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RESEARCH PAPER

Endothelin-1 contributes to endothelial dysfunction and enhanced vasoconstriction through augmented superoxide production in penile arteries from insulin-resistant obese rats: role of ET_A and ET_B receptors

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BACKGROUND AND PURPOSE

We assessed whether endothelin-1 (ET-1) inhibits NO and contributes to endothelial dysfunction in penile arteries in a model of insulin resistance-associated erectile dysfunction (ED).

EXPERIMENTAL APPROACH

Vascular function was assessed in penile arteries, from obese (OZR) and lean (LZR) Zucker rats, mounted in microvascular myographs. Changes in basal and stimulated levels of superoxide (O_2^-) were detected by lucigenin-enhanced chemiluminescence and ET receptor expression was determined by immunohistochemistry.

KEY RESULTS

ET-1 stimulated acute O_2^- production that was blunted by tempol and the NADPH oxidase inhibitor, apocynin, but markedly enhanced in obese animals. ET-1 inhibited the vasorelaxant effects of ACh and of the NO donor S-nitroso-N-acetyl-DL-penicillamine in arteries from both LZR and OZR. Selective ET_A (BQ123) or ET_B receptor (BQ788) antagonists reduced both basal and ET-1-stimulated superoxide generation and reversed ET-1-induced inhibition of NO-mediated relaxations in OZR, while only BQ-123 antagonized ET-1 actions in LZR. ET-1-induced vasoconstriction was markedly enhanced by NO synthase blockade and reduced by endothelium removal and apocynin. In endothelium-denuded penile arteries, apocynin blunted augmented ET-1-induced contractions in OZR. Both ET_A and ET_B receptors were expressed in smooth muscle and the endothelial layer and up-regulated in arteries from OZR.

CONCLUSIONS AND IMPLICATIONS

ET-1 stimulates ET_A-mediated NADPH oxidase-dependent ROS generation, which inhibits endothelial NO bioavailability and contributes to ET-1-induced contraction in healthy penile arteries. Enhanced vascular expression of ET_B receptors contributes to augmented ROS production, endothelial dysfunction and increased vasoconstriction in erectile tissue from insulin-resistant obese rats. Hence, antagonism of ET_B receptors might improve the ED associated with insulin-resistant states.

Abbreviations

DPI, diphenylene iodonium; ED, erectile dysfunction; ET-1, endothelin-1; KPSS, high K^+-physiological saline solution; LZR, lean Zucker rat; O_2^-, superoxide radical; OZR, obese Zucker rat; PDBu, phorbol 12,13-dibutyrate; ROS, reactive oxygen species; SNAP, S-nitroso-N-acetyl-DL-penicillamine; VSM, vascular smooth muscle

Tables of Links

TARGETS		LIGANDS		
ET_A receptor	Nitric oxide synthase (NOS)	Acetylcholine (ACh)	Endothelin-1 (ET-1)	Phenylephrine
ET_B receptor		Angiotensin II	Methacholine	Prostacyclin
		BQ123	NADPH	TNF-α
		BQ788	Nitric oxide (NO)	

These Tables list key protein targets and ligands in this document, which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al*., 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al*., 2013a,b).

Introduction

Endothelial dysfunction is an early pathogenic event in the vascular complications associated with the insulin-resistant states of diabetes and obesity and has traditionally been ascribed to the reduced bioavailability of vasodilators such as NO and prostacyclin (Galili *et al*., 2007; Vanhoutte *et al*., 2009; Prieto *et al*., 2014). However, earlier evidence established the importance of enhanced endogenous activity of the vasoconstrictor, pro-inflammatory and mitogenic endothelial peptide endothelin-1 (ET-1) in humans who are overweight, obese and/or have type 2 diabetes. ET-1-induced vasoconstrictor tone was augmented and blockade of ET_A receptors improved basal and blunted methacholine-elicited increases in blood flow in obese adults and patients with type 2 diabetes, thus suggesting that ET-1 contributes to endothelial dysfunction (Mather *et al*., 2002; 2004; Weil *et al*., 2011).

Dysregulation of reactive oxygen species (ROS) signalling and oxidative stress seriously interfere with the synthesis and actions of NO and prostacyclin and reduce endothelium-dependent vasodilatation (Montezano and Touyz, 2012). NADPH oxidase is one of the main enzymatic sources of superoxide radical (O_2^-) generation in the vascular wall and ROS production can be augmented via activation of NADPH oxidase by some inducing factors such as ET-1, angiotensin II and TNF-α (Münzel *et al*., 2010; Montezano and Touyz, 2012). ET-1 has been demonstrated to significantly increase O_2^- production in human arteries (Cerrato *et al*., 2012) and animal vessels (Elmarakby *et al*., 2005; Loomis *et al*., 2005; Romero *et al*., 2009; 2010), and ET-1 infusion in the forearm of healthy individuals (Böhm *et al*., 2007) or *in vitro* exposure of intact arteries to ET-1 have been shown to produce endothelial dysfunction (Romero *et al*., 2009; 2010). Furthermore, ET receptor blockade improves endothelial function in human coronary arteries (Verma *et al*., 2001) and endothelium-dependent vasodilatation in patients with insulin resistance (Mather *et al*., 2002; Shemyakin *et al*., 2006; Rafnsson *et al*., 2012) and experimental models of atherosclerosis (Barton *et al*., 1998) and type 2 diabetes (Abdelsaid *et al*., 2014). ET-1 vascular actions are mediated by two G-protein coupled membrane receptors, ET_A and ET_B, and both receptor types have been suggested to contribute to ET-1-induced vascular ROS generation (Duerrschmidt *et al*., 2000; Li *et al*., 2003; Dai *et al*., 2004; Dong *et al*., 2005; Fellner and Arendshorst, 2007; Just *et al*., 2008; Cerrato *et al*., 2012).

Erectile dysfunction (ED) is considered to be an early manifestation of endothelial dysfunction and vascular disease and it is a highly prevalent condition in diabetic men and patients with cardiovascular risk factors (Vlachopoulos *et al*., 2013). We have recently demonstrated that both changes in the NO signalling (Villalba *et al*., 2009; Contreras *et al*., 2010) and impaired release of vasodilator prostanoids (Sánchez *et al*., 2010) contribute to the pathogenesis of endothelial dysfunction in penile arteries from the obese Zucker rat (OZR), an established model of genetic obesity and prediabetes-associated ED (Kovanecz *et al*., 2006). High levels of oxidative stress in these arteries lead to neuronal (n) NOS uncoupling and nitrergic dysfunction thus also being involved in the pathogenesis of impaired erectile function (Sánchez *et al.*, 2012). On the other hand, ET-1 levels are augmented in diabetic men with ED and up-regulation of both ET_A and ET_B receptors has been demonstrated in erectile tissue in experimental models of diabetes and insulin resistance (Bell *et al.*, 1995; Francavilla *et al.*, 1997; Sullivan *et al.*, 1997; Ritchie and Sullivan, 2011; Contreras *et al.*, 2013).

Although ET-1-NO interactions have been suggested to be key factors in the endothelial dysfunction of obesity and diabetes (Böhm *et al.*, 2002; 2007; Mather *et al.*, 2002; 2004), the exact nature of these interactions is not completely understood and the ET receptors and sources of oxidative stress involved have not yet been investigated in penile erectile tissue. Therefore, the purpose of the present study was to assess whether ET-1 can inhibit endothelial NO bioavailability through its ability to stimulate ROS generation and, if so, determine the ET receptors and vascular sources involved. Furthermore, we sought to investigate whether ET-1–NO interactions may underlie penile endothelial dysfunction in the OZR, a well-established model of obesity/insulin resistance-associated ED.

