Impaired Ca\(^{2+}\) handling in penile arteries from prediabetic Zucker rats: involvement of Rho kinase

Nuria Villalba, Cristina Contreras, Medardo Hernández, Albino García-Sacristán, and Dolores Prieto

Departamento de Fisiología, Facultad de Farmacia, Universidad Complutense, Madrid, Spain

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Villalba N, Contreras C, Hernández M, García-Sacristán A, Prieto D. Impaired Ca\(^{2+}\) handling in penile arteries from prediabetic Zucker rats: involvement of Rho kinase. Am J Physiol Heart Circ Physiol 300: H2044–H2053, 2011. First published February 4, 2011; doi:10.1152/ajpheart.01204.2010.—Diabetes is associated with an increased vascular tone usually involved in the pathogenesis of diabetic cardiovascular complications such as hypertension, stroke, coronary artery disease, or erectile dysfunction (ED). Enhanced contractility of penile erectile tissue has been associated with augmented activity of the RhoA/Rho kinase (RhoK) pathway in models of diabetes-associated ED. The present study assessed whether abnormal vasoconstriction in penile arteries from prediabetic obese Zucker rats (OZRs) is due to changes in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]) and/or in myofilament Ca\(^{2+}\) sensitivity. Penile arteries from OZRs and lean Zucker rats (LZRs) were mounted on microvascular myographs for simultaneous measurements of [Ca\(^{2+}\)\(_i\)] and tension. The relationships between [Ca\(^{2+}\)\(_i\)] and contraction for the \(\alpha_1\)-adrenergic vasoconstrictor phenylephrine (PE) were left shifted and steeper in OZRs compared with LZRs, and this increase was associated with an increase in both the sensitivity and maximum responses to Ca\(^{2+}\). The RhoK inhibitor Y-27632 (10 \(\mu\)M) reduced the vasoconstriction induced by PE to a greater extent in OZRs than in LZRs, without altering Ca\(^{2+}\). Y-27632 inhibited with a greater potency the contraction elicited by high KCl in arteries from OZRs compared with LZRs without changing [Ca\(^{2+}\)]. RhoK-II expression was augmented in arteries from OZRs. These results suggest receptor-specific changes in the Ca\(^{2+}\) handling of penile arteries under conditions of metabolic syndrome. Whereas augmented vasoconstriction upon activation of the thromboxane A\(_2\) receptor is coupled to enhanced Ca\(^{2+}\) entry, a RhoK-mediated enhancement of myofilament Ca\(^{2+}\) sensitivity is coupled with the \(\alpha_1\)-adrenergic vasoconstriction in penile arteries from OZRs.

calcium handling; \(\alpha_1\)-adrenergic; thromboxane A\(_2\) receptor; obese zucker rat

Diabetes is associated with an increased vascular tone usually involved in the pathogenesis of diabetic cardiovascular complications such as hypertension, stroke, or coronary artery disease (3, 22, 43). Both augmented smooth muscle contractility and endothelial dysfunction have been shown to underlie the abnormal vasoconstriction in arteries from diabetic and metabolic syndrome patients and animal models (16, 18, 19, 28). The enhanced vascular smooth muscle contractile responses in diabetes have, in turn, been ascribed to increases in Ca\(^{2+}\) entry and/or Ca\(^{2+}\) release (1, 6, 16, 36), although elevation in the Ca\(^{2+}\) sensitivity of the contractile proteins has alternatively been proposed as the mechanism underlying the increased vascular contractility associated with diabetes (12, 23, 26).

Elevation of the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) in vascular smooth muscle cells is considered a classic trigger for force development through the activation of myosin light chain (MLC) kinase (MLCK) and the subsequent phosphorylation of regulatory MLC, resulting in shortening of vascular smooth muscle and contraction (35). In addition, regulation of the activity of MLC phosphatase (MLCP) mediates the myofilament sensitivity to Ca\(^{2+}\), allowing changes in the magnitude of force development for any given level of [Ca\(^{2+}\)], a mechanism of Ca\(^{2+}\) sensitization (35). RhoA is a small monomeric G protein that activates Rho kinase (RhoK), and inhibition of MLCP activity by phosphorylation through the RhoA/RhoK pathway is a main mechanism of Ca\(^{2+}\) sensitization, although other kinases, such as PKC, can also phosphorylate MLCP and thus regulate myofilament Ca\(^{2+}\) sensitivity. RhoK and PKC pathways can converge to phosphorylate an inhibitory protein of MLCP, CPI-17 (35).

