hyperinsulinemic OZR
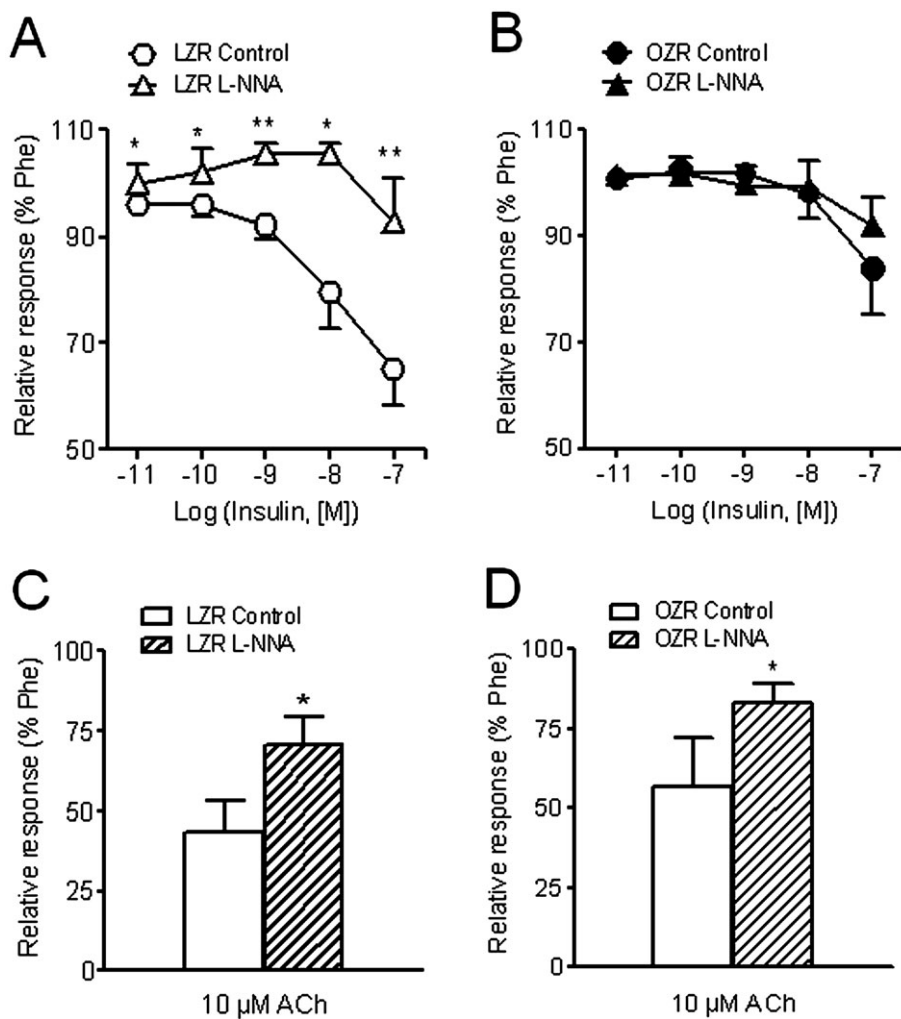
Figure 3

Effects of the NOS inhibitor, L-NNA (100 μM) on the relaxant responses to insulin (A, B) and (C, D) ACh (10 μM) in penile arteries from LZR and OZR. Data are shown as the means ± SEM of 6–8 arteries, 1–2 per animal. *$P < 0.05$, **$P < 0.01$, versus control arteries. Unpaired Student's *t*-test. ACh, acetylcholine; L-NNA, N$^\omega$-nitro-L-arginine; LZR, lean Zucker rat; NOS, nitric oxide synthase; OZR, obese Zucker rat; Phe, phenylephrine.

used in the present study exhibited penile endothelial dysfunction and vascular insulin resistance, along with ED (Wingard *et al.*, 2007) and abnormal structure but before the onset of hypertension (Villalba *et al.*, 2009). These findings reinforce the view of ED as an early sign of subclinical vascular disease (Montorsi *et al.*, 2003).