Methods

Animal model

All animal care and experimental protocols conformed to the European Union Guidelines for the Care and the Use of Laboratory Animals (European Union Directive 2010/63/EU) and were approved by the Institutional Animal Care and Use Committee of the Madrid Complutense University. Male OZR (fa/fa, $n = 42$) and their control strain, lean Zucker rats (LZR) (fa/–, $n = 42$), were purchased from Charles River Laboratories (Barcelona, Spain) at 8–10 weeks of age. Animals were housed at the Pharmacy School animal care facility and maintained on standard chow and water *ad libitum*, until they were used for study, at 17–18 weeks of age. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Dissection of microvessels, mounting and force measurement

After the animals were killed, the penis was quickly removed and placed in cold physiological saline solution (PSS). The penile arteries, first- or second-order branches of the rat dorsal penile artery from LZR and OZR rats, were carefully dissected by removing the connective and fat tissue, as described previously (Sánchez *et al.*, 2012) and mounted in parallel in double microvascular myographs (Danish Myotechnology, Aarhus, Denmark) by inserting two 40 µm tungsten wires into the vessel lumen. After being mounted, the arteries were equilibrated for 30 min in PSS maintained at 37°C of the following composition (mM): NaCl 119, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.17, $MgSO_4$ 1.18, $CaCl_2$ 1.5, EDTA 0.027 and glucose 11, continuously gassed with a mixture of 5% CO_2/95% O_2 to maintain pH at 7.4. The relationship between passive wall tension and internal circumference was determined for each individual artery and from this, the internal diameter, l_1, that yielded a circumference equivalent to 90% of that given by an internal pressure of 100 mmHg was calculated, and arteries were set to an l_1 at which all experiments were performed (Sánchez *et al.*, 2012).

Experimental procedure for the functional experiments

To assess a possible influence of ET-1 on the endothelium-dependent and endothelium-independent relaxing responses of penile arteries, the effect of a threshold concentration of ET-1 (0.3 nM) was tested on the relaxant responses induced by ACh, the NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP) and the adenylate cyclase activator forskolin in arteries precontracted with phenylephrine (1 µM) from LZR and OZR, by incubating the arteries with ET-1 for 30 min before cumulative addition of these agents.

The involvement of ET_A and/or ET_B receptors in the ET-1 effects on the relaxant responses to ACh and SNAP was assessed by incubation with the selective antagonists of the ET_A receptor (BQ123, 1 µM) or the ET_B receptor (BQ788, 0.1 µM). These drugs were introduced 30 min before a second concentration-response curve for either ACh or SNAP in the presence of 0.3 nM ET-1 was constructed. ACh and SNAP relaxant responses were reproducible in a second stimulation and two concentration-response curves for these agonists were performed in each artery. Due to tachyphylaxis to ET-1, arteries were exposed to the peptide only once during the experiment. Phenylephrine concentration was adjusted to match the contraction during the first control curve. Experiments with the NO donor, SNAP, were performed under conditions of NOS blockade with N^G-nitro-L-arginine (L-NOARG; 100 µM).

Cumulative concentration-response curves to ET-1 (0.01 nM–0.1 µM) were performed in the presence and absence of the NOS inhibitor L-NOARG (100 µM) or the NADPH oxidase inhibitor apocynin (30 µM). 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 ACh (10 µM).

Measurement of superoxide production by lucigenin-enhanced chemiluminescence

Changes in basal or ET-1-stimulated levels of superoxide were detected in the corpus cavernosum by lucigenin-enhanced chemiluminescence, as previously described in erectile tissue (Prieto *et al.*, 2010; Sánchez *et al.*, 2012). Corpora cavernosa (4–5 mm long strips) from LZR and OZR were dissected and equilibrated in Krebs buffer for 30 min at room temperature and then incubated in the absence (controls) and presence of ET-1 (1 nM), the specific antagonists of the ET_A receptors, BQ123 (1 µM), and ET_B receptors, BQ788 (0.1 µM), the superoxide scavenger tempol (30 µM), the NADPH oxidase inhibitors apocynin (30 µM) and diphenylene iodonium (DPI) (10 µM), or the PKC activator 12,13-dibutyrate (PDBu, 10 µM) for 30 min at 37°C. The corpus cavernosum was then transferred to microtitre plate wells containing 5 µM lucigenin (bis-N-methylacridinium nitrate) in air-equilibrated Krebs solution buffered with 10 mM HEPES-NaOH. Chemiluminescence was measured in a luminometer (BMG Fluostar

Optima, BMG LABTECH, Ortenberg, Germany), and for calculation baseline values were subtracted from the counting values under the different experimental conditions and superoxide production was normalized to tissue weight.

Immunohistochemistry

Tissue samples from the penis containing the dorsal penile artery were immersion-fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), cryoprotected in 30% sucrose in PB and snap frozen in liquid nitrogen and stored at –80°C. Transverse sections of 10 µm were obtained by means of a cryostat and pre-incubated in 10% normal goat serum in PB containing 0.3% Triton-X-100 for 2–3 h. Then, sections were incubated with either a rabbit anti-ET_A receptor antibody (Alomone Labs, Jerusalem, Israel) diluted at 1:100 or a rabbit anti-ET_B receptor antibody (Alomone Labs) diluted at 1:100 for 48 h at 4°C. Location of ET receptors in perivascular nerve fibres was visualized by coimmunostaining with a mouse anti-eNOS (Chemicon International Inc., Millipore Corporation, MA USA; 1:500 dilution). Sections were then washed and reacted with the second antibodies for 2 h at room temperature. Secondary antibodies used were Alexa Fluor 594 (red) goat anti-rabbit (Invitrogen, Life Technologies, Madrid, Spain; 1:200 dilution) and Alexa Fluor 488 (green) goat anti-mouse (Invitrogen, Life Technologies; 1:200 dilution). The slides were covered with a specific mounting medium with the nuclear stain DAPI (Invitrogen, Life Technologies). No immunoreactivity could be detected in sections incubated in the absence of the primary antisera. Preadsorption with ET_A and ET_B receptors showed no cross-reactivity for the antibodies.

Drugs and materials

ACh, apocynin (acetovanillone), DPI, L-NOARG, noradrenaline (arterenol), phenylephrine and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (tempol) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). ET-1, forskolin, BQ123 and BQ788 sodium salt, SNAP, PDBu were from Tocris Cookson (Bristol, UK). For *in vitro* experiments, drugs were dissolved in distilled water, except SNAP, apocynin, PDBu and BQ788 which were dissolved at 10 mM concentration in DMSO. The subsequent dilutions were made in distilled water.

Data presentation and statistical analysis

Results are expressed as either Nm^{-1} of tension or as a % of the responses to either phenylephrine or high K^+-physiological saline solution (KPSS) in each artery, as means ± SEM of *n* number of animals (one to two arteries from each animal were used). Statistically significant differences between means were analysed by one-way ANOVA or by using Student's paired or unpaired *t*-test when appropriate. Probability levels of $P < 0.05$ were considered statistically significant.

Results

General parameters

At the time of the experiment (17–18 weeks of age), OZR were significantly heavier than LZR (473 ± 7 g vs. 361 ± 6 g, $P < 0.001$; $n = 42$). We have reported that animals from the OZR group exhibit mild non-fasting hyperglycaemia, hyperinsulinaemia and dyslipidaemia with elevated total cholesterol and triglycerides levels (Villalba *et al.*, 2009). The normalized internal lumen diameters, l_1, were significantly smaller in penile arteries from OZR (127 ± 3 µm, $n = 32$) compared with LZR (142 ± 3 µm, $P < 0.01$; $n = 32$) indicating vascular remodelling. Contractions to KPSS were also reduced in the OZR group (1.6 ± 0.2 Nm^{-1} vs. 2.1 ± 0.1 Nm^{-1} in LZR, $P < 0.01$; $n = 32$), as reported previously (Villalba *et al.*, 2009).

ET-1 stimulates superoxide production in erectile tissue and inhibits NO-mediated relaxations in penile arteries

In order to determine whether ET-1 stimulates ROS generation in penile erectile tissue, strips of corpus cavernosum were acutely stimulated with 10 nM ET-1 for 30 min and superoxide generation was measured by lucigenin-enhanced chemiluminescence. Because basal NO release has been reported to counteract basal superoxide release in healthy penile arteries (Prieto *et al.*, 2010), these experiments were performed in the absence and the presence of the NOS inhibitor L-NOARG (100 µM). While ET-1-induced increase in superoxide production was negligible in erectile tissue from LZR, this effect was magnified and blunted by tempol treatment under conditions of NOS blockade in both LZR and OZR, indicating that ET-1-stimulated superoxide is counteracted by NO release. Both basal and basal plus ET-1-stimulated ROS levels were significantly augmented in OZR compared with LZR (Figure 1), which suggests that ET-1 contributes to the higher levels of oxidative stress in obese animals.