RhoA/RhoK is involved in the major vasoconstrictor pathways responsible for the maintenance of penile flaccidity (11, 24, 31), and alterations in RhoA/RhoK activity have been proposed to play a role in the pathogenesis of diabetes-associated ED (7, 44, 46). Thus, RhoK regulates the contraction of erectile tissue in response to endothelin-1 and \(\alpha_1\)-adrenergic agonists (24, 41, 45), and enhanced vasoconstriction of corporal tissue to these agonists has been demonstrated to be associated with augmented expression of RhoK in the prediabetic obese Zucker rat (OZR) (45, 46). Furthermore, the beneficial effect of RhoK inhibition on the impaired erectile responses to nerve stimulation has been shown in models of both type 1 (7) and type 2 (46) diabetes-associated ED.

Metabolic syndrome is a cluster of metabolic and cardiovascular abnormalities that usually precedes type 2 diabetes, including obesity, insulin resistance, dyslipidemia, and hypertension, all of which act as independent risk factors for ED (17). We (33, 39) have recently demonstrated blunted endothelium-dependent vasodilatation and augmented vasoconstriction due in part to impaired basal release of nitric oxide (NO)

Penile Erection is a predominantly vascular event that requires integrity of the nerves to the penis and an intact endothelium (4, 30). Due to its high prevalence in patients with cardiovascular risk factors such diabetes, hypertension, and hyperlipidemia, erectile dysfunction (ED) is currently considered an early sign of endothelial dysfunction and vascular disease (25). ED is a common complication and frequent cause of decreased quality of life in diabetic men, with its prevalence being three times higher in type 1 and type 2 diabetic patients than in the general population (38).

Address for reprint requests and other correspondence: D. Prieto, Departamento de Fisiología, Facultad de Farmacia, Universidad Complutense, Madrid 28040, Spain (e-mail: dprieto@farm.ucm.es).

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in penile arteries from OZRs, a model of prediabetes/metabolic syndrome. However, whether the enhanced vasoconstriction of erectile tissue in this and other models of diabetes-associated ED is due to changes in the Ca\(^{2+}\)/H\(_{11001}\) handling of penile vascular smooth muscle cells is currently unknown. Therefore, the present study assessed whether abnormal vasoconstriction was associated with changes in [Ca\(^{2+}\)/H\(_{11001}\)] and/or in the sensitivity of the contractile proteins to Ca\(^{2+}\) in penile arteries from OZRs.

**METHODS**

**Animals.** All animal protocols conformed with the United States National Guide for the Care and the Use of Laboratory Animals and were approved by the Animal Experimentation Ethics Committee of Complutense University. Adult male OZRs (n = 41) and their control counterparts, lean Zucker rats (LZRs; n = 37), were purchased from Charles River Laboratories (Barcelona, Spain) at 8–10 wk of age and maintained on standard chow and water ad libitum until they were used for study at 17–18 wk of age. On the day of the experiment, rats were weighed, blood samples were collected, and plasma was frozen for the determination of nonfasting glucose levels using commercially available kits. Systolic blood pressure (SBP) and heart rate were measured weekly by tail-cuff plethysmography.

**Dissection, mounting, and force measurement.** The penis was excised and transferred into cold physiological saline solution (PSS) of the following composition (in mM): 119 NaCl, 4.7 KCl, 1.18 KH\(_2\)PO\(_4\), 1.17 MgSO\(_4\), 1.5 CaCl\(_2\), 24.9 NaHCO\(_3\), 0.027 EDTA, and 11 glucose (pH 7.4). The penile arteries, first- or second-order branches of the rat dorsal penile artery, were carefully dissected by removing the connective and fat tissue and mounted in a microvascular myograph (Danish Myotechnology) as ring preparations by inserting two 40-μm tungsten wires into the vessel lumen. The arteries were equilibrated for 30 min in PSS at 37°C while continuously gassed with a mixture of 95% O\(_{2}\)-5% CO\(_2\), and the relationship between passive wall tension and internal circumference was then determined for each individual artery. From this, the internal circumference corresponding to a transmural pressure of 100 mmHg (L\(_{100}\)) for a relaxed vessel in situ was calculated. The arteries were set to an internal circumference (L\(_i\)) equal to 0.9 times L\(_{100}\) (L\(_i\) = 0.9 × L\(_{100}\)) at which tension development is maximal in small arteries. The normalized internal lumen diameter (l\(_i\)) of penile arteries was 163 ± 6 μm (n = 26) in the LZR group and 146 ± 8 μm (n = 28) in the OZR group.

