Published in final edited form as:

J Sex Med. 2013 June ; 10(6): 1502–1515. doi:10.1111/jsm.12134.

Activated Rho Kinase Mediates Diabetes-Induced Elevation of Vascular Arginase Activation and Contributes to Impaired Corpora Cavernosa Relaxation: Possible Involvement of p38 MAPK Activation

Haroldo A. Toque, PhD^{*}, Kenia P. Nunes, PhD[†], Lin Yao, PhD^{*}, James K. Liao, MD, PhD[¶], R. Clinton Webb, PhD^{*†}, Ruth B. Caldwell, PhD^{‡,§}, and R. William Caldwell, PhD^{*†}

^{*}Department of Pharmacology & Toxicology, Georgia Health Sciences University, Augusta, GA, USA

[†]Department of Physiology, Georgia Health Sciences University, Augusta, GA, USA

[‡]Department of Cell Biology and Anatomy, Georgia Health Sciences University, Augusta, GA, USA

[§]Vascular Biology Center, Georgia Health Sciences University, Augusta, GA, USA

[¶]Department of Cardiology, University of Chicago Medicine, Chicago, IL, USA

Abstract

Introduction—Activated RhoA/Rho kinase (ROCK) has been implicated in diabetes-induced erectile dysfunction. Earlier studies have demonstrated involvement of ROCK pathway in the activation of arginase in endothelial cells. However, signaling pathways activated by ROCK in the penis remain unclear.

Aim—We tested whether ROCK and p38 MAPK are involved in the elevation of arginase activity and subsequent impairment of corpora cavernosal (CC) relaxation in diabetes.

Methods—Eight weeks after streptozotocin-induced diabetes, vascular functional studies, arginase activity assay, and protein expression of RhoA, ROCK, phospho-p38 MAPK, p38 MAPK, phospho-MYPT-1^{Thr850}, MYPT-1 and arginase levels were assessed in CC tissues from nondiabetic wild type (WT), diabetic (D) WT (WT + D), partial ROCK 2^{+/-} knockout (KO), and ROCK 2^{+/-} KO + D mice.

Main Outcome Measures—The expression of RhoA, ROCK 1 and 2, phosphorylation of MYPT-1^{Thr850} and p38 MAPK, arginase activity/expression, endothelial- and nitric-dependent relaxation of CC was assayed.

Results—Diabetes significantly reduced maximum relaxation (E_{max}) to both endothelium-dependent acetylcholine (WT + D: E_{max} ; $61 \pm 4\%$ vs. WT: E_{max} ; $75 \pm 2\%$) and nitric nerve stimulation. These effects were associated with increased expression of active RhoA, ROCK 2, phospho-MYPT-1^{Thr850}, phospho-p38 MAPK, arginase II, and activity of corporal arginase (1.6-fold) in WT diabetic CC. However, this impairment in CC of WT + D mice was absent in

heterozygous ROCK 2^{+/-} KO + D mice for acetylcholine ($E_{\max}$: 80 ± 5%) and attenuated for nitrgic nerve-induced relaxation. CC of ROCK 2^{+/-} KO + D mice showed much less ROCK activity, did not exhibit p38 MAPK activation, and had reduced arginase activity and arginase II expression. These findings indicate that ROCK 2 mediates diabetes-induced elevation of arginase activity. Additionally, pretreatment of WT diabetic CC with inhibitors of arginase (ABH) or p38 MAPK (SB203580) partially prevented impairment of ACh- and nitrgic nerve-induced relaxation and elevation of arginase activity.

Conclusion—ROCK 2, p38 MAPK and arginase play key roles in diabetes-induced impairment of CC relaxation.

Keywords

Diabetes; Rho Kinase; p38 MAPK; Arginase

Introduction

Erectile dysfunction (ED) is a common condition in type 1 diabetes. Penile erection and flaccidity are regulated mainly by neurophysiological process involving the relaxation and contraction of cavernosal smooth muscle. Strong evidence indicates nitric oxide (NO) as the principal mediator of penile erection [1]. NO released from sinusoidal endothelial cells or from nitrgic nerves cause corpora cavernosal (CC) smooth muscle relaxation [1,2]. Cavernosal tissue and penile vessels receive a rich adrenergic innervation that maintains the penis in the flaccid state mainly via a tonic activity. A balance between the production of relaxing and contractile factors is critical in maintaining the normal process of erectile function.

Given that NO synthase (NOS) and arginase share L-arginine as their common substrate, elevation of arginase activity can limit availability of L-arginine for NOS, thereby reducing NO production and impairing vascular function. Previous studies indicate that diabetes-induced ED involves increased vascular arginase activity [3]. Cavernosal tissues from human diabetic patients with ED exhibit elevated arginase activity and diminished NO synthesis, with reduced cavernosal relaxation [3]. Additionally, arginase inhibition or deletion of arginase gene has been shown to enhance NO production [4] and reduce endothelial dysfunction in diabetic models [5–7]. Two isoforms of arginase exist, arginase I and II, and their distribution appears to be tissue and species dependent [8–10]. Both arginase isoforms have been identified in vascular tissue and endothelial cells and displayed an important role in endothelial dysfunction in diabetes and other diseases [11].

Much evidence indicates that an activated RhoA/Rho-kinase (ROCK) pathway is needed to maintain penile flaccidity [12]. Also, complete gene deletion of eNOS reportedly leads to amplification of the RhoA/ROCK pathway [13] and suppressed expression and activity of penile eNOS is observed in diabetes-induced RhoA/ROCK activation [14]. ROCK is expressed in human, rabbit, rat, and mice cavernosal smooth muscle [13–16], and inhibition of ROCK pathway improves CC relaxation in diabetes and hypertension-induced ED [14,17–19]. Diabetes and endothelial cells treated with high glucose increased arginase activity through activated RhoA/ROCK function [5]. Also, the RhoA/ROCK pathway is linked to up-regulation of arginase in endothelial cells exposed to oxidative species [20], angiotensin II [21], thrombin [10], as well as in inflammatory bowel disease [22]. Furthermore, RhoA/ROCK has been shown to be an upstream regulator of mitogen-activated protein kinase (MAPK) family members such as p38 MAPK [21,23]. p38 MAPK has been shown to have a central role in cardiovascular dysfunctions including vascular inflammation, endothelial dysfunction, and endothelial cell proliferation/ apoptosis [24,25],

and activated p38 MAPK is implicated in enhanced arginase activity in models of elevated angiotensin II and activated macrophages [21,26,27]. This evidence suggests that activation of the RhoA/ROCK and p38 MAPK pathway is a critical step in elevated arginase activity and expression in the vasculature and endothelial cells.

Two isoforms of ROCK (1 and 2) have been identified in mammalian tissues and both isoforms are expressed in vascular smooth muscle and endothelial cells. Despite the up-regulation of activity and expression of ROCK in pathological conditions such as diabetes, hypertension, aging, and ED [5,14,21,28], the functional role of ROCK isoforms in modulating increased cavernosal arginase activity during diabetes remains to be determined. To address this issue, we used heterozygous ROCK 1^{+/-} or ROCK 2^{+/-} knockout (KO) mice having one allele of ROCK 1 or 2. We could not examine complete deletion of ROCK 1 (ROCK 1^{-/-}) or ROCK 2 (ROCK 2^{-/-}) alleles in mice (homozygous knockout), as many do not survive in early stages of life [29,30]. Use of partial gene deletion ROCK mice may provide better understanding of impaired CC relaxation in diabetes. Our initial findings in this study demonstrated that partial KO of ROCK 2^{+/-}, but not ROCK 1^{+/-}, completely prevents impaired CC relaxation and attenuated elevation of arginase activity in streptozotocin (STZ)-induced type 1 diabetes. We hypothesized that heterozygous ROCK 2^{+/-} KO mice prevents diabetes-induced vascular dysfunction by decreasing vascular arginase activity.