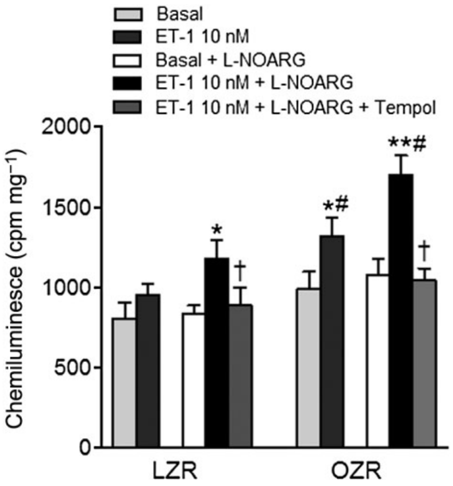

Figure 1

Basal superoxide production in corpus cavernosum tissue from LZR and OZR detected by lucigenin-enhanced chemiluminescence. Effect of ET-1 (10 nM) on basal superoxide production, and of tempol (100 µM) on the ET-1-induced superoxide production, in the absence and presence of the NOS inhibitor L-NOARG (100 µM). Superoxide production is expressed in counts per minute (cpm) mg^{-1} of tissue. Data are shown as the means ± SEM of $n = 4$ animals (two corpus cavernosum samples from each animal). $^*P < 0.05$, $^{**}P < 0.01$ versus basal, $^†P < 0.05$ versus ET-1-treated, $^\#P < 0.05$ versus LZR. Student's *t*-test for unpaired observations.

The acute effects of a threshold concentration ET-1 (0.3 nM) on the endothelium-dependent and endothelium-independent relaxant responses of penile arteries were investigated next. Incubation with ET-1 for 30 min markedly reduced the relaxations elicited by ACh in arteries from LZR (Figure 2A, Table 1) and showed a trend for inhibition in arteries from OZR (Figure 2B, Table 1), in which ACh control responses were already blunted compared with LZR indicating endothelial dysfunction, as earlier reported (Villalba *et al.*, 2009).

Pretreatment with 0.3 nM ET-1 blunted the NO-mediated vasodilator responses to the exogenous NO donor SNAP in penile arteries from both OZR and LZR, this inhibition being more pronounced in OZR (Figure 2C and D, Table 1), suggesting that ET-1 interferes with the relaxant action of NO. In contrast, ET-1 (0.3 nM) did not alter the relaxant responses elicited by the adenylate cyclase activator forskolin in either LZR (pD_2 7.76 ± 0.16 and 7.86 ± 0.22, $n = 3$ before and after ET-1 respectively) or OZR (pD_2 7.40 ± 0.04 and 7.50 ± 0.11, $n = 3$ before and after ET-1 respectively) (Figure 2E and F).

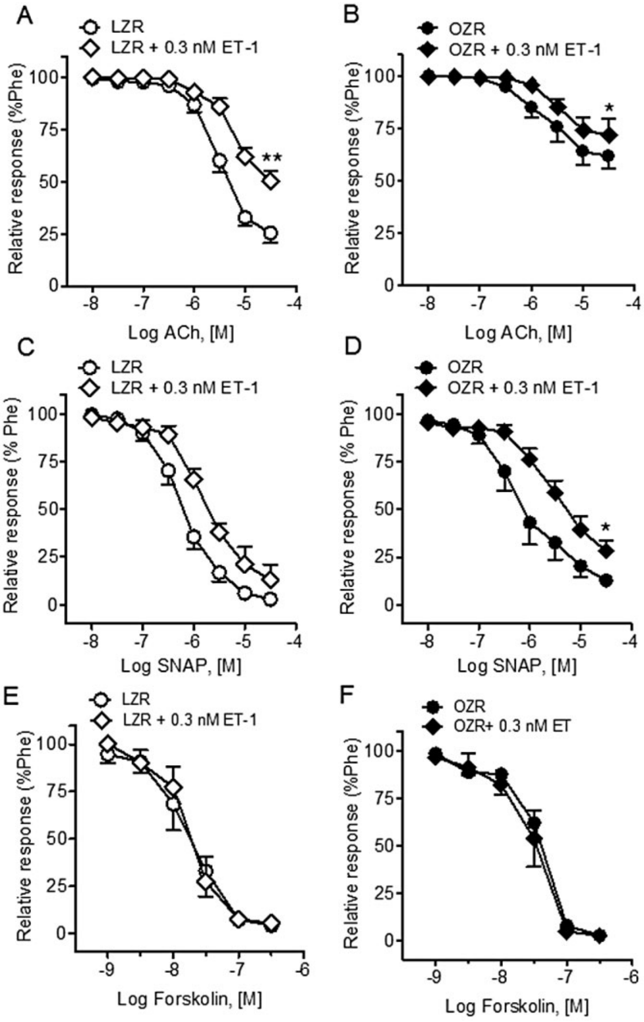

Figure 2

Threshold concentrations of ET-1 inhibited NO-mediated relaxant responses in penile arteries from LZR and OZR. Effect of ET-1 (0.3 nM) on (A, B) the average contractions response curves to ACh, (C, D) the concentration-response curves to the NO donor SNAP and (E, F) the concentration-response curves to the AC activator forskolin in penile arteries precontrated with phenylephrine (Phe; 1 µM) from LZR (A, C, E) and OZR (B, D, F). Results are means ± SEM of $n = 3$–5 animals (one to two arteries from each animal). $^*P < 0.05$, $^{**}P < 0.01$ versus control E_{max}; Student's *t*-test for paired observations.

Effect of NADPH inhibition on the ET-1-induced superoxide formation in erectile tissue

To determine the mechanisms of the ET-1-induced superoxide production in erectile tissue, the effects of NADPH inhibition and NADPH stimulation were assessed. Treatment with the NADPH oxidase inhibitors apocynin (30 µM) or DPI (10 µM) significantly reduced superoxide production induced by ET-1 in corpus cavernosum from LZR and OZR, but apocynin did not change basal superoxide generation (Figure 3). Because PKC is known to activate NADPH oxidase, the effect of non-receptor activation of PKC with PDBu (10 µM) was investigated next. PDBu markedly increased superoxide generation in both LZR and OZR, this augmentation being higher in OZR (Figure 3).

Effect of NOS and NADPH oxidase inhibition and role of endothelium in the ET-1-induced vasoconstriction of penile arteries

To determine the interactions between endothelial-derived NO and superoxide generation stimulated by ET-1, the vasoconstrictor effect of ET-1 was assessed under conditions of NOS or NADPH oxidase blockade in the absence and the presence of endothelium. Treatment with L-NOARG (100 µM) markedly enhanced the constriction induced by the lower concentrations of ET-1 in penile arteries from both LZR and to a lesser extent those in OZR in which ET-1 contractions were already enhanced compared with LZR (Figure 4A and B, Table 2). Mechanical removal of the endothelium suppressed the augmentation induced by NOS blockade of the ET-1 contractions in arteries from both control and obese rats, although this procedure significantly enhanced E_{max} for ET-1 in obese rats (Figure 4C and D, Table 2). On the other hand, inhibition of NADPH oxidase with apocynin (30 µM) largely reduced the augmented vasoconstriction induced by ET-1 under conditions of NOS blockade in both LZR and OZR (Figure 4C and D, Table 2). In endothelium-denuded arteries, apocynin did not reduce any longer ET-1-induced contractions in LZR, while markedly inhibited ET-1 vasoconstriction in OZR (Figure 4E and F, Table 2), suggesting that vascular smooth muscle (VSM) superoxide contributes to the augmented ET-1 contractile responses in obese animals.