**Fig. 1.** Increased Ca\(^{2+}\) sensitivity upon \(\alpha_1\)-adrenergic stimulation in penile arteries from obese Zucker rats (OZRs). A and C: representative traces illustrating the changes in both intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)/H\(_{11001}\]; top) and tension (bottom) induced by high-K\(^+\) physiological saline solution (KPSS; 124 mM KCl) and by cumulative addition of the \(\alpha_1\)-adrenergic agonist phenylephrine (PE; Phe) in arteries from lean Zucker rats (LZRs; A) and OZRs (C). B and D: summarized data showing the changes in [Ca\(^{2+}\)/H\(_{11001}\)] (top) and tension (bottom) in response to cumulative doses of PE. Values are means ± SE; n = 6 arteries. F\(_{340}/F_{380}\), 340- and 380-nm fluorescence ratio. Responses are percentages of the maximal rise in F\(_{340}/F_{380}\) and contraction elicited by KPSS.
Simultaneous measurements of \([\text{Ca}^{2+}]_i\) and tension. Simultaneous measurements of \([\text{Ca}^{2+}]_i\) and tension were performed in intact arterial segments by fura-2 AM fluorescence as previously described (40). Briefly, arteries were loaded in the dark in PSS containing 8 \(\mu\)M fura-2 AM and 0.05% Cremophor EL for 3 h at 37°C. They were washed three times in PSS, and the solution was changed to PSS with fresh fura-2 AM after 90 min. The myograph chamber was mounted on a Zeiss inverted microscope equipped for dual-excitation wavelength fluorimetry (Deltascan, Photon Technology). Arteries were illuminated with alternating 340- and 380-nm light, and the intensity of the emitted fluorescence was collected at a wavelength of 510 nm using a photomultiplier and monitored together with the tension. At the end of each experiment, \([\text{Ca}^{2+}]_i\)-insensitive signals were determined after quenching with Mn²⁺, and the values obtained were subtracted.

Table 1. Effects of PE and U-46619 on the sensitivity and maximal responses of \([\text{Ca}^{2+}]_i\), and tension in penile arteries from LZR and OZR

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<th>LZR Group</th>
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<td>(\text{pD}_2)</td>
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<td>[\text{Ca}^{2+}]_i, % of KPSS</td>
<td>6.40 ± 0.10</td>
<td>92.2 ± 12.1</td>
<td>6</td>
<td>6.51 ± 0.41</td>
<td>37.0 ± 8.2†</td>
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<td>Tension, % of KPSS</td>
<td>6.40 ± 0.15</td>
<td>146.8 ± 11.8</td>
<td>6</td>
<td>6.22 ± 0.18</td>
<td>147.3 ± 20.7</td>
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<td>U-46619 [\text{Ca}^{2+}]_i, % of KPSS</td>
<td>7.22 ± 0.19</td>
<td>100.5 ± 18.8</td>
<td>8</td>
<td>8.05 ± 0.08†</td>
<td>145.3 ± 18.2*</td>
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<tr>
<td>Tension, % of KPSS</td>
<td>7.14 ± 0.16</td>
<td>120.7 ± 11.8</td>
<td>8</td>
<td>7.69 ± 0.11†</td>
<td>189.4 ± 28.6*</td>
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Data are means ± SE; \(n\), number of arteries (1 artery/animal). Results are expressed as percentages of the increases in intracellular \(\text{Ca}^{2+}\) concentration (\([\text{Ca}^{2+}]_i\)) and tension induced by high-K⁺ physiological saline solution (KPSS) in each artery. LZR, lean Zucker rats; OZR, obese Zucker rats; PE, phenylephrine; \(E_{\text{max}}\), maximal response. Significant differences were analyzed by an unpaired Student’s \(t\)-test. *\(P < 0.05\) and †\(P < 0.01\) vs. the LZR group.

Fig. 2. Enhanced increase in \([\text{Ca}^{2+}]_i\) entry and vasoconstriction upon thromboxane A₂ (TXA₂) receptor stimulation in penile arteries from OZR. A and C: representative traces illustrating the changes in both \([\text{Ca}^{2+}]_i\), (top) and tension (bottom) induced by KPSS (124 mM KCl) and by cumulative addition of the TXA₂ analog U-46619 in arteries from LZR(A) and OZR(C). B and D: summarized data showing the changes in \([\text{Ca}^{2+}]_i\), (top) and tension (bottom) in response to cumulative doses of U-46619. Values are means ± SE; \(n = 7–8\) arteries. Responses are percentages of the maximal rise in \(F_{340}/F_{380}\) and contraction elicited by KPSS.
from those obtained during the experiment. The ratio of fluorescence at 340 and 380 nm (F340/F380) corrected for auto fluorescence was taken as a measure of [Ca2+].