Methods

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the Georgia Health Sciences University (Protocol Number: 2010-0230). All surgeries were performed under a mixture of ketamine/xylazine (100:10 mg/kg, intraperitoneal [i.p.]) anesthesia, and all efforts were made to minimize suffering.

Animals and Diabetes Induction

A total of 68 10-week-old male C57BL/6J KO mice lacking one copy of the ROCK 1^{+/-} or ROCK 2^{+/-} gene and wild type (WT) of the same genetic background mice were used in this study. Breeding pairs of ROCK heterozygous KO mice were obtained as a gift from Dr. James K. Liao of Harvard Medical School. Mice were divided into six groups: nondiabetic WT, WT diabetic (WT + D), haploinsufficient ROCK 1^{+/-} (ROCK 1^{+/-} KO) nondiabetic, ROCK 1^{+/-} diabetic (ROCK 1^{+/-} KO + D), and ROCK 2^{+/-} (ROCK 2^{+/-} KO) nondiabetic and ROCK 2^{+/-} diabetic (ROCK 2^{+/-} KO + D). Mice diabetic groups received i.p. injections of STZ (65 mg/kg) every other day for up to three injections. In nondiabetic groups, citrate buffer (pH 4.5), the vehicle of STZ, was injected in the same manner as in diabetic groups. Mice with blood glucose levels >350 mg/dL were considered diabetic. Body weight and glucose levels of each mouse were measured at the time of injections and 8 weeks after treatment. Systolic arterial blood pressure was determined by the noninvasive tailcuff plethysmography.

Genotyping Protocol for ROCK 1 and 2

Genotyping was performed by polymerase chain reaction (PCR) amplification, and DNA extraction from ear punch of mouse was performed using an Extract-N-Amp™ tissue PCR Kit (XNAT2 Kit, Sigma, St Louis, MO, USA). For PCR analysis, the primers for ROCK 1 were 5'-AGG CAG GGC TAC ACA GAG AA-3' (forward primer), 5'-ACA GCT GCC ATG GAG AAA AC-3' (reverse primer). The primers for ROCK 2 were 5'-GTT TCT CAG CAT TAT GTT GG-3' (primer 1), 5'-CTG GGT TGT TTC TCA GAT GA-3' (primer 2),

and 5'-CGC TTT CAT CTG TAA ACC TC-3' (primer 3). The molecular weight bands were 544 bp for ROCK 1, 918 bp for WT, 800 bp for ROCK 2, and 1 kb for WT.

CC Membrane Protein Isolation

Briefly, CC tissues were pulverized, homogenized in lysis extraction buffer (100 mM Tris-HCl, 1 mM EDTA and 1 mM EGTA containing phenylmethylsulfonyl fluoride [PMSF], protease inhibitor and phosphatase inhibitors), and centrifuged at $100,000 \times g$ for 20 minutes at 4°C. Supernatant was collected as cytosolic fraction, and pellet was suspended in extraction buffer containing 1% Triton X-100 to obtain the membrane fraction. Protein was estimated using a commercially available kit from Bio-Rad Laboratories (Hercules, CA, USA), and equal amounts of protein were loaded for Western blot.

Western Blot Analysis

Cavernosal tissues were homogenized in lysis buffer containing protease and phosphatase inhibitors, PMSF 0.1 mM, and centrifuged at $14,000 \times g$ for 20 minutes at 4°C. The supernatant was collected and protein concentration was determined. An aliquot of 20 µg of protein from each sample was loaded per lane and resolved by SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories). Nonspecific binding sites were blocked with 5% of bovine serum albumin in Trisbuffered saline/Tween for 1 hour at 24°C. Membranes were incubated with primary antibodies against arginase I (1:1,000), arginase II (1:250), p38 MAPK (1:1,000), phosphorylated p38 MAPK (1:1,000), ROCK 1 (1:1,000), ROCK 2 (1:1,000), RhoA (1:1,000), phosphorylated MYPT-1^{Thr850} (1:1,000), MYPT-1 (1:1,000), total actin (1:5,000), or α -actin (1:5,000). After overnight exposure at 4°C, the membranes were washed and incubated with a horseradish peroxidase-labeled secondary antibody. Immunoreactivity was detected by enhanced chemiluminescence kit (Amersham, Piscataway, NJ, USA), and the protein expression was normalized to the actin content.

Measurement of Cavernosal Arginase Activity

Cavernosal tissues were collected and frozen in liquid nitrogen. Tissues were pulverized, homogenized in ice-cold lysis buffer (combined 1:4 w/v with 50 mmol/L, Tris-HCl, 100 µmol/L, EDTA and EGTA, pH 7.5) containing protease inhibitor, phosphatase inhibitors cocktail 1 and 2. Homogenates were sonicated and centrifuged at $14,000 \times g$ for 20 minutes at 4°C and supernatants were collected for enzyme assay. There was 25 µL of the supernatants in triplicate added to 25 µL of Tris-HCl 121 (5×10^{-2} M, pH 7.5) containing MnCl_2 (10^{-2} M) and the mixture was activated by heating for 10 minutes at 55–60°C. Arginase activity was assayed by measuring urea production from L-arginine as previously described [27].

Cavernosal Vascular Functional Studies

After 8 weeks of diabetes, animals were anesthetized with ketamine/xylazine (100:10 mg/kg; i.p.) and penis was removed and placed in chilled Krebs solution of the following composition (in mM): NaCl, 118; NaHCO_3 , 25; glucose, 5.6; KCl, 4.7; KH_2PO_4 , 1.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.17; and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.5. After removal of the vein and urethra, the penile tissue was cleaned from connective and adventitial tissue, and the fibrous septum separating the CC was opened from its proximal extremity toward the penile shaft. A slit was made in the tunica albuginea along the shaft to obtain two strips (approximately $11 \times 1 \times 1$ mm) of CC from each animal. Each strip was mounted under resting tension of 2.5 mN in 4-mL myograph chambers filled with Krebs solution at 37°C (pH 7.4) and continuously bubbled with a mixture of 95% O_2 and 5% CO_2 . Isometric force was recorded using a PowerLab/8SP

data acquisition system (Software Chart, version 5, AD Instrument, Colorado Springs, CO, USA). The tissues were allowed to equilibrate for 1 hour before starting the experiments.

After equilibration, the ability of the preparation to develop contraction was assessed by a high KCl solution (80 mM). Cumulative concentration-response curve to acetylcholine (ACh; 10^{-9} to 10^{-5} M, an endothelium-dependent vasodilator) was obtained in CC tissues contracted with phenylephrine (PE; 10^{-5} M, α_1 -adrenergic receptor agonist). Then, CC tissues were washed three times every 15 minutes for 1 hour. Next, cavernosal strips from WT mice were incubated with SB203580 (10^{-5} M, p38 MAPK inhibitor) for 45 minutes before a second curve was generated. Cumulative concentration-response curve to sodium nitroprusside (SNP; 10^{-8} to 10^{-4} M, NO donor) and the contractile agent PE (10^{-9} to 10^{-4} M) were also performed in the cavernosal tissue.