Role of ET_A and ET_B receptors in ET-1-induced oxidative stress

Contribution of ET receptors to the higher superoxide levels in erectile tissue from obese animals was assessed in strips of corpus cavernosum incubated with BQ123 or BQ788 under basal conditions and before acute exposure to ET-1. Both the ET_A receptor antagonist BQ123 (1 µM) and the ET_B receptor antagonist BQ788 (0.1 µM) significantly reduced basal and

Table 1

Effect of ET-1 (0.3 nM) and selective antagonism of ET$_A$ (BQ123, 1 μM) and ET$_B$ (BQ788, 0.1 μM) receptors on the concentration-relaxation curves to ACh and to the NO donor in penile arteries from LZR and OZR

	ACh							
	LZR				OZR			
	Phe	pEC$_{50}$	E$_{max}$	*n*	Phe	pEC$_{50}$	E$_{max}$	*n*
Control	2.4 ± 0.5	5.56 ± 0.08	75 ± 5	7	1.1 ± 0.2	5.61 ± 0.15	38 ± 6	7
+ET-1	2.5 ± 0.6	5.27 ± 0.10^b	49 ± 5^b	4	1.3 ± 0.2	5.54 ± 0.07	28 ± 7^b	4
+ET-1 + BQ123	2.6 ± 0.5	5.72 ± 0.19^c	65 ± 6^c	3	1.3 ± 0.1	5.57 ± 0.13	40 ± 4^c	5
+ET-1 + BQ788	2.2 ± 0.3	5.52 ± 0.07	55 ± 8^a	4	1.5 ± 0.3	5.43 ± 0.11	38 ± 7	4
Control	2.4 ± 0.3	5.74 ± 0.12	83 ± 4	7	1.8 ± 0.2	5.28 ± 0.24	54 ± 6	9
+BQ123	2.2 ± 0.2	5.82 ± 0.13	79 ± 9	3	1.7 ± 0.3	5.52 ± 0.10	58 ± 11	4
+BQ788	2.7 ± 0.3	5.88 ± 0.24	82 ± 4	4	1.5 ± 0.3	4.76 ± 0.12	63 ± 8^b	4
+BQ123 + BQ788	2.1 ± 0.3	5.88 ± 0.16	87 ± 6	3	2.0 ± 0.2	5.06 ± 0.13	82 ± 6^a	4

	SNAP							
	LZR				OZR			
	Phe	pEC$_{50}$	E$_{max}$	*n*	Phe	pEC$_{50}$	E$_{max}$	*n*
Control	1.6 ± 0.4	6.23 ± 0.12	97 ± 2	8	1.7 ± 0.6	6.19 ± 0.19	89 ± 3	8
+ET-1	1.3 ± 0.4	5.79 ± 0.08^b	86 ± 8	4	1.5 ± 0.2	5.57 ± 0.11^b	75 ± 5^a	4
+ET-1 + BQ123	1.3 ± 0.3	6.28 ± 0.17^c	96 ± 2	4	1.3 ± 0.1	5.90 ± 0.19	90 ± 3^d	4
+ET-1 + BQ788	1.7 ± 0.3	5.86 ± 0.20	87 ± 6	4	1.1 ± 0.4	6.20 ± 0.12^d	77 ± 8	4

Values represent mean ± SEM of the number *n* of animals (one to two individual arteries were used from each animal). Significant differences from controls were analysed by paired Student's *t*-test and one-way ANOVA followed by Bonferroni *a posterio* test. Phe precontraction (Nm^{-1}).

$^a P < 0.05$; $^b P < 0.01$ versus control.

$^c P < 0.05$; $^d P < 0.01$; versus ET-1-treated.

ET-1-induced enhancement of superoxide levels in OZR (Figure 5), indicating that both ET$_A$ and ET$_B$ receptors mediate the elevated levels of oxidative stress induced by ET-1 in obese rats. In erectile tissue from LZR, only the ET$_A$ receptor antagonist BQ123 reduced the ET-1-elicited generation of superoxide.

Role of ET$_A$ and ET$_B$ receptors in the ET-1 induced impairment of NO-mediated relaxations

To determine the ET receptor/s involved in the acute inhibitory effect of ET-1 on the NO-mediated relaxant responses of penile arteries, the action of selective antagonists of ET$_A$ and ET$_B$ receptors was assessed on the relaxant responses to ACh and SNAP in the presence of ET-1 (10 nM). Figure 6A shows that the antagonist of ET$_A$ receptors BQ123 (1 μM) reversed the inhibitory effect elicited by ET-1 (0.3 nM) on the relaxations induced by ACh in penile arteries from LZR and also in arteries from OZR (Figure 6A and B, Table 1). In contrast, the antagonist of ET$_B$ receptors BQ788 (0.1 μM) failed to significantly change the ET-1-induced inhibition of ACh relaxant responses in arteries from LZR, but reversed the modest inhibition in arteries from OZR (Figure 6C and D, Table 1).

Incubation with the selective antagonist of ET$_A$ receptors BQ123 restored the blunted concentration-response curves to the NO donor SNAP elicited by ET-1 in penile arteries from both LZR and OZR (Figure 7A and B, Table 1). On the other hand, treatment with the ET$_B$ receptor antagonist BQ788 restored SNAP relaxant responses to values before inhibition with ET-1 only in arteries from OZR (Figure 7D) but not in LZR (Figure 7C, Table 1).

In order to investigate whether ET endogenous activity may interfere with the endothelium-dependent relaxations in penile arteries, the effect of selective ET$_A$ and ET$_B$ receptor blockade was assessed on the ACh relaxant responses. Figure 8 shows that while the ET$_A$ receptor antagonist BQ123 did not alter the ACh-induced relaxations in either LZR or OZR (Figure 8A and B), treatment with the antagonist of ET$_B$ receptors BQ788 or combined blockade of ET$_A$ and ET$_B$ receptors improved relaxant responses to ACh in arteries from obese animals (Figure 8C–F, Table 1).

Localization of ET receptors in the endothelium of penile arteries

To further investigate the interactions between endothelial NO and ET-1-derived superoxide, colocalization of ET receptors and eNOS was assessed in penile arteries. Immunoreactivity for ET$_A$ receptors was found in VSM and in the endothelial layer of penile arteries from both LZR and OZR (Figure 9A and D), where it was colocalized with eNOS

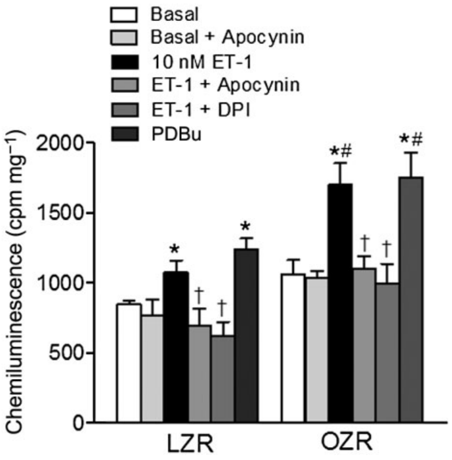

Figure 3

Basal superoxide production in corpus cavernosum tissue from LZR and OZR detected by lucigenin-enhanced chemiluminescence. Effect of the inhibitors of NADPH oxidase, apocynin (30 μM) and diphenylene iodonium (DPI) (10 μM) on both basal and ET-1 (10 nM)-stimulated superoxide production, and of the activator of PKC PDBu (10 μM) on the basal superoxide generation in corpus cavernosum tissue from LZR and OZR detected by lucigenin-enhanced chemiluminescence and expressed in counts per minute (cpm) mg^{-1} of tissue. Experiments were performed in the presence of the NOS inhibitor L-NOARG (100 μM). Data are shown as the means ± SEM of $n = 3$–4 animals (two corpus cavernosum samples from each animal). $^{*}P < 0.05$ versus basal, $^{\dagger}P < 0.05$ versus ET-1-treated, $^{\#}P < 0.05$ versus LZR; Student's *t*-test for unpaired observations.

(Figure 9B, C, E and F). Intensity of immunoreactivity for the ET$_A$ receptor was increased in obese animals, as reported earlier (Contreras *et al*., 2013). The ET$_B$ receptor was found to be primarily expressed in the endothelium of penile arteries from LZR colocalized with eNOS (Figure 9G–I), while it was expressed in both endothelium and smooth muscle layer in arteries from OZR (Figure 9J–L).