Experimental procedure for the functional experiments. After normalization, the contractile ability of the arteries was tested by stimulating them with a high-K+ solution (KPSS; equivalent to PSS except that NaCl was exchanged for KCl on an equimolar basis, giving a final concentration of 123.7 mM K+). Vasorelaxant responses to the α1-adrenoceptor agonist phenylephrine (PE) or to the thromboxane A2 (TXA2) receptor analog U-46619 were assessed by constructing cumulative concentration-response curves to these agonists. The effects of a selective inhibitor of RhoK, Y-27632, were evaluated on the adrenergic contractions elicited by electrical field stimulation (EFS) and on the changes in isometric tension and [Ca2+]i induced by the α1-adrenoceptor agonist PE and high-K+ depolarization in rat penile small arteries.

EFS was performed with a pair of thick platinum square electrodes measuring 2 × 2 mm fixed in plastic mounting jaws of the myograph and connected to an electrical stimulator (Cibertec, CS20, Barcelona, Spain) with a constant current output adjusted to 35 mA. Frequency-response curves to EFS were obtained at baseline tension over a range of 1–32 Hz by delivering 20-s pulse trains (0.3-ms pulse duration) at intervals of 5 min (34). To test the influence of RhoK inhibition on the contractions elicited by electrical stimulation of perivascular nerves in the arterial wall, frequency-response curves were performed in the absence and presence of a submaximal concentration of Y-27632 (10 μM) (20, 41). These experiments were carried out in the presence of the NO synthase (NOS) inhibitor Nω-nitro-L-arginine (L-NNA; 100 μM) to avoid the effects of neural-released NO.

In a second set of experiments, the effects of RhoK inhibition on the changes in isometric [Ca2+]i and tension induced by high-K+ depolarization were evaluated by adding increasing concentrations of Y-27632 (0.1–100 μM) on arteries precontracted with KPSS. In these experiments, phentolamine (0.3 μM) was used to avoid the effects of neural-released norepinephrine.

Protein expression of RhoK. To examine levels of total RhoK, dorsal penile arteries from 17- to 18 wk-old LZRs (n = 11) and OZRs (n = 13) were dissected free and snap frozen in liquid N2. Each sample was homogenized in 10 mM Tris-HCl (pH 7.4) in buffer containing 1% SDS and 1 mM Na3VO4. Samples were centrifuged at 14,000 g for 10 min at 4°C to remove insoluble debris. The supernatant was collected, and sample protein concentrations were determined by the Lowry method. Equal amounts of proteins (15 μg) were loaded in wells and subjected to electrophoresis on a 10% polyacrylamide-SDS gel followed by blotting to a polyvinylidene difluoride membrane. Membranes were blocked for 1 h at room temperature with blocking buffer containing 0.01% Tween 20. Blots were then incubated overnight at 4°C with a mouse monoclonal antibody for RhoK (1:200, Santa Cruz Biotechnology). For immunochemical labeling, blots were incubated for 1 h at room temperature with anti-rabbit IgG (1:4,000, Biosciences). Subsequent to RhoK detection, blots were washed in PBS containing 0.1% Tween 20 and incubated for 1 h with rabbit monoclonal antibody for α-actin (1:2,500, Sigma). Immunoblotting was achieved by incubating blots for 1 h at room temperature with anti-rabbit IgG (1:5,000, Sigma). Quantification of the bands was accomplished by densitometric analysis of scanned images using ImageJ software. Bands for RhoK were normalized to those of α-actin. Each artery (~1 cm in length) from each animal was used to determine the expression of total RhoK.

Statistics and data analysis. In the functional experiments, the mechanical responses of the arteries were measured as force and expressed as active wall tension, which is the increase in force divided by twice the segment length. The results are expressed as either absolute values [in mN of tension or in units of ratio of fluorescence (F340/F380)] or as a percentage of the responses induced by KPSS. Data are expressed as means ± SE; n represents the number of arteries. Differences between means were analyzed using paired or unpaired Student’s t-tests when appropriate. P values of ≤0.05 were considered significant. All calculations were made using a standard software package (Prism 5.0, GraphPad Software).

RESULTS

General parameters. At 17–18 wk of age, OZRs displayed a significant increase in body weight compared with LZRs (495 ± 6 vs. 373 ± 5 g, n = 37, P < 0.001) and exhibited mild hyperglycemia (140 ± 15 mg/ml, n = 13, vs. 101 ± 4 mg/ml, n = 12, P < 0.05). SBP was similar in LZRs and OZRs (121 ± 2 and 128 ± 6 mmHg, n = 6).