In another set of experiments, electrical field stimulation (EFS) was applied in cavernosal strips placed between two platinum ring electrodes connected to a grass S88 stimulator (Astro-Med, Industrial Park, RI, USA), and EFS was conducted at 20 V, 1-ms pulse width and trains of stimuli lasting 10 seconds at varying frequencies (1–32 Hz, 90-second interval between each stimulation). To evaluate nitrenergic relaxations, cavernosal tissues were pretreated with bretylium tosylate (3×10^{-5} M) and atropine (10^{-6} M) to deplete the catecholamine stores and to block the muscarinic receptors, respectively. Involvement of NO on EFS-induced cavernosal relaxations was confirmed by using N^G-nitro-L-arginine methyl ester (L-NAME) (10^{-4} M, NO synthesis inhibitor). Frequency-response curves were performed in the absence or in the presence of 2(S)-amino-6-boronohexanoic acid (ABH, 10^{-4} M, arginase inhibitor) or SB203580 (10^{-5} M) for 45 minutes in cavernosal tissues. To evaluate adrenergic nervemediated responses, the strips were incubated with L-NAME (10^{-4} M) plus atropine (10^{-6} M), before EFS was performed.

Drugs and Chemicals

ACh, atropine, bretylium tosylate, L-NAME, SNP, STZ, PE, phosphatase cocktail 1 and 2, and protease inhibitor were purchased from Sigma Aldrich (St. Louis, MO, USA). The antibodies against arginase I, ROCK 1 and 2 isoforms, and MYPT-1 were obtained from BD Transduction Laboratories (San Jose, CA, USA). The RhoA antibody was purchased from Abcam Laboratories (Cambridge, MA, USA). The antibodies against p38 MAPK and phosphorylated p38 MAPK were purchased from Cell Signaling Technology (Danvers, MA, USA), and pMYPT-1^{Thr850} was purchased from Millipore (Billerica, MA, USA). The antibody against arginase II was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All reagents used were of analytical grade. Stock solutions were prepared in deionized water or ethanol and stored in aliquots at -20°C ; dilutions were prepared immediately before use.

Statistical Analysis

Experimental values of relaxation or contraction were calculated relative to the maximal changes from the contraction produced by PE and KCl, respectively taken as 100% in each tissue. Curves were fitted to all the data using nonlinear regression, and half-maximum response (pEC_{50}) of each drug expressed as $-\log$ molar (M) was used to compare potency. Data are expressed as the mean $\pm$ SEM of n experiments. Two-way analysis of variance was used to evaluate the results followed by Bonferroni post hoc test. $P < 0.05$ was considered significant. A program package was used for the statistical analysis of all data (Graph-Pad Instat, version 5.00; GraphPad Software Inc., San Diego, CA, USA).

Results

Blood Glucose, Body Weight, and Blood Pressure Levels

STZ-induced diabetic mice exhibited increased in blood glucose levels (~3.0-fold) and decreased body weights in WT, ROCK 1^{+/-}, and ROCK 2^{+/-} KO mice of 20%, 22%, and 18%, compared with their normoglycemic controls, respectively (Table 1). The average dry cavernosal tissue weight weights (mg) did not differ from nondiabetic and STZ-diabetic mice. Diabetes moderately increased systolic blood pressure compared with WT mice (129 ± 5 vs. 107 ± 4 mm Hg). Blood pressure of ROCK 1^{+/-} and ROCK 2^{+/-} KO mice were not elevated by diabetes (93 ± 2 and 95 ± 3 mm Hg), and these KO mice had lower basal blood pressure level than WT mice (92 ± 2 and 90 ± 3 mm Hg, respectively).

Protein Expression of ROCK 2 Isoform is Increased in CC from Diabetic Mice

In order to determine whether RhoA and its downstream effect ROCK are associated with diabetes-induced vascular dysfunction in CC tissues, we determined the effect of diabetes on levels of active RhoA and expression of ROCK 1 and ROCK 2 isoform in CC from diabetic WT mice. Western blot analysis of cavernosal tissues from diabetic WT mice showed markedly increased levels of active RhoA (by 1.77-fold) bound to plasma membrane over nondiabetic WT mice (Figure 1A). Protein expression of ROCK 2 in CC from diabetic WT mice was also significantly increased compared with nondiabetic WT mice (Figure 1B), but no differences were observed in ROCK 1 levels (Figure 1C). These results suggest involvement of ROCK 2 in the penile vascular dysfunction of diabetic mice.

Haploinsufficiency of ROCK 2 Reduces Phosphorylation of MYPT-1^{Thr850} in Diabetic Cavernosal Tissue

Levels of phosphorylated MYPT-1 at the inhibitory site (Thr850) were examined in CC samples. MYPT-1^{Thr850} has been shown to be a selective ROCK phosphorylation site [31]. Our results show that diabetes significantly increased phosphorylation of MYPT-1^{Thr850} in CC tissue after being normalized to total MYPT-1 content (Figure 2). Levels of phospho-MYPT-1^{Thr850} were much less in CC of diabetic ROCK 2^{+/-} KO mice ($P < 0.01$).

Haploinsufficiency of ROCK 2 Attenuates Diabetes-Stimulated CC Arginase Activity

To assess the role of ROCK-induced enhancement of arginase activity in diabetes-induced impairment of vascular endothelial function, arginase activity levels were measured in CC strips from all groups. A 2.3-fold increase in arginase activity was observed in CC tissues from the WT + D mice compared with WT mice (Figure 3A). The diabetes-induced elevation of corporal arginase activity was prevented in mice with haploinsufficiency of ROCK 2. Levels of arginase activity in CC of nondiabetic ROCK 1^{+/-} or ROCK 2^{+/-} KO mice were not different compared with nondiabetic WT mice. These data suggest that in diabetes the activated ROCK 2 isoform is mainly involved in elevation of corporal arginase activity.

Haploinsufficiency of ROCK 2 Prevents Diabetes-Induced Enhancement of CC Arginase Protein Levels

In order to determine whether the increase in arginase activity in diabetic mice is associated to elevated protein levels of arginase isoforms, we determined protein expression of arginase I and II in CC of all groups. Protein expression of arginase II was increased by 2.1-fold in cavernosal tissues from WT + D mice (Figure 3B), while arginase I levels were not significantly altered as compared with nondiabetic WT mice (not shown). Diabetes-induced elevation of arginase II protein levels was blunted in the ROCK 2^{+/-} KO mice. Additionally,

basal protein levels of arginase II isoforms in the nondiabetic ROCK 2^{+/-} KO mice were not different as compared with the WT mice (Figure 3B).

Haploinsufficiency of ROCK 2 Maintains Endothelium-Dependent Relaxation in Diabetes

Cumulative addition of ACh evoked a graded endothelium-dependent relaxation of CC preparations precontracted with PE. Caverosal strips from diabetic WT mice exhibited markedly decreased maximal relaxation response ($E_{\max}$) and reduced potency (pEC_{50}) values to ACh compared with nondiabetic WT mice ($E_{\max}$: $60 \pm 4\%$ and pEC_{50} : 6.56 ± 0.07 for diabetic WT mice, vs. $E_{\max}$: $75 \pm 5\%$ and pEC_{50} : 7.03 ± 0.03 for nondiabetic WT, Figure 4A). However, no differences were observed in the $E_{\max}$ and pEC_{50} values between nondiabetic WT and ROCK 2^{+/-} KO ($E_{\max}$: $86 \pm 7\%$ and pEC_{50} : 6.89 ± 0.12) mice. There was no impairment of ACh-induced relaxation response in the diabetic ROCK 2^{+/-} KO mice compared with diabetic WT mice as indicated by the $E_{\max}$ ($80 \pm 5\%$) and pEC_{50} (6.87 ± 0.09) values. Furthermore, the maximal relaxation response of CC from diabetic ROCK 1^{+/-} KO mice ($74 \pm 3\%$) was significantly greater than in the diabetic WT mice (Figure 4B). These data suggest that ROCK 1 and ROCK 2 have a role in CC endothelial dysfunction, but that the role of ROCK 2 is greater. Based on these findings and that only the ROCK 2 isoform is up-regulated in diabetes, we only examined ROCK 2 KO mice for the next protocols.