Discussion

Enhanced activity and/or levels of ET-1 have been associated to endothelial dysfunction in the insulin-resistant states of obesity and type 2 diabetes (Mather *et al*., 2002; 2004; Pernow *et al*., 2012). In the present study, we demonstrate that ET-1 stimulates NADPH oxidase-derived ROS production which inhibits NO-mediated endothelial relaxations and contributes to contraction through superoxide–NO interactions in penile small arteries. ROS generation by ET-1 was mediated by ET$_A$ receptors in healthy arteries, while in arteries from obese animals both ET$_A$ and ET$_B$ receptors significantly contributed to the augmented ET-1-induced superoxide vascular production and to the blunting of the NO relaxant responses. Acute treatment with the selective ET$_B$ receptor antagonist or combined ET$_A$ and ET$_B$ receptor blockade improved penile endothelial dysfunction while both endothelium- and VSM-derived superoxide contributed to the enhanced vasoconstriction under conditions of obesity-associated insulin resistance.

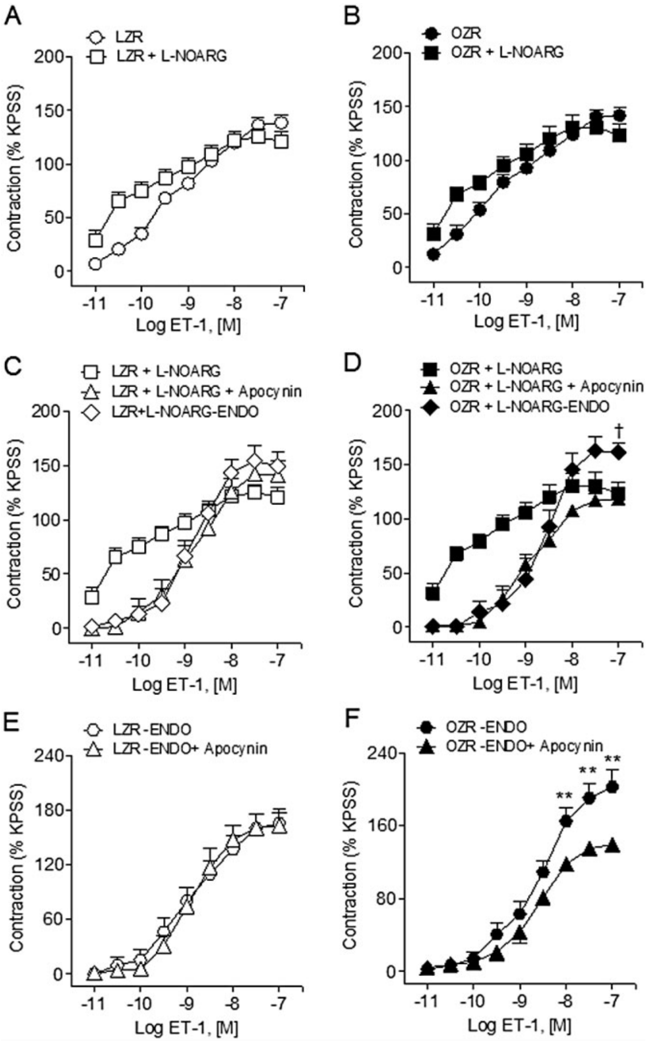

Figure 4

Effect of NOS inhibition, endothelium removal and NADPH blockade on the ET-1-induced vasoconstriction of penile arteries. (A, B) Effect of L-NOARG (100 μM) treatment on the contractile response to ET-1 in penile arteries from LZR (A) and OZR (B). (C, D) Average effect of apocynin (30 μM) and endothelium removal in the presence of L-NOARG on the vasoconstriction induced by ET-1 in penile arteries from LZR and OZR. (E, F) Average effects of apocynin (30 μM) on the ET-1-induced contractions in endothelium-denuded (-ENDO) penile arteries from LZR (E) and OZR (F). Results are means ± SEM of $n = 3$–5 animals (one to two arteries from each animal). $^{\dagger}P < 0.05$ versus L-NOARG-treated E$_{max}$; one-way ANOVA followed by Bonferroni as *a posterio* test. $^{**}P < 0.01$ versus -ENDO; Student's *t*-test for unpaired observations.

Enhanced ET-1 activity reported in insulin-resistant states has been ascribed to the concurrent hyperinsulinaemia resulting in overstimulation of the MAPK pathway and increased production of ET (Potenza *et al*., 2009), but also to the augmented expression of ET receptors in the vascular wall (Mundy *et al*., 2007; Kobayashi *et al*., 2008; Kelly-Cobbs *et al*., 2011; Contreras *et al*., 2013), all this leading to enhanced vasoconstriction, inflammation and oxidative stress. We have recently demonstrated up-regulation of ET$_A$ and ET$_B$ receptors

Table 2

Effect of inhibition of NOS (L-NOARG, 100 μM), endothelium removal (-ENDO) and inhibition of NADPH oxidase (apocynin, 30 μM) on the vasoconstriction elicited by ET-1 in penile arteries from LZR and OZR

	ET-1					
	LZR			OZR		
	pEC$_{50}$	E$_{max}$	*n*	pEC$_{50}$	E$_{max}$	*n*
Control	9.22 ± 0.12	138 ± 7	6	9.56 ± 0.18[f]	142 ± 7	7
+L-NOARG	10.14 ± 0.18[b]	121 ± 9	5	10.44 ± 0.22[a]	123 ± 10	5
+L-NOARG -ENDO	8.95 ± 0.15[d]	150 ± 13	3	8.70 ± 0.18[a,d]	163 ± 9[c]	4
+L-NOARG + apocynin	8.92 ± 0.25[d]	141 ± 14	4	8.92 ± 0.14[d]	119 ± 7	4
Control-ENDO	9.04 ± 0.20[a]	165 ± 15[a]	5	8.68 ± 0.16[b]	203 ± 19[b]	5
-ENDO + apocynin	8.91 ± 0.16	156 ± 14	4	8.78 ± 0.18	139 ± 7[e]	4

Values represent mean ± SEM of the number *n* of animals (one to two individual arteries from each animal were used). Significant differences from controls were analysed by one-way ANOVA followed by Bonferroni as *a posterio* test or Student's *t*-test for unpaired observations.

[a] $P < 0.05$; [b] $P < 0.01$ versus control.

[c] $P < 0.05$; [d] $P < 0.01$ versus L-NOARG-treated.

[e] $P < 0.01$ versus control-ENDO.

[f] $P < 0.05$ versus LZR.

in penile arteries from insulin-resistant OZR linked to both increased contraction and augmented VSM intracellular Ca^{2+} mobilization (Contreras *et al.*, 2013). Our current data show that enhanced expression of ET receptors in penile arteries is additionally coupled to increased ROS production, oxidative stress and endothelial dysfunction in obese animals.

ET-1 can stimulate superoxide generation in both endothelial and VSM cells (Duerrschmidt *et al.*, 2000; Dong *et al.*, 2005; Loomis *et al.*, 2005; Fellner and Arendshorst, 2007; Just *et al.*, 2008; Matsuo *et al.*, 2009; Romero *et al.*, 2009; 2010) through a mechanism involving up-regulation of the main NADPH oxidase cytosolic subunit p47^{phox} (Romero *et al.*, 2009; 2010). This contributes to vasoconstriction (Loomis *et al.*, 2005; Fellner and Arendshorst, 2007; Matsuo *et al.*, 2009) and endothelial dysfunction (Böhm *et al.*, 2007; Romero *et al.*, 2009; 2010) in healthy vessels, and promotes proliferation and reduces apoptosis in human endothelial cells (Dong *et al.*, 2005). Accordingly, the current data demonstrate that acute exposure to ET-1 induces NADPH oxidase-mediated superoxide generation, inhibits relaxations to both ACh and to the NO donor SNAP and causes endothelium-dependent vasoconstriction involving ROS in penile small arteries. Superoxide acts as a vasoconstrictor by directly acting on VSM or by quenching NO. In penile arteries, blockade of NOS greatly enhanced ET-1-induced ROS generation and contractions, and both endothelial removal and NADPH oxidase inhibition with apocynin blunted enhanced constriction which suggests that endothelial NO is buffering the constrictor influence of superoxide released by ET-1 from the endothelium in healthy arteries. These findings are consistent with our recent data demonstrating that ET-1 releases a contractile factor from the penile endothelium (Contreras *et al.*, 2013), and likewise support that reported in both large arteries (Loomis *et al.*, 2005) and in renal and retinal microvessels (Fellner and Arendshorst, 2007; Just *et al.*, 2008), where NADPH oxidase-derived superoxide importantly contributes to the acute vasoconstriction upon ET receptor stimulation.