PE-induced changes in [Ca2+]i and tension. Activation of the α1-adrenoceptor with PE produced a concentration-dependent vasoconstriction in dorsal penile arteries from both LZRs and OZRs, with no differences in either the sensitivity or maximal response to PE between the two strains (Fig. 1 and Table 1). PE also produced simultaneous concentration-dependent increases in smooth muscle [Ca2+]i in arteries from LZRs. In contrast, PE-induced Ca2+ entry was significantly reduced in arteries from OZRs (Fig. 1 and Table 1). The changes in the Ca2+ dependence of PE-mediated vasoconstriction in penile arteries of obese animals are shown in Fig. 3A. The relationships between

Fig. 3. Effect of the α1-adrenergic agonist PE (Δ) and the TXA2 analog U-46619 (B) on the relationship between tension and [Ca2+]i in penile arteries from LZRs and OZRs. Values are means ± SE; n = 6–8 arteries. Responses are percentages of the maximal rise in F340/F380 and contraction elicited by KPSS.
[Ca^{2+}], and contraction for the α₁-adrenergic vasoconstrictor PE were left shifted and steeper in OZRs compared with LZRs, although the magnitude of the contraction was similar in both groups, thus indicating an increase in the Ca^{2+} sensitization processes linked to the α₁-adrenoceptor in arteries from obese animals.

**U-46619-induced changes in [Ca^{2+}], and tension.** Figure 2 shows the changes in [Ca^{2+}]i and contraction elicited by activation of the TXA2 receptor in penile arteries from LZRs and OZRs. Contractions evoked by U-46619 were accompanied by simultaneous increases in smooth muscle [Ca^{2+}]i in control arteries (Fig. 2, A and B, and Table 1). Both Ca^{2+} entry and vasoconstriction evoked by this agonist were greatly enhanced in terms of the sensitivity and maximum response in penile arteries from OZR (Fig. 2, C and D, and Table 1). While the slope of the relationships of [Ca^{2+}]i, to contraction was not significantly different, the enhanced vasoconstriction in penile arteries from OZRs was associated with both an enhanced sensitivity and an increase in the maximum smooth muscle Ca^{2+} response (Fig. 3B and Table 1).

**Effect of RhoK inhibition on EFS and Phe-induced vasoconstrictor and [Ca^{2+}], responses.** To evaluate the mechanisms involved in the augmented Ca^{2+} sensitization of penile arteries from obese rats, we next investigated the effect of RhoK inhibition on the vasoconstrictor responses evoked by activation of the α₁-adrenoceptor with either PE or by endogenous norepinephrine released from nerve endings. EFS (1–32 Hz) performed at resting tension elicited frequency-dependent contractions of dorsal penile arteries from both LZRs and OZRs (Fig. 4). The selective RhoK inhibitor Y-27632 (10 μM) markedly reduced the electrically induced contractions in arteries from both groups, with maximal responses at 32 Hz in LZRs being 1.07 ± 0.3 and 0.2 ± 0.05 Nm⁻¹ (n = 7, P < 0.0001) in the absence and presence of Y-27632, respectively (Fig. 4, A and C), and in OZRs being 0.9 ± 0.15 and 0.3 ± 0.07 Nm⁻¹ (n = 7, P < 0.0001) in the absence and presence of Y-27632, respectively (Fig. 4, B and D).

The effects of RhoK inhibition on the [Ca^{2+}],-contraction relationships for the α₁-adrenoceptor agonist PE in penile arteries from LZRs and OZRs are shown in Fig. 5. Pretreatment with Y-27632 (10 μM) caused a profound inhibition of the contractions elicited by PE in arteries from LZRs and OZRs, with maximal responses in the presence of the inhibitor being 55 ± 10% (n = 6, P < 0.001 vs. control) and 26 ± 7% of the KPSS response (n = 8, P < 0.001 vs. control and P < 0.05 vs. LZRs), respectively (Fig. 5, C and D). Concerning the changes in [Ca^{2+}], Y-27632 (10 μM) inhibited and augmented Ca^{2+} entry elicited by submaximal (1 μM) and maximal (10 μM) concentrations of PE, respectively, in penile arteries from LZRs (Fig. 5, A and E). However, in OZRs, pretreatment with Y-27632 (10 μM) did not alter the reduced PE-induced increases in [Ca^{2+}], entry compared with control conditions (Fig. 5, B and F).
Effects of RhoK inhibition on the changes in \([Ca^{2+}]_i\) and tension elicited by KCl depolarization. Figure 6 shows simultaneous measurements of \([Ca^{2+}]_i\) and contraction and the effects of Y-27632 (0.1–100 μM) on the steady-state responses induced by a high-KCl solution. Change of the bathing medium from PSS to KPSS evoked a rapid rise in both \([Ca^{2+}]_i\) and tension followed by sustained levels of slower rise (Fig. 6). Cumulative addition of the RhoK inhibitor Y-27632 (0.1–10 μM) on the steady-state increases in \([Ca^{2+}]_i\) and force induced by KPSS concentration dependently reduced tension to a greater degree in arteries from OZRs compared with LZRs without changing \([Ca^{2+}]_i\). The inhibition obtained at the 1 μM concentration of Y-27632 was 86 ± 6% (n = 6) and 57 ± 12% (n = 5, P < 0.05 vs. LZRs) in penile arteries from LZRs and OZRs, respectively. However, as shown in Fig. 6, the relaxation produced by Y-27632 was not associated with significant decreases in \([Ca^{2+}]_i\), although the L-type Ca\(^{2+}\) channel blocker nifedipine (0.3 μM) inhibited the Ca\(^{2+}\) entry elicited by KPSS depolarization by 58 ± 10% (n = 5) in LZRs and 80 ± 12% (n = 4) in OZRs. These results confirm that RhoK is involved in Ca\(^{2+}\) sensitization mechanisms in both groups of animals, but its involvement is greater in OZRs due to enhanced inhibitory potency of Y-27632 on vasoconstriction induced by KPSS in penile small arteries.