Endothelium-independent maximum relaxations in CC tissue induced by the NO donor, sodium SNP, were not different among nondiabetic and diabetic group from WT and ROCK 2^{+/-} KO mice (pEC_{50} and $E_{\max}$ values: WT, 6.32 ± 0.04 , $79 \pm 4\%$; WT + D, 6.43 ± 0.05 , $85 \pm 6\%$; ROCK 2^{+/-} KO, 6.49 ± 0.06 , $89 \pm 3\%$; ROCK 2^{+/-} KO + D, 6.21 ± 0.04 , $93 \pm 3\%$, respectively).

Haploinsufficiency of ROCK 2 Maintains Normal Nitroergic Nerve-Induced Relaxation in Diabetes

EFS-induced relaxations in cavernosal segments from diabetic WT mice were significantly reduced at all frequencies (1–32 Hz) compared with those from nondiabetic WT mice ($P < 0.05$, Figure 5A). ROCK 2^{+/-} KO tissue exhibited similar relaxant responses to EFS compared with CC from non-diabetic WT mice. Interestingly, CC from diabetic ROCK 2^{+/-} KO exhibited less impairment of nitroergic dysfunction at 2, 4, 8, 16, and 32 Hz compared with CC from diabetic WT mice (Figure 5A). Representative traces to EFS for WT tissue and diabetic WT and ROCK 2^{+/-} KO tissues are shown in Figure 5B

Additionally, the EFS-induced relaxation in CC from diabetic WT mice was increased by acute treatment with an arginase inhibitor ABH (10^{-5} M) at frequencies of 1 to 8 Hz (Figure 5C). The relaxation induced by EFS in cavernosal strips from nondiabetic WT mice was not significantly altered by the addition of ABH. These results confirm that diabetes-induced impairment of nitroergic nerve-mediated relaxation involves arginase activity in diabetic cavernosal tissue.

Haploinsufficiency of ROCK 2 Prevents Diabetes-Induced Augmented Contractile Responses to PE and Adrenergic Nerve Stimulation

The contractions of CC induced by KCl (80 mM) were not significantly different among the groups (WT, 1.0 ± 0.2 mN; WT + D, 1.2 ± 0.1 mN; ROCK 2^{+/-} KO, 1.1 ± 0.1 mN; ROCK 2^{+/-} KO + D, 1.2 ± 0.2 mN). Isolated cavernosal tissues were contracted with PE (10^{-5} M) to achieve a level of tension representing ~80% of the KCl-induced maximum contraction.

Application of increasing concentrations of PE developed progressive contractile responses in CC from all groups, which then reached a plateau level (tonic response). This general

pattern was similar between the control and KO mice. The concentration-response curve values measured to PE were tonic contractile responses. Cavernosal strips from diabetic WT mice tended to have higher contractile responses to PE at higher concentrations ($0.1-1 \times 10^{-4}$ M), but they were not significantly different compared with CC of WT mice. Similarly, cavernosal strips of ROCK $2^{+/-}$ KO mice tended to have decreased in CC from diabetic WT mice compared with those from non-diabetic WT and ROCK $2^{+/-}$ KO contractile responses to PE at these concentrations compared with CC in WT mice. However, in diabetes, CC of ROCK $2^{+/-}$ KO + D mice clearly displayed lower E_{max} (reduced by 24%) values compared with the WT + D mice ($P < 0.05$; Figure 6A). EFS-induced contractions were enhanced mice (Figure 6B). ROCK $2^{+/-}$ KO + D mice exhibited significantly lower contractile response in CC from those of the diabetic WT mice at 8, 16, and 32 Hz, respectively.

ROCK Mediates p38 MAPK Activation by Diabetes in Cavernosal Tissue

To further understand the mechanisms by which ROCK mediates diabetes-induced activation of arginase, we used a p38 MAPK inhibitor and assessed its effect on corporal arginase activity. As shown in Figure 7A, SB203580 (10^{-5} M), a specific inhibitor of p38 MAPK, significantly attenuated diabetes-mediated arginase activation. We therefore sought to examine the involvement of ROCK 2 on phosphorylation (activation) of p38 MAPK. Western blot analysis showed a significant increase of the phospho-p38 MAPK/p38 MAPK ratio in CC from diabetic mice compared with nondiabetic WT tissues. However, cavernosal tissues from diabetic ROCK $2^{+/-}$ KO mice showed less phosphorylation of p38 MAPK than tissues from diabetic WT mice (Figure 7B). These observations indicate that ROCK 2 activation is upstream to p38 MAPK in mediating diabetes-induced elevation of CC arginase activity and expression.

Inhibition of p38 MAPK Enhances Endothelium-Dependent and Nitric Relaxation Response in Diabetic Cavernosal Tissue

We next evaluated the effect of p38 MAPK inhibition on vascular functional in CC tissues from nondiabetic and diabetic WT mice. Acute treatment with SB203580 partially attenuated the impairment of ACh-induced relaxation in CC from diabetic WT mice (from $51 \pm 5\%$ to $69 \pm 5\%$, $P < 0.05$, Figure 7C), and increased the EFS-induced nitric nerve-mediated relaxation at 1, 2, 4, and 8 Hz by 327%, 126%, 81%, and 33%, respectively, in CC from diabetic WT mice (Figure 7D). No differences were observed in relaxations of CC from nondiabetic WT mice in response to ACh or EFS of nitric nerves with or without treatment with SB203580 (Figure 7C,D).

Discussion

Emerging evidence indicates that elevated arginase activity is linked to vascular endothelial dysfunction in a variety of cardiovascular diseases, including diabetes, by mechanisms involving decreased availability of L-arginine substrate for NOS, elevated reactive oxygen species (ROS) production, and decreased NO bioavailability [2]. Previous studies have demonstrated involvement of the RhoA/ROCK pathway in the activation of arginase in endothelial cells [5,10,20,21]. This study investigated mediators of diabetes-induced arginase activation in mouse corpora cavernosum. Our findings show that elevated arginase activity in diabetes occurs through the RhoA/ROCK pathway and subsequent p38 MAPK activation. We observed that diabetes increases ROCK activity, protein expression of ROCK 2 and arginase II, enhances vascular arginase activity and p38 MAPK activation, and decreases endothelial-dependent and nitric nerve relaxation responses in CC tissues. All these events favor impaired erectile function. Moreover, studies with CC from haplo-insufficient ROCK $2^{+/-}$ mice exhibit less diabetes-induced increased arginase activity as well as arginase II and ROCK 2 expression, activation of p38 MAPK, and vascular endothelium-

dependent and nitric dysfunction. The use of heterozygous ROCK 1^{+/-} and ROCK 2^{+/-} KO mice provides a comprehensive view of the involvement of ROCK-dependent signaling pathways in diabetes-induced impairment of CC relaxation.