Oxidative stress is a key pathogenic factor in vascular diseases, such as hypertension and atherosclerosis, and also in the vascular complications of obesity and insulin-resistant states (Sonta *et al.*, 2004; Montezano and Touyz, 2012). Up-regulation of the ET-1 precursor has been found to be associated with enhanced NADPH activity, oxidative stress and expression of the NF-κB in endothelial cells from obese individuals suggesting that ET-1 may induce endothelial dysfunction through NADPH-derived ROS production (Silver *et al.*, 2007). The current data demonstrate that ET-1 is a considerable source of oxidative stress and might contribute to endothelial dysfunction in penile arteries from insulin-resistant rats and confirm the key contribution of NADPH oxidase to vascular ROS generation in obesity-associated insulin resistance (Sonta *et al.*, 2004; Silver *et al.*, 2007; Sánchez *et al.*, 2012). Thus, both basal and basal plus ET-1-stimulated superoxide levels were markedly increased in penile erectile tissue from OZR, blunted by NADPH oxidase inhibition with apocynin and DPI and mimicked by PKC activation with PDBu, the latter being also markedly enhanced in obese animals, which suggests that ET-1 contributes to oxidative stress in arteries from obese animals through NADPH oxidase PKC-dependent ROS generation.

Impaired NO availability as a result of enhanced ET activity was earlier suggested to contribute to endothelial dysfunction in human obesity although the underlying mechanisms were unclear (Mather *et al.*, 2004). In the present study, we found that ET-1 inhibited the relaxations induced by the NO donor SNAP in arteries from OZR, consistent with its contribution to the higher ROS production in erectile tissue, and in agreement with recent clinical studies showing that acute infusion of ET-1 did not only reduce basal forearm blood

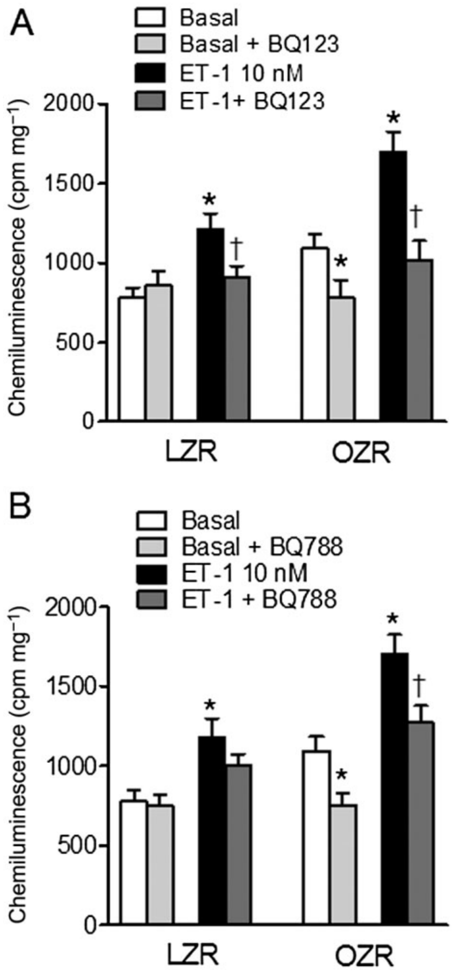

Figure 5

Effect of ET receptor antagonists on the ET-1-induced superoxide production in corpus cavernosum from LZR and OZR detected by lucigenin-enhanced chemiluminescence. Average effects of (A) the ET_A receptor antagonist BQ123 (1 μM) and (B) the ET_B receptor antagonist BQ788 (0.1 μM) on basal and on the ET-1 (10 nM)-stimulated superoxide generation in LZR and OZR. Superoxide production is expressed as counts per minute (cpm) mg^{-1} of tissue. Experiments were performed in the presence of the NOS inhibitor L-NOARG (100 μM). Data are shown as the means ± SEM of $n = 3–5$ animals (two corpus cavernosum samples from each animal). Five to 10 corpus cavernosum samples. $^*P < 0.05$ versus basal, $^{\dagger}P < 0.05$ versus ET-1-treated; Student's *t*-test for unpaired observations.

blow but also impaired endothelium-dependent and endothelium-independent vasodilatations both in healthy individuals (Böhm *et al.*, 2007) and in patients with insulin resistance (Shemyakin *et al.*, 2011). In our study, ET-1 exhibited a lesser inhibitory effect on the endothelium-dependent relaxations to ACh that were already blunted in penile arteries from OZR (Villalba *et al.*, 2009). This suggests that other mechanisms probably dependent on chronic oxidative stress, such as eNOS uncoupling (Münzel *et al.*, 2005; 2010; Sánchez *et al.*, 2012) or prostacyclin synthase oxidation (Du *et al.*, 2006; Sánchez *et al.*, 2010), might additionally contribute to penile endothelial dysfunction in obese animals.

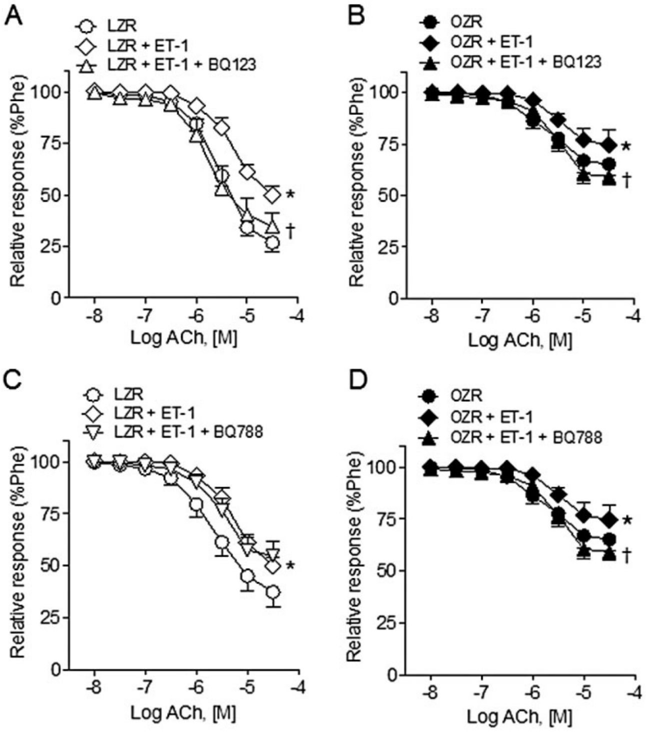

Figure 6

Effects of ET_A and ET_B receptor antagonists on the ET-1-elicited blunting of ACh-induced relaxation of penile arteries from LZR and OZR. (A, B) Effects of ET-1 (0.3 nM) and the ET_A receptor antagonist BQ123 (1 μM) on the relaxant responses elicited by ACh in penile arteries from LZR (A) and OZR (B). (C, D) Average effects of ET-1 and of the ET_B receptor antagonist BQ788 (0.1 μM) on the relaxant responses to ACh in penile arteries from LZR (C) and OZR (D). Results are means ± SEM of $n = 3–5$ animals (one to two arteries from each animal). $^*P < 0.05$ versus control E_{max}, $^{\dagger}P < 0.05$ versus ET-1-treated E_{max}; Student's *t*-test for unpaired observations.

Enhanced ET levels and ROS vascular generation have been linked to increased ROS-mediated ET-1-induced venoconstriction in mineralocorticoid hypertension, (Li *et al.*, 2003). Here, we also demonstrate that dysregulated ROS production in erectile tissue from OZR results in a higher constrictor influence of ROS released by ET-1 not only from the endothelium but also from VSM. Thus, enhanced contractions to lower concentrations of ET-1 under conditions of NOS inhibition were largely reduced by endothelial removal or apocynin confirming the constrictor influence of ET-1-stimulated endothelial ROS in arteries from OZR, this influence exhibiting a trend to be higher than in lean rats. Interestingly, removal of the endothelium unmasked an augmented vasoconstriction to the highest concentrations of ET-1 in OZR (Contreras *et al.*, 2013) that was blunted by inhibition of NADPH oxidase with apocynin. These findings suggest an increased ROS release by ET-1 from VSM counterbalanced by an increased NO relaxing influence from the endothelium in obese rats, as earlier observed in obese individuals where ET antagonism unmasked an augmented NO synthesis capacity that counterbalanced enhanced ET vasoconstrictor activity (Mather *et al.*, 2004).