Protein expression of RhoK. To determine whether the apparent increased effect of RhoK inhibition in penile arteries from OZRs was caused by an enhanced expression of the enzyme, Western blot analysis was performed for RhoK isoform II. A single ~160-kDa immunoreactive band was present in both OZR and control LZR homogenates probed with an

**Fig. 5.** Effect of RhoK inhibition on PE-induced vasoconstriction and changes in \([Ca^{2+}]_i\) in penile arteries from LZRs and OZRs. A–D: average data showing the changes in \([Ca^{2+}]_i\) (A and B) and contraction (C and D) in response to PE in LZRs (A and C) and OZRs (B and D) in the absence and presence of Y-27632 (10 μM). E and F: effect of Y-27632 (10 μM) on the relationships between \([Ca^{2+}]_i\), and tension for PE in penile arteries from LZRs (E) and OZRs (F). Responses are percentages of the rise in F\(_{340}/F_{380}\) and contraction elicited by KPSS. Values are means ± SE; n = 6–8 arteries. *P < 0.05, **P < 0.01, and ***P < 0.001, significantly different from PE-induced responses in the absence of Y-27632; †P < 0.05 vs. LZRs.
antibody against RhoK (Fig. 7). RhoK protein content was significantly higher in penile arteries from OZRs, thus suggesting that the enhanced effects of Y-27632 on PE-induced contraction in OZR arteries may be due to increased RhoK activation.

**DISCUSSION**

The present study demonstrates impaired Ca\(^{2+}\)/H\(_{11001}\) handling and differential receptor-specific changes in the Ca\(^{2+}\)/H\(_{11001}\) entry and Ca\(^{2+}\)/H\(_{11001}\) sensitization processes underlying penile vasoconstriction in a rat model of prediabetes/metabolic syndrome. While augmented TXA\(_2\)-induced contraction was associated with increased Ca\(^{2+}\) entry, RhoK-mediated enhancement of myofilament Ca\(^{2+}\) sensitivity was coupled with unchanged \(\alpha_1\)-adrenergic contractile responses in penile arteries from OZRs. TXA\(_2\) is involved in the regulation of penile smooth muscle tone, and enhanced reactivity to contractile prostanoids has been shown to impair endothelium-dependent responses in the corpus cavernosum of diabetic patients and has been proposed as a cause of ED in these patients (5). Furthermore, in penile arteries from OZRs, a shift in arachidonic acid metabolism toward enhanced formation of contractile prostanoids along with increased cyclooxygenase-2-mediated basal vasoconstrictor activity has been reported (33). In the present study, the enhanced vasoconstriction upon activation of the TXA\(_2\) receptor was associated with a marked increase in both the sensitivity and maximum response to Ca\(^{2+}\) in penile arteries from OZRs. Due the minor role of intracellular Ca\(^{2+}\) stores in the agonist-induced vasoconstriction of these arteries (40), the present findings suggest that the augmented TXA\(_2\) receptor-mediated vasoconstriction in OZRs is likely to be due to the augmented Ca\(^{2+}\) entry in penile arteries from prediabetic rats. Activation of the TXA\(_2\) receptor is coupled with G protein-mediated stimulation of phospholipase C, which generates inositol (1,4,5)-trisphosphate and diacylglycerol, with the latter activating PKC. Enhanced PKC activity has been demonstrated to underlie the enhanced TXA\(_2\) receptor-mediated contractility of the corpus cavernosum from diabetic patients (5), and both PKC expression (\(\alpha\)- and \(\beta\)-isoforms) and activity have recently been shown to be augmented in corporal tissue from OZRs.
Gated the changes in Ca\(^{2+}\) renergic relaxations of erectile tissue through the Akt pathway (13, 27), the enhanced Ca\(^{2+}\) through entries contracts penile trabecular and arterial smooth muscle mainly in the flaccid state through the release of norepinephrine, which is elicited by the magnitude of the contraction induced by PE was similar in penile tissue from OZRs. Nevertheless, further studies are needed to clarify the involvement of PKC in the augmented Ca\(^{2+}\) responses and vasoconstriction of prediabetic penile arteries.