Interestingly, the endothelial dysfunction involving the PI3K/eNOS pathway observed in the present study affected the vasodilator effect of β-adrenoceptor agonists, as shown by the blunted relaxations elicited by isoprenaline in penile arteries from OZR. This impairment could initially be ascribed to a failure of the cAMP/protein kinase A pathway. However, relaxations to the adenylate cyclase activator forskolin were unaffected, whereas inhibition of PI3K did not further reduce relaxations

to isoprenaline in arteries from insulin-resistant animals suggesting an impairment of the PI3K/ eNOS pathway. Our findings are consistent with those of a recent study showing reduced cardiac output and depressor responses to isoprenaline in OZR, due to an impairment of the β-adrenoceptor signalling pathway in both cardiac and vascular myocytes from OZR (D'Angelo *et al.*, 2006). Thus, impairment of the β-adrenoceptor-mediated vasodilatation results in an impaired ability to regulate blood pressure after stress, in pre-diabetic OZR. The β$_2$-adrenoceptor is the predominant receptor subtype in most vascular smooth muscles and mediates relaxations that are completely or partly dependent on the release of endothelial NO through activation of the PI3K/Akt pathway (Ferro *et al.*, 2004). Concerning erectile tissue, β$_2$-adrenoceptors
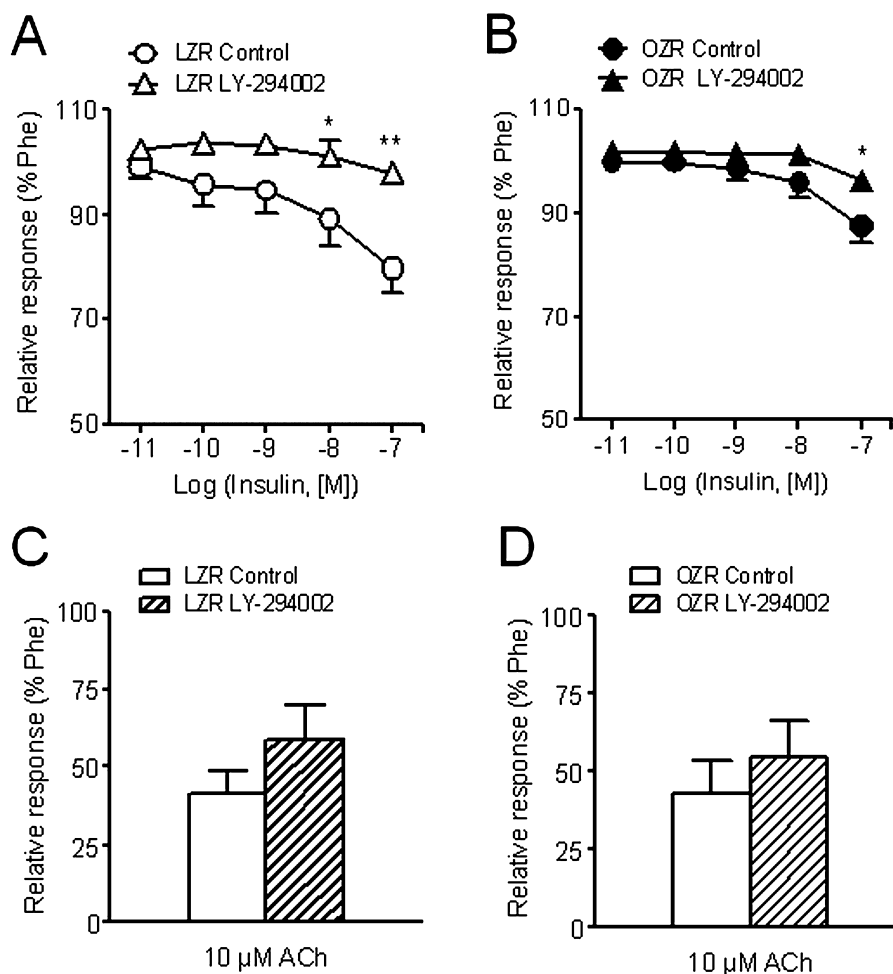
Figure 4

Effect of the PI3K inhibitor LY-294002 (3 μM) on the vasodilator responses induced by insulin (A, B) and ACh (C, D) in penile arteries from LZR and OZR. Data are shown as the means ± SEM of 7–8 arteries, 1–2 per animal. *$P < 0.05$, **$P < 0.01$, versus control arteries. Unpaired Student's *t*-test. ACh, acetylcholine; LZR, lean Zucker rat; OZR, obese Zucker rat; Phe, phenylephrine.

are involved in the relaxation of penile arteries (Simonsen *et al.*, 1997) and β-adrenoceptor-mediated vasodilatation has been proposed to play a role in the erectile response to the adrenaline release associated with sexual activity (Simonsen *et al.*, 1997; Prieto, 2008). Therefore, the blunted responses to β-adrenoceptor agonists found for the first time in penile arteries from OZR would further contribute to the endothelial and ED associated with insulin-resistant states.