Elevated arginase activity and expression are associated with oxidative stress and activation of ROCK pathway in STZ-diabetic rats and endothelial cells exposed to high glucose [5], thrombin [10], or ROS [20]. Treatment with an inhibitor of arginase or deletion of arginase gene has been shown to reduce vascular dysfunction in hypertensive rats [32] and diabetic mice [5,7]. However, identifying the upstream regulators of arginase activation can provide more specific targets to limit arginase activity in disease states like diabetes. A mediator involved in the elevation of arginase expression and activity in endothelial cells exposed to thrombin or angiotensin II is the RhoA/ROCK pathway [10,21]. Activated ROCK function is linked to ED and penile detumescence in cavernosal tissues from type 1 [14,33,34] or type 2 diabetes [35]. Inhibition of this pathway restores erectile function in aging and diabetic animals [36–38]. ROCK consists of two isoforms, ROCK 1 and ROCK 2, which are found in corporal tissue [16,19,33]. To circumvent potential nonspecific effect of ROCK inhibitors and their identical actions on the two ROCK isoforms, haploinsufficient mouse models with partial deletion of ROCK 1^{+/-} and ROCK 2^{+/-} genes have been used in this study to address the specific role of each ROCK isoform in the activation of corporal arginase. Complete KO of the ROCK 1^{-/-} gene in mice most die postnatally [29], while deficiency of ROCK 2^{-/-} is embryonically lethal because thrombus formation in the placenta and decreased blood flow to the labyrinth layer lead to growth retardation [30]. ROCK has been indicated as an important mediator of insulin signaling and glucose metabolism. We did not observe significant changes in glucose levels among all groups. However, diabetes-induced elevation of blood pressure observed in WT mice was not observed in either the ROCK 1^{+/-} or ROCK 2^{+/-} diabetic mice. Additionally, lower basal blood pressure levels were observed in nondiabetic ROCK 1^{+/-} or 2^{+/-} KO mice compared with WT mice. Our current results are in agreement with previous study in which inhibition of ROCK decrease blood pressure in diabetic rats [39].

Activated RhoA/ROCK has been observed in diabetic rats to reduce NOS activity, cGMP levels, and penile relaxation [14]. Additionally, reduced NO availability has been observed in diabetic human CC compared with nondiabetic tissue, and treatment with an arginase inhibitor increased NO production in diabetic CC tissues [3]. Our current study shows increased vascular arginase activity and ROCK 2 expression in CC of diabetic WT mice compared with nondiabetic CC, with no differences observed in ROCK 1 expression. Additionally, elevation of arginase activity is much lower in CC tissues from ROCK 2^{+/-} KO vs. diabetic WT mice. These observations indicate that ROCK 2 is the isoform primarily involved in the up-regulation of CC arginase activity in diabetic mice. RhoA, a small GTP binding protein, is made active by conversion from the GDP- to the GTP- bound state. When RhoA is activated, it activates ROCK, a key regulator of erectile function [19]. In this study, we observed increased levels of active RhoA (membrane bound) and protein expression of ROCK 2, but no changes in ROCK 1 protein levels in CC of diabetic WT mice compared with nondiabetic mice. Previous studies have described increased CC levels of active RhoA and ROCK 2 expression in diabetic rats [14] and increased CC expression of ROCK 1 in diabetic rabbits [33]. These differences in ROCK isoform expression may be due to different diabetic models or species. Our result indicates that active RhoA protein levels are increased in CC of diabetic WT mice, and that the expression and activity of its downstream effector, the ROCK 2, are increased. These events appear to be responsible for the increased cavernosal arginase activity and impaired endothelium-dependent and nitric nerve relaxation observed in CC of diabetic mice.

Activated ROCK inactivates myosin phosphatase through the specific phosphorylation of myosin phosphatase target subunit 1 (MYPT-1), allowing myosin light chain to remain phosphorylated, thereby enhancing contraction [40]. Different sites of phosphorylation induced by ROCK have been identified (Ser^{849/854}, Thr^{850/855}, and Thr^{695/697}), but the main site involved in the inhibition of MLC phosphatase activity is Thr^{850/855} [40], whereas Thr^{695/697} is thought to be phosphorylated by other kinases [31]. We measured the expression of phospho-MYPT-1^{Thr850}/MYPT-1 ratio in CC of WT and ROCK 2^{+/-} KO mice. Our data show that diabetes increased the expression of phospho-MYPT-1^{Thr850} in CC tissues compared with nondiabetic CC tissues. The effect of diabetes on pMYPT-1^{Thr850} expression was markedly decreased in CC of ROCK 2^{+/-} KO mice. Indeed, increased phosphorylation of MYPT-1 has been associated with decreased NO bioavailability shown in several conditions such as diabetes and aging [14,19]. We speculate that less ROCK activity in partial ROCK 2^{+/-} KO mouse induces less increase of arginase and better release of NO from endothelial cell and smooth muscle relaxation.

Strong evidence indicates NO as the main mediator of penile erection [1]. NO released from sinusoidal endothelial cells or from nitrergic nerves activates the cGMP/protein kinase G signaling pathway causing corporal smooth muscle relaxation. In our vascular functional studies, an impairment of ACh-induced relaxation was observed in STZ-diabetic WT mice. However, this impairment was largely absent in CC of ROCK 2^{+/-} KO mice but was only partially reduced in CC of ROCK 1^{+/-} KO diabetic mice. Also, impaired CC relaxation responses to nitrergic nerve stimulation were observed for diabetic WT mice, but a lesser impairment was observed in the CC of the ROCK 2^{+/-} KO mice. These findings, along with the data on arginase activity/ expression, indicate that ROCK 2 is the main ROCK isoform involved in diabetes-induced enhancement of corporal arginase activity and endothelial and nitrergic dysfunction.

Decreased plasma L-arginine concentrations have been reported in diabetic animals [41] and patients [42], with elevation of plasma ornithine (product of arginase) also observed [11]. Because arginase competes with NOS for L-arginine, increased activity/expression of arginase in diabetic CC can limit L-arginine availability for NOS and reduce NO production and smooth muscle relaxation. Our data show that exposure of diabetic CC strips to the arginase inhibitor ABH markedly enhanced nitrergic nerve-mediated relaxation. This finding is in concert with other human or animal models reporting enhanced NO-dependent nitrergic nerve-mediated relaxation of penile CC with the use of other arginase inhibitors [7,43].

Previous studies have revealed that RhoA and its effector ROCK regulate cavernosal smooth muscle tone [12,44]. Activation of RhoA/ROCK pathway increases vascular smooth muscle contraction and penile detumescence either by direct phosphorylation of smooth muscle myosin or by deactivating myosin phosphatase [12,45]. Our data show that PE-induced CC contractions in nondiabetic ROCK 2^{+/-} KO tissue tended to be lower at the highest concentrations (0.3 and 1 × 10⁻⁴ M), but were not significantly different from those of WT mice. Diminished cavernosal contraction in ROCK 2^{+/-} KO diabetic mice may be due to lower ROCK activity which results to a lesser increase of arginase and better endothelial cell release of NO and smooth muscle relaxation. We point out that similar findings to CC were observed in aortic vessels [46]. We observed that nondiabetic vessels from ROCK 1^{+/-}, but not ROCK 2^{+/-} KO mice, had significantly blunted contractile responses to PE compared with aorta from WT mice. However, responses in ROCK 2^{+/-} vessels only tended to be lower. Also, increased contractile responses to PE observed with diabetic WT vessels were markedly diminished in diabetic ROCK 1^{+/-} or ROCK 2^{+/-} vessels. Our current data in CC and taking together findings in aorta suggest that the ROCK 1 isoform contributes to ROCK activity and plays an important role in cavernosal contractile function.