Sympathetic adrenergic nerves are responsible for the detumescence of the erect penis and for the maintenance of the flaccid state through the release of norepinephrine, which contracts penile trabecular and arterial smooth muscle mainly through \(\alpha_1\)-adrenoceptors (4, 34). The present study investigated the changes in Ca\(^{2+}\) handling of penile vascular smooth muscle cells under specific activation of the \(\alpha_1\)-adrenoceptor in OZRs. Our results demonstrate that despite the fact that the magnitude of the contraction induced by PE was similar in arteries from OZRs compared with LZR, vasoconstriction elicited by the \(\alpha_1\)-adrenoceptor agonist was much less dependent on the increase in smooth muscle [Ca\(^{2+}\)]\(_i\), in arteries from OZRs. The lack of enhanced vasoconstrictor responses to PE in penile arteries from OZRs is consistent with that reported in the penile corpus cavernosum in other rodent models of type 2 diabetes-associated ED, where PE-elicted contractions were either unchanged (9) or even decreased (8), which probably represents a compensatory mechanism in cavernosal tissue to overcome restricted prepenile blood supply or altered venoocclusive mechanisms in mechanisms. Nevertheless, adrenergic tone has been shown to be increased in skeletal muscle arterioles from OZRs (18), and vasoconstrictor responses to both exogenous and nerve-released norepinephrine were augmented in penile arteries from the same strain (39). The latter may be ascribed to the impairment of the NO pathway, which usually counteracts noradrenergic vasoconstriction and also mediates the norepinephrine-activated \(\beta\)-adrenergic relaxations of erectile tissue through the Akt pathway (14, 39).

The relationships between [Ca\(^{2+}\)]\(_i\) and contraction for the \(\alpha_1\)-adrenoceptor agonist PE were left shifted and steeper in penile arteries from OZRs compared with LZR, suggesting an enhanced role for Ca\(^{2+}\) sensitization at the expense of reduced Ca\(^{2+}\) influx. Different mechanisms have been reported to underlie the changes in Ca\(^{2+}\) signaling in response to adrenergic vasoconstrictor stimuli in animal models of diabetes and metabolic syndrome. Thus, the increased reactivity to \(\alpha_1\)-adrenoceptor stimulation was found to be largely dependent on the increase in cytosolic [Ca\(^{2+}\)]\(_i\) in arteries from type 1 diabetic humans (16) and rats (1, 2). Furthermore, enhanced extracellular Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels has been suggested to contribute to the enhanced contraction to norepinephrine in type 1 diabetic arteries (21, 36, 42), although an apparent increased release of intracellular Ca\(^{2+}\) was also reported (1, 2). In contrast to the latter studies, enhanced contractile responses to norepinephrine were shown to be largely independent of changes in [Ca\(^{2+}\)]\(_i\), but rather due to the enhanced sensitivity of the contractile proteins to Ca\(^{2+}\) in mesenteric arteries from streptozotocin-induced diabetic rats (12). The augmented Ca\(^{2+}\) sensitivity of the \(\alpha_1\)-adrenergic vasoconstriction found in penile arteries from OZRs is consistent with that reported by Naik et al. (26) in skeletal muscle arterioles from the same model, where PE-induced vasoconstriction was largely independent of changes in Ca\(^{2+}\), with enhanced Ca\(^{2+}\) sensitivity in obese animals being selective for the \(\alpha_1\)-adrenoceptor, like in penile arteries from OZRs.

Growing experimental evidence demonstrates that increased smooth muscle Ca\(^{2+}\) sensitivity mediated by RhoK plays a main role in the augmented vasoconstriction of the diabetic vasculature (15, 23, 26, 46). Concerning erectile tissue, enhanced activity of the RhoA/RhoK pathway has been demonstrated to be involved in the impaired erectile function of rodent models of both type 1 diabetes (7) and metabolic syndrome (44, 46). In the present study, inhibition of RhoK markedly reduced the contractions induced by both stimulation of adrenergic nerves and selective activation of the \(\alpha_1\)-adrenergic receptor with PE, with inhibition of PE responses by Y-27632 being greater in penile arteries from OZRs compared with LZR. At the concentration used (10 \(\mu\)M), the effects of Y-27632 on the contraction and Ca\(^{2+}\) sensitivity of rat arteries have been previously shown not to be mimicked or prevented by selective inhibition of PKC (20, 41), which suggests that the effects of the RhoK inhibitor are mediated through its interaction with Rho-dependent kinase. The present findings therefore suggest that a larger component of the \(\alpha_1\)-adrenergic vasoconstriction is mediated through the activation of RhoK in prediabetic arteries, which is in agreement with results reported by Naik et al. (26) in skeletal muscle arterioles from OZRs and consistent with the more potent erectile responses to intracavernous injection of RhoK inhibitors in type 1 diabetic rats compared with controls (7) and with the ability of these compounds to improve the voltage-dependent erectile responses to stimulation of the cavernous nerves in prediabetic OZRs (46).