The MAPK-dependent branch of the insulin signalling pathway promotes pro-hypertensive and pro-atherogenic actions in part mediated through the production of ET-1, but usually opposed by the PI3K/Akt pathway and NO in healthy individuals (Kim *et al.*, 2006). In insulin-resistant states, there is a selective impairment of the PI3K/Akt branch (Jiang *et al.*, 1999) and the compensatory hyperin-

sulinemia activates the MAPK pathway and enhances pro-hypertensive and pro-atherogenic actions of insulin (Kim *et al.*, 2006). Accordingly, in the present study, inhibition of MAPK significantly increased the relaxant effect of insulin in penile arteries from hyperinsulinemic OZR but not LZR, thus suggesting that insulin could exert constrictor effects through the MAPK branch, probably unmasked by the impairment of the PI3K/eNOS pathway. However, antagonism of ET-1 receptors did not change insulin relaxant responses in penile arteries of either LZR or OZR. This is in contrast to the findings in skeletal muscle arterioles, where insulin lacked a net vasoactive effect in arteries from LZR, but ET-1 antagonism uncovered insulin-induced vasodilatation and inhibits insulin-induced vasoconstriction in arteries from OZR (Eringa *et al.*, 2007). The discrepancies between our
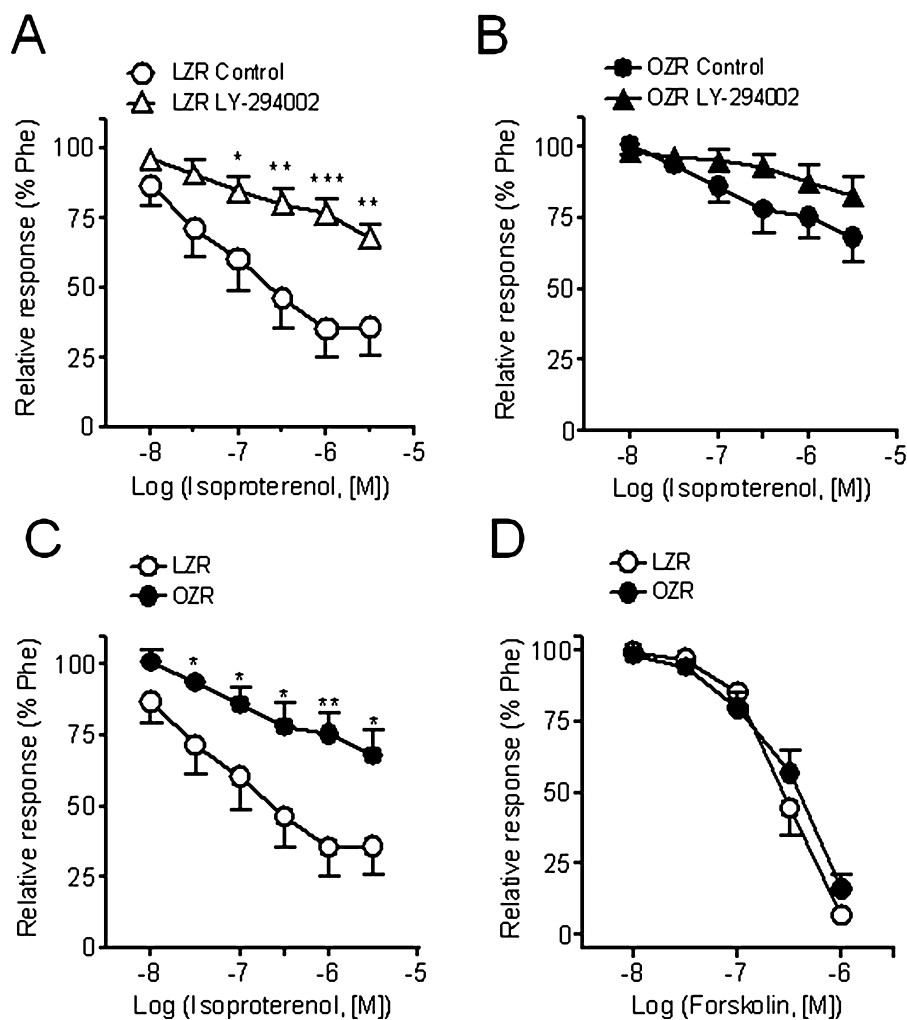
Figure 5