To further examine mechanisms by which ROCK 2^{+/-} KO in CC could improve diabetes-induced ED, we measured levels of phospho (activated)-p38 MAPK in CC tissues from diabetic WT and ROCK 2^{+/-} KO mice. Activation of p38 MAPK observed in CC of diabetic WT mice was absent in CC of diabetic ROCK 2^{+/-} KO mice. Accumulating evidence indicates that the RhoA/ ROCK is an upstream regulator of MAPK family members such as p38 MAPK [21,23]. p38 MAPK is considered a potential therapeutic target in several diseases including hypertension [21,47], arthritis [48], and diabetic retinopathy [49]. As RhoA/ROCK activates p38 MAPK, activation of this pathway by diabetes may lead to enhanced corporal arginase activity and induce vascular dysfunction. Inhibition of p38 MAPK can correct nitrgic nerve relaxation in CC of diabetic mice [50]. Additionally, we recently showed that reduction of arginase activity by p38 MAPK inhibition improves CC relaxation in angiotensin-II treated mice [30]. Our present data show that diabetes increases corporal arginase activity followed with impaired endothelium-dependent and nitrgic nerve-mediated relaxation in CC tissues. Additionally, elevated CC arginase activity is markedly attenuated by SB203580, an inhibitor of p38 MAPK. Further, enhancement of endothelial and nitrgic nerve-mediated relaxation is observed after incubation with p38 inhibitor in CC of diabetic mice.

In summary, our data confirm that ROCK is involved with elevated CC arginase activity and vascular dysfunction in diabetes. Moreover, ROCK 2 plays a major role in enhancing arginase activity in CC of diabetic mice. Diabetic heterozygous ROCK 2^{+/-} KO mice have less ROCK activity, decreased levels of CC arginase activity/ expression, activated p38 MAPK, and less impairment of endothelium-dependent and nitrgic nerve-mediated relaxation than diabetic WT mice. We propose that increased corporal arginase activity caused by diabetes involves the ROCK and p38 MAPK pathways, which contribute to penile vascular dysfunction.

Acknowledgments

“Other” NIH funding body: This study was funded by Sexual Medicine Society of North America (SMSNA) Research Award and Postdoctoral Fellowship from the American Heart Association (AHA; 7990086) to HAT and by grants from the National Institute of Health, HL-70215 (RWC), EY-11766 (RBC) and HL052233 and DK085006 (JKL).

References

1. Andersson KE. Pharmacology of penile erection. *Pharmacol Rev.* 2001; 53:417–450. [PubMed: 11546836]
2. Dean RC, Lue TF. Physiology of penile erection and pathophysiology of erectile dysfunction. *Urol Clin North Am.* 2005; 32:379–395. [PubMed: 16291031]
3. Bivalacqua TJ, Hellstrom WJ, Kadowitz PJ, Champion HC. Increased expression of arginase II in human diabetic corpus cavernosum: In diabetic-associated erectile dysfunction. *Biochem Biophys Res Commun.* 2001; 283:923–927. [PubMed: 11350073]
4. Durante W, Johnson FK, Johnson RA. Arginase: A critical regulator of nitric oxide synthesis and vascular function. *Clin Exp Pharmacol Physiol.* 2007; 34:906–911. [PubMed: 17645639]
5. Romero MJ, Platt DH, Tawfik HE, Labazi M, El-Remessy AB, Bartoli M, Caldwell RB, Caldwell RW. Diabetes-induced coronary vascular dysfunction involves increased arginase activity. *Circ Res.* 2008; 102:95–102. [PubMed: 17967788]
6. Grönroos J, Jung C, Lundberg JO, Cerrato R, Ostenson CG, Pernow J. Arginase inhibition restores in vivo coronary microvascular function in type 2 diabetic rats. *Am J Physiol Heart Circ Physiol.* 2011; 300:H1174–H1181. [PubMed: 21297024]
7. Toque HA, Tostes RC, Yao L, Xu Z, Webb RC, Caldwell RB, Caldwell RW. Arginase II deletion increases corpora cavernosa relaxation in diabetic mice. *J Sex Med.* 2011; 8:722–733. [PubMed: 21054801]

8. Zhang C, Hein TW, Wang W, Chang CI, Kuo L. Constitutive expression of arginase in microvascular endothelial cells counteracts nitric oxide-mediated vasodilatory function. *FASEB J*. 2001; 15:1264–1266. [PubMed: 11344108]
9. Bachetti T, Comini L, Francolini G, Bastianon D, Valetti B, Cadei M, et al. Arginase pathway in human endothelial cells in pathophysiological conditions. *J Mol Cell Cardiol*. 2004; 37:515–523. [PubMed: 15276021]
10. Ming XF, Barandier C, Viswambharan H, Kwak BR, Mach F, Mazzolai L, et al. Thrombin stimulates human endothelial arginase enzymatic activity via RhoA/ROCK pathway: Implications for atherosclerotic endothelial dysfunction. *Circulation*. 2004; 110:3708–3714. [PubMed: 15569838]
11. Morris SM Jr. Recent advances in arginine metabolism: Roles and regulation of the arginases. *Br J Pharmacol*. 2009; 157:922–930. [PubMed: 19508396]
12. Chitaley K, Wingard CJ, Clinton Webb R, Branam H, Stopper VS, Lewis RW, Mills TM. Antagonism of Rho-kinase stimulates rat penile erection via a nitric oxide-independent pathway. *Nat Med*. 2001; 7:119–122. [PubMed: 11135626]
13. Priviero FB, Jin LM, Ying Z, Teixeira CE, Webb RC. Up-regulation of the RhoA/Rho-kinase signaling pathway in corpus cavernosum from endothelial nitric-oxide synthase (NOS), but not neuronal NOS, null mice. *J Pharmacol Exp Ther*. 2010; 333:184–192. [PubMed: 20093396]
14. Bivalacqua TJ, Champion HC, Usta MF, Celtek S, Chitaley K, Webb RC, Lewis RL, Mills TM, Hellstrom WJ, Kadowitz PJ. RhoA/Rho-kinase suppresses endothelial nitric oxide synthase in the penis: A mechanism for diabetes-associated erectile dysfunction. *Proc Natl Acad Sci U S A*. 2004; 101:9121–9126. [PubMed: 15184671]
15. Mills TM, Chitaley K, Wingard CJ, Lewis RW, Webb RC. Effect of Rho-kinase inhibition on vasoconstriction in the penile circulation. *J Appl Physiol*. 2001; 91:1269–1273. [PubMed: 11509525]
16. Rees RW, Ziessen T, Ralph DJ, Kell P, Moncada S, Celtek S. Human and rabbit cavernosal smooth muscle cells express Rho-kinase. *Int J Impot Res*. 2002; 14:1–7. [PubMed: 11896471]
17. Higashi M, Shimokawa H, Hattori T, Hiroki J, Mukai Y, Morikawa K, Ichiki T, Takahashi S, Takeshita A. Long-term inhibition of Rho-kinase suppresses angiotensin II-induced cardiovascular hypertrophy in rats in vivo: Effect on endothelial NAD(P)H oxidase system. *Circ Res*. 2003; 93:767–775. [PubMed: 14500337]
18. Shah DI, Singh M. Effect of fasudil on macrovascular disorder-induced endothelial dysfunction. *Can J Physiol Pharmacol*. 2006; 84:835–845. [PubMed: 17111028]
19. Jin L, Liu T, Lagoda GA, Champion HC, Bivalacqua TJ, Burnett AL. Elevated RhoA/Rho-kinase activity in the aged rat penis: Mechanism for age-associated erectile dysfunction. *FASEB J*. 2006; 20:536–538. [PubMed: 16396994]
20. Chandra S, Romero M, Shatanawi A, Caldwell R, Caldwell R. Oxidative species increase arginase activity through the RhoA/ Rho kinase (RAK) pathway. *Br J Pharmacol*. 2011; 165:506–519. [PubMed: 21740411]
21. Shatanawi A, Romero MJ, Iddings JA, Chandra S, Umapathy NS, Verin AD, Caldwell RB, Caldwell RW. Angiotensin II-induced vascular endothelial dysfunction through RhoA/ Rho kinase/p38 mitogen-activated protein kinase/arginase pathway. *Am J Physiol Cell Physiol*. 2011; 300:C1181–C1192. [PubMed: 21289285]
22. Horowitz S, Binion DG, Nelson VM, Kanaa Y, Javadi P, Lazarova Z, Andrekopoulos C, Kalyanaraman B, Otterson MF, Rafiee P. Increased arginase activity and endothelial dysfunction in human inflammatory bowel disease. *Am J Physiol Gastrointest Liver Physiol*. 2007; 292:G1323–G1336. [PubMed: 17218473]
23. Rodrigues-Díez R, Carvajal-González G, Sánchez-López E, Rodríguez-Vita J, Rodrigues Díez R, Selgas R, Ortiz A, Egido J, Mezzano S, Ruiz-Ortega M. Pharmacological modulation of epithelial mesenchymal transition caused by angiotensin II. Role of ROCK and MAPK pathways. *Pharm Res*. 2008; 25:2447–2461. [PubMed: 18633694]
24. Behr TM, Berova M, Doe CP, Ju H, Angermann CE, Boehm J, Willette RN. p38 mitogen-activated protein kinase inhibitors for the treatment of chronic cardiovascular disease. *Curr Opin Investig Drugs*. 2003; 4:1059–1064.