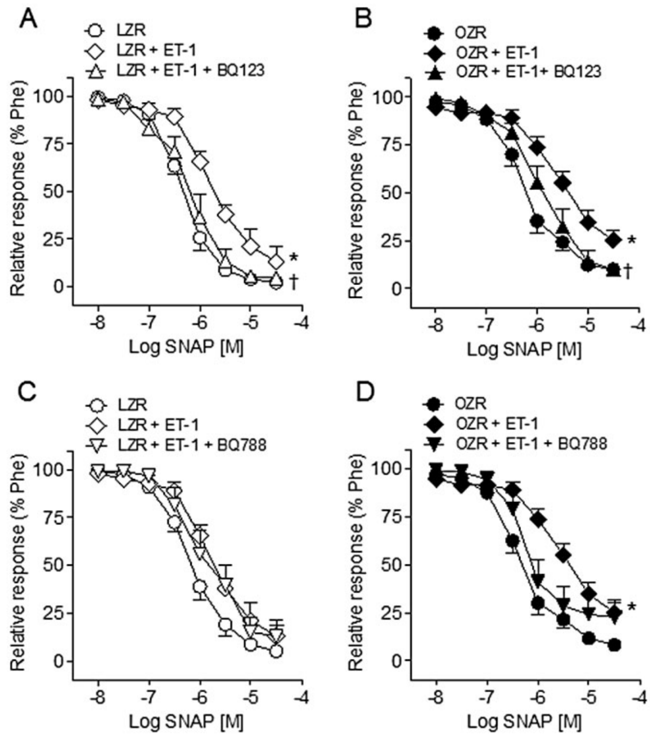

Figure 7

Effects of ET_A and ET_B receptor antagonists on the ET-1-elicited blunting of the relaxations induced by the NO donor SNAP in penile arteries of LZR and OZR. (A, B) Effects of ET-1 (0.3 nM) and of the ET_A receptor antagonist BQ123 (1 μM) on the relaxant responses elicited by SNAP in penile arteries from LZR (A) and OZR (B). (C, D) Average effects of ET-1 and of the ET_B receptor antagonist BQ788 (0.1 μM) on the relaxant responses to SNAP in penile arteries from LZR (C) and OZR (D). Results are means ± SEM of $n = 4–8$ animals (one to two arteries from each animal). $^{*}P < 0.05$ versus control E_{max}, $^{\dagger}P < 0.05$ versus ET-1-treated E_{max}; Student's *t*-test for unpaired observations.

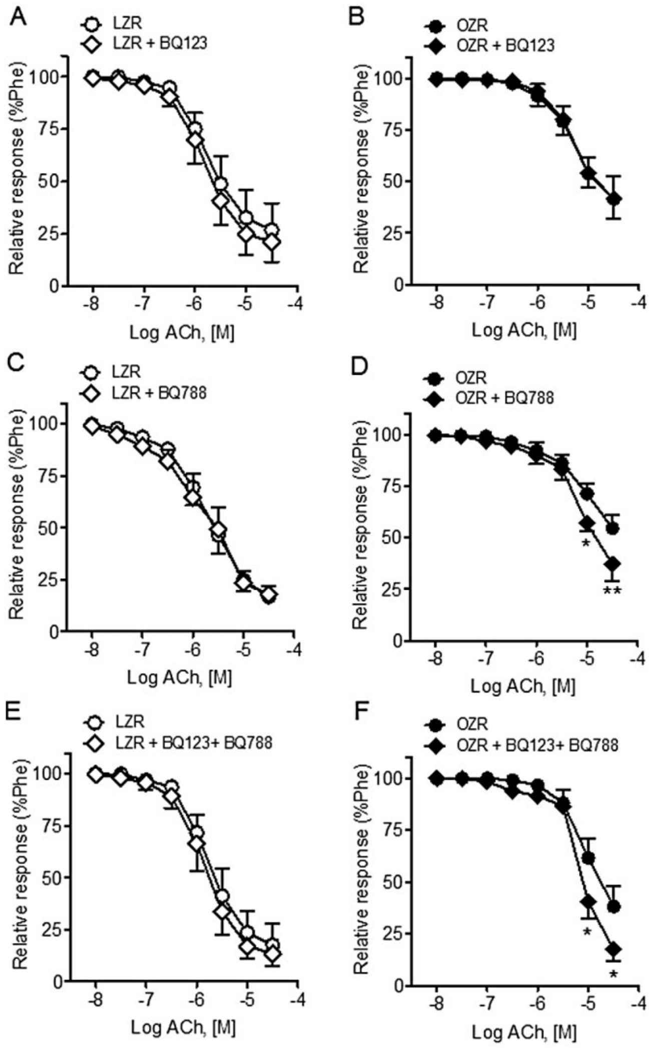

Figure 8

Effects of ET_A and ET_B receptor antagonists on the ACh-induced relaxations of penile arteries of LZR and OZR. Average effects of (A, B) the ET_A receptor antagonist BQ123 (1 μM), (C, D) the ET_B receptor antagonist BQ788 (0.1 μM) or (E,F) combined blockade of ET_A receptors (BQ123, 1 μM) and ET_B receptor (BQ788, 0.1 μM) on the relaxant responses elicited by ACh in penile arteries from LZR (A, C, E) and OZR (B, D, F). Results are means ± SEM of 6–8 arteries. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; Student's *t*-test for paired observations.

ET receptor blockade has been reported to improve endothelial function in obese and type 2 diabetic individuals (Mather *et al.*, 2002; Weil *et al.*, 2011; Rafnsson *et al.*, 2012) and experimental studies have confirmed the involvement of ET-1 through ET_A receptors in the blunted NO-mediated endothelium-dependent relaxations of arteries from diet-induced obese mice (Traupe *et al.*, 2002) and mouse models of atherosclerosis (Barton *et al.*, 1998; Böhm *et al.*, 2002). ET_A receptors have likewise been involved in the enhanced vascular oxidative stress in experimental hypertension (Callera *et al.*, 2003; Li *et al.*, 2003; Laplante *et al.*, 2005; Viel *et al.*, 2008).

In the present study, ET_A receptor-mediated effects were functionally comparable in erectile tissue from LZR and OZR, although up-regulation of these receptors in penile arteries of OZR (Contreras *et al.*, 2013) along with the ability of BQ123 to restore augmented basal and ET-1-stimulated superoxide to levels similar to those in LZR suggest a role for ET_A receptors in oxidative stress and endothelial dysfunction in obese rats. Interestingly, we also demonstrate here that while ET_B receptors do not significantly contribute to superoxide generation in healthy arteries, up-regulation of ET_B receptors is associated to enhanced oxidative stress, blunting of NO-mediated relaxant responses and endothelial dysfunction in penile arteries from insulin-resistant OZR. ET_B receptors have earlier been shown to stimulate superoxide release in human umbilical endothelial cells (Duerrschmidt *et al.*, 2000; Dong *et al.*, 2005) through a mechanism involving enhanced expression of the NADPH oxidase subunits $gp91^{phox}$ (Duerrschmidt *et al.*, 2000) and $gp47^{phox}$ (Dong *et al.*, 2005) and subsequent increased NADPH oxidase activity with submaximal effects after 30 min (Duerrschmidt *et al.*, 2000). This might explain the acute effects of ET-1 on endothelial dysfunction observed here and in clinical studies (Böhm *et al.*, 2007; Shemyakin *et al.*, 2011), and also the beneficial effect of acute treatment with the ET_B receptor antagonist on the endothelium-