The relaxant responses to the β-adrenoceptor agonist isoprenaline were partly dependent on the PI3K/Akt pathway in penile arteries from LZR but not from OZR. (A, B) Effect of the PI3K inhibitor LY-294002 (3 μM) on the vasodilator responses induced by isoprenaline in penile arteries from LZR (A) and OZR (B). (C, D) Relaxations induced by isoprenaline (C) and by the adenylate cyclase activator forskolin (D) in penile arteries from LZR and OZR. Data are shown as the means ± SEM of 10–15 arteries, 1–2 per animal. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$. Unpaired Student's t-test. LZR, lean Zucker rat; OZR, obese Zucker rat; Phe, phenylephrine.

results on the insulin signalling pathways in penile arteries and those in skeletal muscle arteries are likely to reflect tissue-specific differences in the regulation of blood flow by the hormone. On the other hand, the relaxant responses uncovered by MAPK blockade in penile arteries from OZR were blunted by combined blockade of NOS and cyclooxygenase, suggesting the release of a relaxant prostanoid probably to compensate the impaired PI3K/NO pathway. Insulin-induced endothelium-dependent relaxation in healthy rabbit corpus cavernosum involves the release of prostacyclin but not NO (Myung *et al.*, 2006), whereas in penile arteries from healthy LZR, this relaxation is mainly due to NO release, which suggests differences in the signalling pathways of insulin between penile erec-

tile tissues. Furthermore, in small mesenteric arteries from Wistar rats, insulin-induced endothelium-dependent relaxations were unaffected by NOS inhibitors but were blunted by cyclooxygenase blockade and were due to prostacyclin release (Miller *et al.*, 2002). In insulin resistant fructose-fed rats, insulin-induced vasodilatation was impaired and unmasked by ET$_A$ receptor blockade, but the hormone-evoked release of prostacyclin persisted (Miller *et al.*, 2002). These data again indicate differences in the signalling pathways of insulin among the different vascular beds, in both healthy and insulin resistant animals.

Earlier studies have demonstrated that insulin attenuates vascular smooth muscle cells (VSMC) contractility by reducing vasoconstrictor
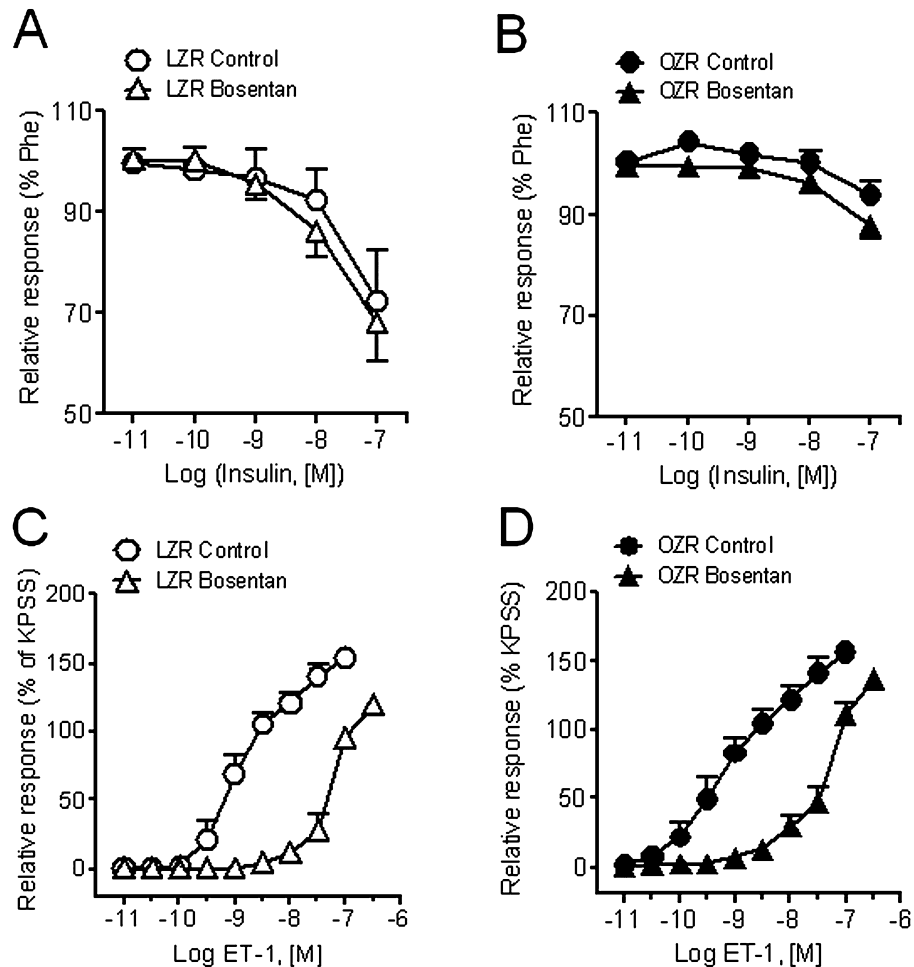
Figure 6