25. Takahashi M, Okazaki H, Ogata Y, Takeuchi K, Ikeda U, Shimada K. Lysophosphatidylcholine induces apoptosis in human endothelial cells through a p38-mitogen-activated protein kinase-dependent mechanism. *Atherosclerosis*. 2002; 161:387–394. [PubMed: 11888522]
26. Liscovsky MV, Ranocchia RP, Gorlino CV, Alignani DO, Morón G, Maletto BA, Pistoresi-Palencia MC. Interferongamma priming is involved in the activation of arginase by oligodeoxynucleotides containing CpG motifs in murine macrophages. *Immunology*. 2009; 128:159–169.
27. Toque HA, Romero MJ, Tostes RC, Shatanawi A, Chandra S, Carneiro Z, Inscho E, Webb RC, Caldwell RB, Caldwell RW. p38 Mitogen-activated protein kinase (MAPK) increases arginase activity and contributes to endothelial dysfunction in corpora cavernosa from angiotensin-II treated mice. *J Sex Med*. 2010; 12:3857–3867. [PubMed: 20807329]
28. Jin L, Lagoda G, Leite R, Webb RC, Burnett AL. NADPH oxidase activation: A mechanism of hypertension-associated erectile dysfunction. *J Sex Med*. 2008; 5:544–551. [PubMed: 18208505]
29. Rikitake Y, Oyama N, Wang CY, Noma K, Satoh M, Kim HH, Liao JK. Decreased perivascular fibrosis but not cardiac hypertrophy in ROCK1+/- haploinsufficient mice. *Circulation*. 2005; 112:2959–2965. [PubMed: 16260635]
30. Thumkeo D, Keel J, Ishizaki T, Hirose M, Nonomura K, Oshima H, Oshima M, Taketo MM, Narumiya S. Targeted disruption of the mouse rho-associated kinase 2 gene results in intrauterine growth retardation and fetal death. *Mol Cell Biol*. 2003; 23:5043–5055. [PubMed: 12832488]
31. Muranyi A, Derkach D, Erdodi F, Kiss A, Ito M, Hartshorne DJ. Phosphorylation of Thr695 and Thr850 on the myosin phosphatase target subunit: Inhibitory effects and occurrence in A7r5 cells. *FEBS Lett*. 2005; 579:6611–6615. [PubMed: 16297917]
32. Demougeot C, Prigent-Tessier A, Marie C, Berthelot A. Arginase inhibition reduces endothelial dysfunction and blood pressure rising in spontaneously hypertensive rats. *J Hypertens*. 2005; 5:971–978. [PubMed: 15834282]
33. Chang S, Hypolite JA, Changelkar A, Wein AJ, Chacko S, DiSanto ME. Increased contractility of diabetic rabbit corpora smooth muscle in response to endothelin is mediated via Rho-kinase beta. *Int J Impot Res*. 2003; 15:53–62. [PubMed: 12605241]
34. Buyukfarsar K, Un I. Effects of the Rho-kinase inhibitors, Y-27632 and fasudil, on the corpus cavernosum from diabetic mice. *Eur J Pharmacol*. 2003; 472:235–238. [PubMed: 12871759]
35. Chiou WF, Liu HK, Juan CW. Abnormal protein expression in the corpus cavernosum impairs erectile function in type 2 diabetes. *BJU Int*. 2010; 105:674–680. [PubMed: 19751257]
36. Mills TM, Chitaley K, Lewis RW, Webb RC. Nitric oxide inhibits RhoA/Rho-kinase signaling to cause penile erection. *Eur J Pharmacol*. 2002; 439:173–174. [PubMed: 11937108]
37. Rajasekaran M, White S, Baquir A, Wilkes N. Rho-kinase inhibition improves erectile function in aging male Brown-Norway rats. *J Androl*. 2005; 26:182–188. [PubMed: 15713824]
38. Li WJ, Park K, Paick JS, Kim SW. Chronic treatment with an oral rho-kinase inhibitor restores erectile function by suppressing corporal apoptosis in diabetic rats. *J Sex Med*. 2011; 8:400–410. [PubMed: 20233282]
39. Komers R, Oyama TT, Beard DR, Anderson S. Effects of systemic inhibition of Rho kinase on blood pressure and renal haemodynamics in diabetic rats. *Br J Pharmacol*. 2011; 162:163–174. [PubMed: 20840471]
40. Fukata Y, Amano M, Kaibuchi K. Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. *Trends Pharmacol Sci*. 2001; 22:32–39. [PubMed: 11165670]
41. Pieper GM, Dondlinger LA. Plasma and vascular tissue arginine are decreased in diabetes: Acute arginine supplementation restores endothelium-dependent relaxation by augmenting cGMP production. *J Pharmacol Exp Ther*. 1997; 283:684–691. [PubMed: 9353386]
42. Hagenfeldt L, Dahlquist G, Persson B. Plasma amino acids in relation to metabolic control in insulin-dependent diabetic children. *Acta Paediatr Scand*. 1989; 78:278–282. [PubMed: 2929351]
43. Kim NN, Cox JD, Baggio RF, Emig FA, Mistry SK, Harper SL, Speicher DW, Morris SM Jr, Ash DE, Traish A, Christianson DW. Probing erectile function: S-(2-boronoethyl)-L-cysteine binds to arginase as a transition state analogue and enhances smooth muscle relaxation in human penile corpus cavernosum. *Biochemistry*. 2001; 40:2678–2688. [PubMed: 11258879]

44. Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem*. 1996; 271:20246–20249. [PubMed: 8702756]
45. Kureishi Y, Kobayashi S, Amano M, Kimura K, Kanaide H, Nakano T, Kaibuchi K, Ito M. Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *J Biol Chem*. 1997; 272:12257–12260. [PubMed: 9139666]
46. Yao L, Chandra S, Toque HA, Bhatta A, Caldwell RC, Caldwell RW. Prevention of diabetes-induced arginase activation and vascular dysfunction by Rho kinase knockout. *Cardiovasc Res*. 2013; 97:509–519. [PubMed: 23250919]
47. Bao W, Behm DJ, Nerurkar SS, Ao Z, Bentley R, Mirabile RC, Johns DG, Woods TN, Doe CP, Coatney RW, Ohlstein JF, Douglas SA, Willette RN, Yue TL. Effects of p38 MAPK Inhibitor on angiotensin II-dependent hypertension, organ damage, and superoxide anion production. *J Cardiovasc Pharmacol*. 2007; 49:362–368. [PubMed: 17577100]
48. Kloesch B, Liszt M, Steiner G, Bröll J. Inhibitors of p38 and ERK1/2 MAPkinase and hydrogen sulphide block constitutive and IL-1 -induced IL-6 and IL-8 expression in the human chondrocyte cell line C-28/I2. *Rheumatol Int*. 2012; 32:729–736. [PubMed: 21161531]
49. Du Y, Tang J, Li G, Berti-Mattera L, Lee CA, Bartkowski D, Gale D, Monahan J, Niesman MR, Alton G, Kern TS. Effects of p38 MAPK inhibition on early stages of diabetic retinopathy and sensory nerve function. *Invest Ophthalmol Vis Sci*. 2010; 51:2158–2164. [PubMed: 20071676]
50. Nangle MR, Cotter MA, Cameron NE. Correction of nitroergic neurovascular dysfunction in diabetic mouse corpus cavernosum by p38 mitogen-activated protein kinase inhibition. *Int J Impot Res*. 2006; 18:258–263. [PubMed: 16355109]

Figure 1.