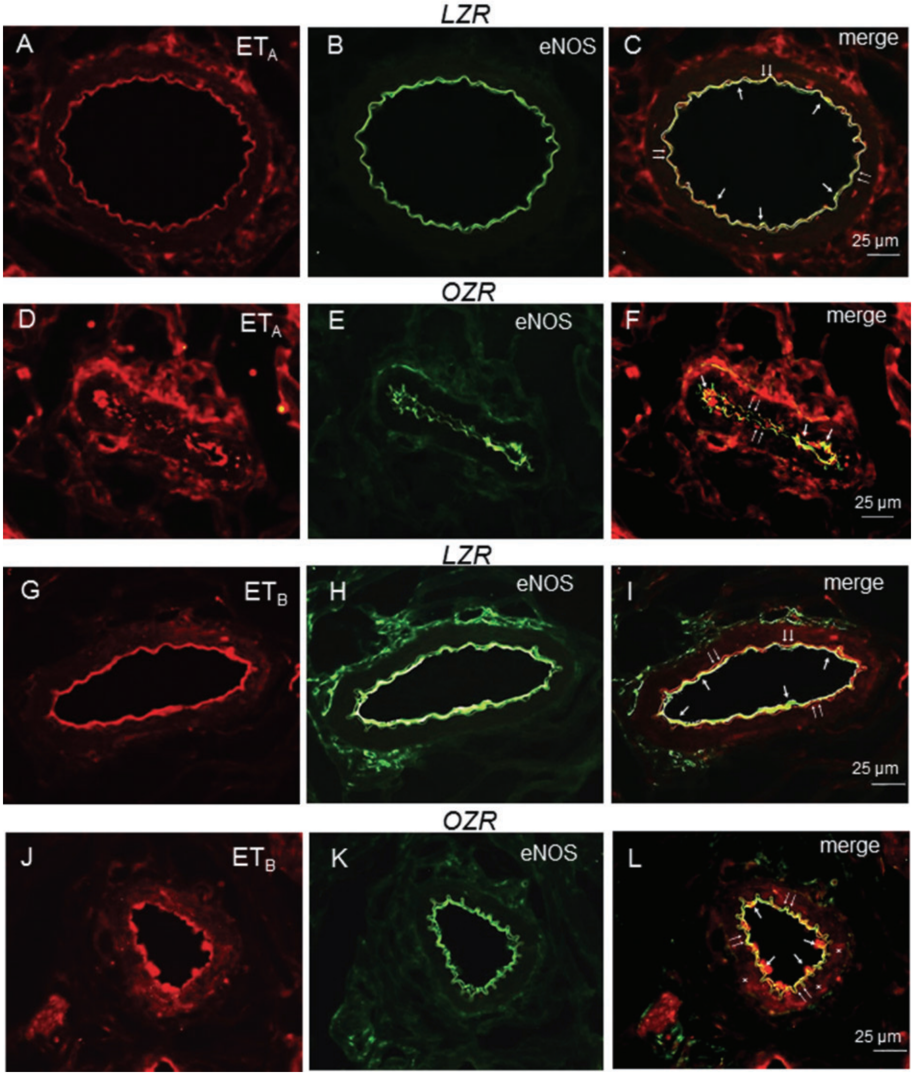

Figure 9

Representative immunohistochemical staining of ET$_A$ and ET$_B$ receptor expression in the endothelium and smooth muscle layer of penile arteries from LZR and OZR. (A, D) Immunofluorescence for ET$_A$ receptors (red areas) is distributed in the endothelial and media layers of the arterial wall in LZR and OZR. (B, E) Endothelial cell layer was visualized with the anti-eNOS marker (green). (C, F) Immunofluorescence double labelling for eNOS marker and ET$_A$ receptor expression in endothelial cell layer demonstrates colocalization in endothelium (yellow areas) in LZR and OZR penile arteries. (G, J) Immunofluorescence for ET$_B$ receptors (red areas) is distributed in the endothelial layer of the arterial wall in LZR and OZR penile arteries and in the smooth muscle layer in OZR (asterisks). (H, K) Endothelial cell layer was visualized with the anti-eNOS marker (green). (I, L) Immunofluorescence double labelling for eNOS marker and ET$_B$ receptor expression in endothelial cell layer demonstrates colocalization in endothelium (yellow areas, arrows) in LZR and OZR. Scale bars indicate 25 µm. Sections are representative of $n = 3$ LZR animals and OZR animals. Double arrows: internal elastic layer.

dependent relaxations and on basal and ET-1-stimulated O_2^- production in penile arteries from OZR. The contribution of ET$_B$ along with ET$_A$ receptors to the ET-1-induced superoxide generation, as shown in the present study, has also been demonstrated in healthy aorta (Loomis *et al.*, 2005) and renal microvessels (Fellner and Arendshorst, 2007; Just *et al.*, 2008) in which these receptors mediate vasoconstriction, and in coronary artery bypass grafts (Cerrato *et al.*, 2012). Furthermore, up-regulation of ET$_B$ receptors coupled to increased superoxide production has been reported in sympathetic neurons of deoxycorticosterone acetate and high-salt diet (DOCA-salt) hypertensive rats (Dai *et al.*, 2004). Enhanced expression of both ET$_A$ and ET$_B$ receptors colocalized with eNOS in the penile endothelium of obese rats, as shown in the present study, would favour superoxide–NO interactions and endothelial dysfunction under conditions of dysregulated ROS signalling and enhanced superoxide production in insulin resistance.

ET$_B$ receptors minimally contribute to vasoconstriction in healthy penile arteries, but their up-regulation in both endothelium and VSM of penile arteries from OZR was associated to augmented ET-1 endothelium-dependent and endothelium-independent contractions respectively (Contreras *et al.*, 2013). ET_B receptor antagonism significantly reduced VSM calcium mobilization and endothelium-independent contractions to ET-1 only in OZR (Contreras *et al.*, 2013) to a degree similar to that obtained with the NADPH oxidase inhibitor apocynin in the present study. Therefore, ET_B receptors might be involved in the ET-1-induced ROS release from VSM in obese rats. Accordingly, augmented expression of VSM ET_B receptors has recently been associated to an attenuation of the endothelial ET_B receptor-mediated prevention of vascular remodelling in arteries from type 2 diabetic animals (Kelly-Cobbs *et al.*, 2011) and ET-1 stimulates proliferation via an ET_B-NADPH oxidase-dependent pathway (Dong *et al.*, 2005).

Taken together, the current findings would be consistent with clinical studies showing that dual ET_A/ET_B receptor blockade, but not selective ET_A blockade, improved endothelium-dependent vasodilatation and peripheral endothelial function in subjects with insulin resistance and type 2 diabetes (Shemyakin *et al.*, 2006; Rafnsson *et al.*, 2012), and restored endothelial cerebral vasodilatation in experimental models of type 2 diabetes (Abdelsaid *et al.*, 2014), thus supporting the concept that ET_B receptor-mediated activation of NADPH oxidase importantly contributes to oxidative stress and endothelial dysfunction under conditions of insulin resistance. Likewise, our results showing that ET_B receptor antagonism reduced oxidative stress and endothelial dysfunction induced by ET-1 in erectile tissue from insulin-resistant obese rats might explain why the use of ET_A receptor antagonists alone has been reported to render variable results in earlier clinical studies, failing to improve erectile function in men with mild to moderate ED of unstated aetiology while enhancing erectile responses in animal models (Kim *et al.*, 2002). Thus, while selective ET_A receptor blockade improved erectile function in experimental models of hypertension (Carneiro *et al.*, 2008), our findings suggest that antagonism of ET_B receptors might be beneficial for endothelial dysfunction in the ED associated with insulin-resistant states.

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Author contributions

A. S. was responsible for acquisition of data, analysis and interpretation of data and drafting of the article. P. M. was responsible for acquisition of data and analysis and interpretation of data. M. M. was responsible for acquisition of data and analysis and interpretation of data. S. B. was responsible for analysis and interpretation of data and revising the article for intellectual content. A. G.-S. was responsible for revising the article for intellectual content. M. H. was responsible for analysis and interpretation of data and revising the article for intellectual content. D. P. was responsible for drafting the article, revising the article for intellectual content and final approval of the completed article.

Conflict of interest

None of the authors have any conflict of interests.

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