Effect of the non-selective ET-1 receptor antagonist, bosentan (10 μM) on the relaxant responses to insulin (A, B) and on the contractions elicited by ET-1 (C, D) of penile arteries from LZR and OZR. Data are shown as the means ± SEM of 6–12 arteries, 1–2 per animal. ET-1, endothelin 1; KPSS, high K+-physiological saline solution; LZR, lean Zucker rat; OZR, obese Zucker rat; Phe, phenylephrine.

agonists-induced increases in $[Ca^{2+}]_i$ through voltage-dependent Ca^{2+} channels (Standley *et al.*, 1991; 1993; Kahn *et al.*, 1994) and by altering the activity of mysosin light chain phosphatases (Begum *et al.*, 2002). Insulin relaxant responses in the corpus cavernosum have been reported to be due to an inhibition of L-type calcium channels (Myung *et al.*, 2006). The present data further demonstrate that the insulin vasodilator effect is paralleled by a decrease in $[Ca^{2+}]_i$ in arterial smooth muscle of healthy penile arteries precontracted with phenylephrine. Because α_1-adrenoceptor-mediated vasoconstriction is mostly dependent on extracellular Ca^{2+} influx in penile arteries (Villalba *et al.*, 2007), the effect of insulin on $[Ca^{2+}]_i$ is probably due to an attenuation of Ca^{2+} influx. The decrease in $[Ca^{2+}]_i$ induced by insulin was found to be blunted and even turned into increase in $[Ca^{2+}]_i$ along with