Western blot analysis demonstrates the effect of diabetes on corporal RhoA and Rho kinase (ROCK) protein expression. Increased levels of active RhoA (membrane bound) were observed in diabetic corpus cavernosum (CC) compared with nondiabetic CC tissue (panel A). Western blot analysis of ROCK 2 (panel B) and ROCK 1 (panel C) in CC tissues of nondiabetic wild type (WT), diabetic WT(WT + D), partial ROCK 2^{+/-} knockout (KO), and ROCK 2^{+/-} KO + D mice. Densitometry values were normalized to actin and expressed as % of WT. Summarized bar graph represents the mean of 4–5 mice per group. * $P < 0.05$, compared with WT group; # $P < 0.05$, compared with WT + D group; ** $P < 0.01$, compared to non-diabetic group

Figure 2.

Measurement of Rho kinase (ROCK) activity as the ratio of phosphorylated MYPT-1^{Thr850} to total MYPT-1 in corpus cavernosum (CC) tissues of nondiabetic wild type (WT), diabetic WT (WT + D), partial ROCK 2^{+/-} knockout (KO), and ROCK 2^{+/-} KO + D mice. Representative immunoblots of p-MYPT-1^{Thr850} and MYPT-1 and summarized bar graph shows that diabetes increased pMYPT-1^{Thr850} in CC of WT + D mice, and that increase was not observed in CC of ROCK 2^{+/-} KO + D mice (N = 4 mice per group). **P* < 0.05 compared with WT group; #*P* < 0.05 compared with WT + D group

Figure 3.

Arginase activity in corpora cavernosa (CC) tissues from nondiabetic wild type (WT), diabetic WT (WT + D), partial ROCK 2^{+/-} knockout (KO), and ROCK 2^{+/-} KO + D mice was determined by urea production (panel A). Western blot analysis of arginase II (panel B) in CC tissues of WT, WT + D, ROCK 2^{+/-} KO, and ROCK 2^{+/-} KO + D mice.

Densitometry values were normalized to actin expressed as % of WT. Summarized bar graph represent the mean of 4–5 mice per group. ** $P < 0.01$, * $P < 0.05$, compared with WT group; # $P < 0.05$, compared with WT + D group

Figure 4.

Endothelium-dependent relaxation to acetylcholine (ACh, 10^{-9} to 10^{-5} M) in cavernosal segments from nondiabetic wild type (WT), diabetic WT (WT + D), partial ROCK 2^{+/-} knockout (KO), and ROCK 2^{+/-} KO + D mice (panel A), and in partial ROCK 1^{+/-} KO and ROCK 1^{+/-} KO + D mice (panel B). Data were calculated relative to the maximal changes from the contraction produced by phenylephrine (10^{-5} M) in each tissue, which was taken as 100% (N = 6–8 mice per group). * $P < 0.05$ compared with WT mice

Figure 5. Nitrgic nerve stimulation by electric field stimulation (EFS, 1–32 Hz) in cavernosal segments from nondiabetic wild type (WT), diabetic WT (WT + D), partial ROCK 2^{+/−} knockout (KO), and ROCK 2^{+/−} KO + D mice (panel A). Representative traces of nitrgic nerve relaxation responses are shown in panel B. Acute effect of the arginase inhibitor ABH (10^{−4} M) on the relaxation induced by EFS (1–32 Hz, panel C) in the cavernosal strips from nondiabetic and diabetic in wild type (WT) mice. Data were calculated relative to the maximal changes from the contraction produced by phenylephrine (10^{−5} M) in each tissue, which was taken as 100% (N = 4–6 mice per group). **P* < 0.05, compared with WT mice; #*P* < 0.05, compared with WT + D group

Figure 6.

Contractile-response curves upon stimulation of α_1 -adrenergic receptor, phenylephrine (PE, 10^{-9} to 10^{-4} M, panel A) or adrenergic nerves (electric field stimulation, EFS 1–32 Hz, panel B) in cavernosal segments from nondiabetic wild type (WT), diabetic WT (WT + D), partial ROCK 2^{+/-} knockout (KO), and ROCK 2^{+/-} KO + D mice. Data were calculated relative to the maximal changes from the contraction produced by KCl (80 mM), which was taken as 100%. Data represent mean of 7–8 mice per group. * $P < 0.05$, compared with WT mice; # $P < 0.05$, compared with WT + D mice; \$ $P < 0.05$, compared with ROCK 2^{+/-} KO + D mice

Figure 7.

Panel A shows the effect of p38 MAPK inhibitor (SB203580, 10^{-5} M) in diabetes-mediated arginase activation in corpus cavernosum (CC) from nondiabetic wild type (WT) and diabetic WT (WT + D). Panel B shows the Western blot analysis of phospho-p38 MAPK/p38 MAPK ratio in CC tissues of nondiabetic WT, WT + D, partial ROCK 2^{+/-} knockout (KO), and ROCK 2^{+/-} KO + D mice. Densitometry values were normalized to actin expressed as % of WT. Summarized bar graph represents the mean of four mice per group. Panel C shows the effect of the p38 MAPK inhibitor (SB203580, 10^{-5} M) on the relaxation induced by acetylcholine (ACh, 10^{-9} to 10^{-5} M) or electrical field stimulation (EFS, 1–32 Hz, panel D) in CC strips of WT and WT + D mice. Data were calculated relative to the maximal changes from the contraction produced by phenylephrine (10^{-5} M) in each tissue, which was taken as 100%. Data represent the mean of 5–8 mice per group. ** $P < 0.01$, * $P < 0.05$, compared with WT group; # $P < 0.05$, compared with WT + D group

Body weight, blood glucose levels, and cavernosal strip weight of nondiabetic wild type (WT), diabetic WT (WT + D), haploinsufficient ROCK 1^{+/-} and ROCK 2^{+/-} knockout (KO), nondiabetic (ROCK 1^{+/-} KO; ROCK 2^{+/-} KO) and diabetic (ROCK 1^{+/-} KO + D; ROCK 2^{+/-} KO + D) mice

Table 1

	WT	WT + D	ROCK 1 ^{+/-} KO	ROCK 1 ^{+/-} KO + D	ROCK 2 ^{+/-} KO	ROCK 2 ^{+/-} KO + D
weight (g)	27.2 ± 1.9	22.1 ± 2.2	27.5 ± 1.3	21.6 ± 1.7	27.7 ± 1.6	22.2.6 ± 1.4
Glucose levels (mg/dL)	148 ± 14	465 ± 30 *	162 ± 17	410 ± 28 *	160 ± 10	387 ± 21 *
Cavernosal strip weight (mg)	1.73 ± 0.4	1.66 ± 0.6	1.70 ± 0.3	1.68 ± 0.2	1.83 ± 0.5	1.70 ± 0.4

* *P* < 0.05