Our previous studies on the Ca\(^{2+}\) signaling mechanisms of the \(\alpha_1\)-adrenergic vasoconstriction in penile arteries have demonstrated that the RhoA/RhoK pathway plays a main role not only in the Ca\(^{2+}\) sensitization mechanisms but also in the regulation of Ca\(^{2+}\) entry through a transient receptor potential cation channel (TRPC) member (41). While pretreatment with Y-27632 had a dual inhibitory/enhancing effect on the increases in [Ca\(^{2+}\)]\(_i\) induced by submaximal and maximal concentrations of PE in penile arteries from LZR, the RhoK inhibitor did not alter the reduced PE-mediated Ca\(^{2+}\) mobilization of arteries from OZRs, thus initially ruling out a role for...

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**Fig. 7.** Expression of RhoK-II protein in penile arteries from LZR and OZR. Results of immunoblot analysis for isofrom II of RhoK protein in penile arteries from LZR and OZR are shown. Protein levels were normalized to \(\alpha\)-actin. Bars represent means ± SE of 10–11 arteries. *\(P < 0.05\) vs. LZR.
RhoK in the regulation of Ca^{2+} fluxes of prediabetic arteries. However, the greater involvement of RhoK in the Ca^{2+} sensitization mechanisms of penile arteries from OZRs was confirmed by the higher inhibitory potency of the RhoK inhibitor Y-27632 on the contractions elicited by KCl depolarization in arteries from OZRs compared with controls. Although RhoK is activated by excitatory agonists via receptors coupled to the G_{11/12/13} family of heterotrimeric G proteins, we (41) and others (20, 32, 37) have demonstrated that Ca^{2+} sensitization involving RhoK activation can also be brought about by membrane depolarization. In the present study, addition of the RhoK inhibitor Y-27632 on the steady-state increases in [Ca^{2+}];_i and force induced by a high-KCl solution reduced tension without changing [Ca^{2+}]; to a greater degree in penile arteries from OZRs, thus suggesting a major role of RhoK-mediated Ca^{2+} sensitization processes in penile vasoconstriction of obese animals.

Consistent with this, changes in the sensitivity of the contractile responses to RhoK inhibitors in arteries from OZRs paralleled changes in the expression of RhoK, with total RhoK protein content in penile arteries being increased in OZRs compared with LZR, which might contribute to the enhanced RhoK activity found in OZRs. This finding is in agreement with the elevated expression of RhoK in the corpus cavernosum from prediabetic OZRs (44) and also from other models of type 1 and 2 diabetes-associated ED (7, 10) along with the improved cavernosal blood pressure in response to RhoK inhibitors in these models (44, 46). A role for enhanced activity of the RhoA/RhoK pathway in the pathogenesis of diabetes-associated ED by reducing endothelial NOS (eNOS) protein and activity has been previously demonstrated in streptozotocin-induced diabetic rats (7). However, this seems unlikely in the prediabetic OZRs since eNOS expression was unchanged in both penile arteries (39) and the corpus cavernosum (44), despite the blunted basal NO activity and NO-mediated endothelium relaxant responses (14, 39). The augmented RhoK-mediated Ca^{2+} sensitivity of the contractile proteins accompanied by the marked induction of RhoK expression in prediabetic Zucker rats supports the concept that upregulation of this intracellular pathway might contribute to the diabetes-associated ED by impairing the contractile tone of erectile tissue.

In summary, the present study shows a receptor-selective impairment of Ca^{2+} handling in prediabetic penile arteries, with enhanced Ca^{2+} entry associated with augmented TXA2 receptor-mediated vasconstriction and RhoK-mediated increased Ca^{2+} sensitivity of the contractile filaments under α1-adrenergic stimulation. The data provide further insight into the intracellular mechanisms underlying the proerectile effects of RhoK inhibitors in models of diabetes-associated ED. The role of PKC in the abnormal Ca^{2+} entry and Ca^{2+} sensitization mechanisms of penile vasconstriction deserves further investigation to broaden the therapeutic strategies for the treatment of diabetes-associated ED by reducing the augmented contractile tone of penile erectile tissue.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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