impaired relaxation in penile arteries from OZR. In isolated VSMC from OZR aorta, however, Ca^{2+} entry induced by vasopressin was similarly attenuated by insulin in LZR and OZR, which suggests that the ability of insulin to reduce Ca^{2+} influx in VSMC is preserved in arteries from insulin resistant animals (Standley *et al.*, 1993). Because the experiments of the present study were performed in arteries with intact endothelium, our results suggest that the blunted effects of insulin on $[Ca^{2+}]_i$ of penile arteries from OZR are likely to reflect the impaired release/effects of endothelial mediators of insulin relaxation, namely NO. Accordingly, the relaxations induced by ACh, which are mediated by NO and prostanoids (Villalba *et al.*, 2009; Sánchez *et al.*, 2010), were found to be impaired along with the ACh-induced decreases in $[Ca^{2+}]_i$ in penile arteries from OZR.
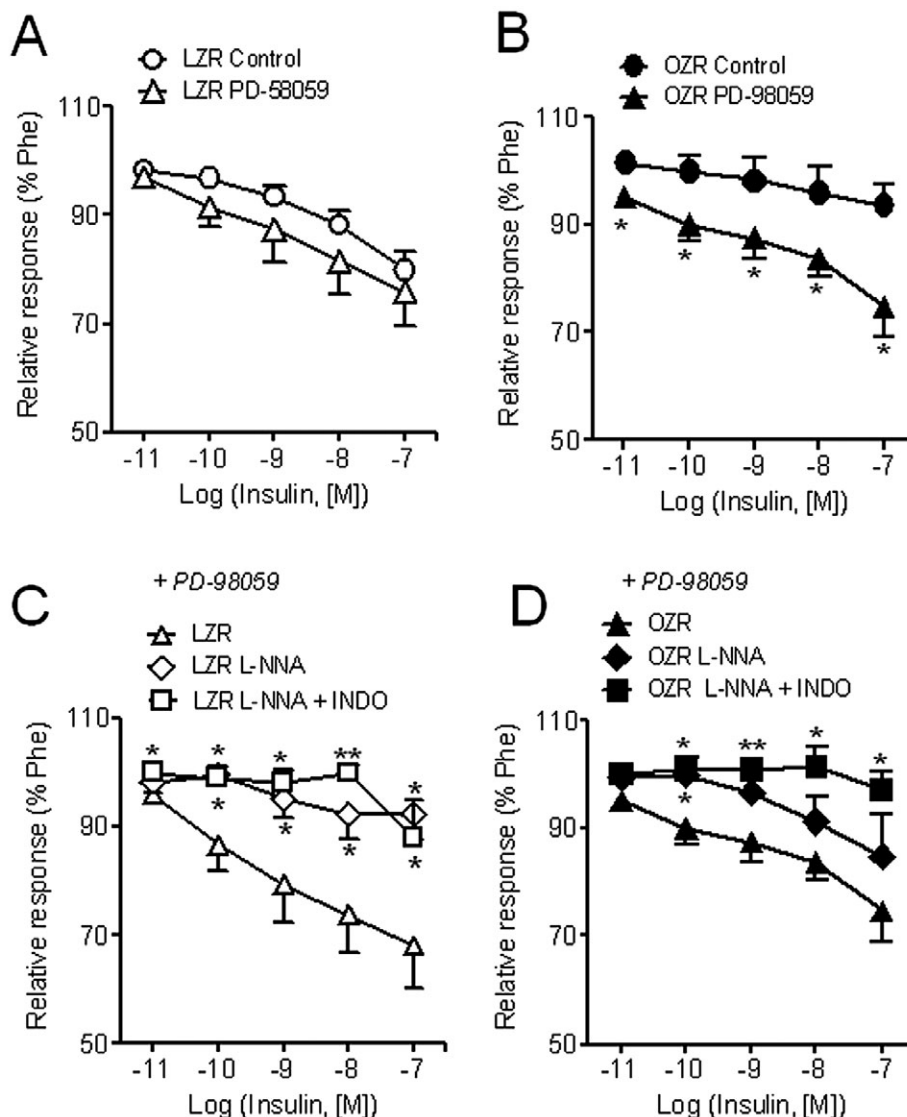
Figure 7

Effect of the MAPK inhibitor, PD-98059 (5 µM) on the relaxant responses to insulin in penile arteries from LZR and OZR (A, B). Effect of the NOS inhibitor, L-NNA (100 µM) and the cyclooxygenase inhibitor indomethacin (1 µM) on the relaxant responses to insulin in penile arteries from LZR (C) and OZR (D) treated with PD-98059. Data are shown as the means ± SEM of 6–12 arteries, 1–2 per animal. *$P < 0.05$, **$P < 0.01$ versus control arteries. (A, B) Unpaired Student's t-test. (C, D) One-way ANOVA. L-NNA, N$^\omega$-nitro-L-arginine; LZR, lean Zucker rat; MAPK, mitogen-activated protein kinase; OZR, obese Zucker rat; Phe, phenylephrine.

In summary, the present results demonstrate that the relaxant responses to insulin were blunted due to altered NO release through the PI3K/eNOS pathway, in penile arteries from the OZR, a model of insulin resistance/metabolic syndrome-associated ED. This endothelial dysfunction further affected the β-adrenoceptor-mediated vasodilatation and correlated with structural abnormalities in penile arteries. Thus, insulin resistance in the penile vasculature is likely to contribute to the endothelial dysfunction observed in insulin-resistant states such as diabetes, obesity and hypertension, all of which act as independent risk factors and jointly increase the risk for ED.
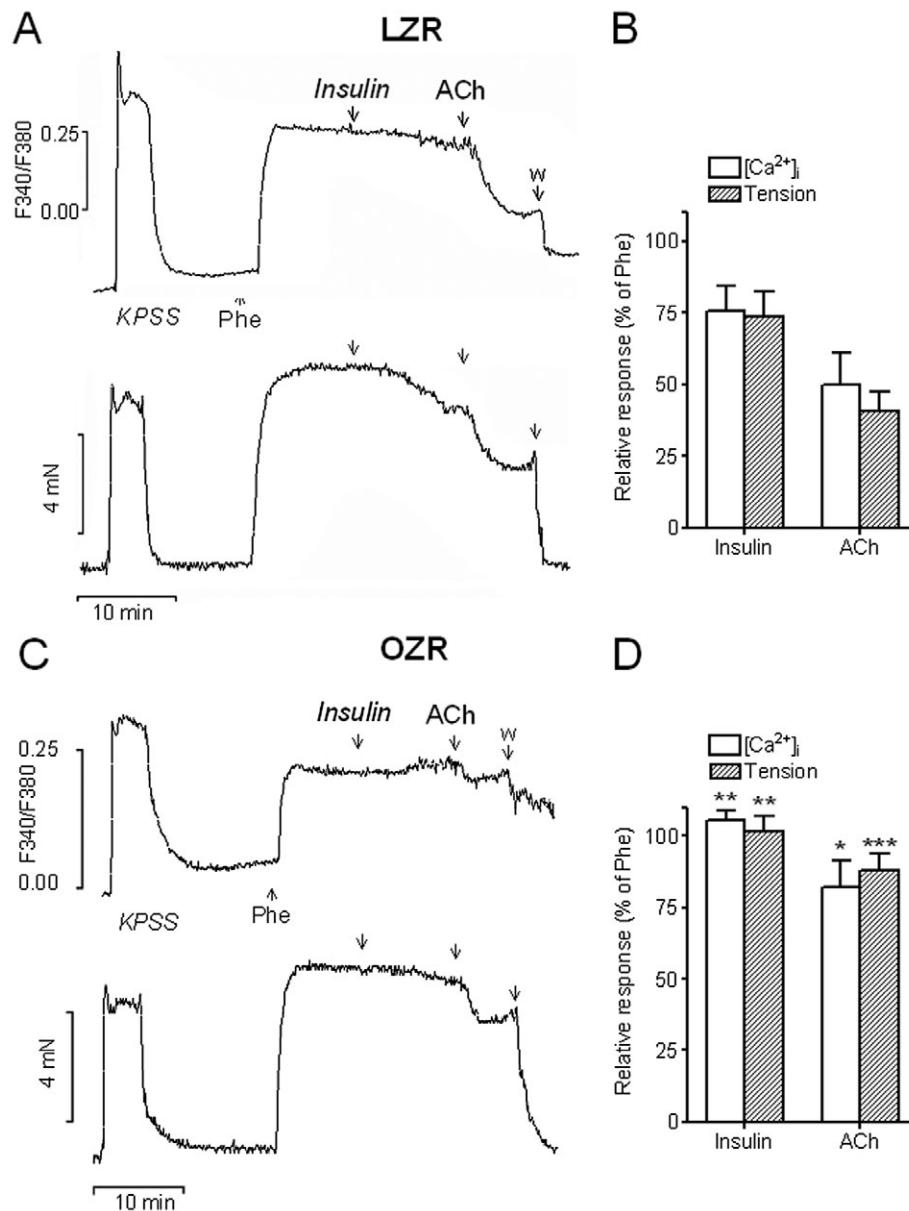
Acknowledgements

Figure 8

Simultaneous recordings of $[Ca^{2+}]_i$ (A, C top) and tension (A, C bottom) showing the effects of a single dose of insulin (10^{-7} M) and ACh (10^{-5} M) on penile arteries from LZR (A) and OZR (C) pre-contracted with Phe (1 μM). Relaxations were accompanied by a substantial decrease of $[Ca^{2+}]_i$ in LZR (A) and both relaxation and decrease in $[Ca^{2+}]_i$ were blunted in OZR (C,D). (B, D) Average effects of insulin and ACh on the increases in $[Ca^{2+}]_i$ and tension induced by Phe in penile arteries from LZR (B) and OZR (D). Data are shown as the means $\pm$ SEM of 5–7 arteries, 1 per animal. *$P < 0.05$, **$P < 0.01$ versus LZR. Unpaired Student's t-test. ACh, acetylcholine; KPSS, high K^+-physiological saline solution; LZR, lean Zucker rat; OZR, obese Zucker rat; Phe, phenylephrine.

Conflict of interest

None to disclose.

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