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**Additive effects of the Rho Kinase Inhibitor Y-27632 and vardenafil on relaxation of corpus cavernosum tissue of patients with erectile dysfunction and clinical phosphodiesterase type 5 inhibitor failure**



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Keywords:	Rho Kinase Inhibitor, Corpus Cavernosum, Erectile Dysfunction, Rho/Rho associated Protein Kinase (ROCK) Pathway, Y-27632, Phosphodiesterase Type 5 Inhibitor
Abstract:	<p><b>Objectives:</b> To evaluate the expression of the Rho/Rho associated protein kinase (ROCK) pathway in corpus cavernosum of patients with severe erectile dysfunction (ED) compared to healthy human corpus cavernosum, and to test the functional effects of two Rho Kinase Inhibitors (RKI) on erectile tissue of patients with severe ED, not responding to phosphodiesterase type 5 inhibitors (PDE5-i).</p> <p><b>Patients and methods:</b> Human corpus cavernosum samples were obtained after consent from</p>

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	<p>individuals undergoing penile prosthesis implantation (n = 7 for organ bath experiments, n = 17 for qPCR). Potent control subjects (n = 5) underwent penile needle biopsy. qPCR was performed for the expression of RhoA and ROCK subtypes 1 and 2. Immunohistochemistry staining against ROCK and <math>\alpha</math> smooth muscle actin (<math>\alpha</math>SMA) was performed on corpus cavernosum of an ED patient. Tissue strips were precontracted with phenylepinephrine and incubated with 1<math>\mu</math>M of the PDE5-i vardenafil or with DMSO (control). Subsequently, increasing concentrations of the RKIs azaindole or Y-27632 were added and relaxation of tissue was quantified.</p> <p>Results:</p> <p>The expression of ROCK1 was unchanged (p &gt; 0.05), while ROCK2 (p &lt; 0.05) was significantly upregulated in ED patients, compared to controls. ROCK 1 and 2 protein colocalized with <math>\alpha</math>SMA, confirming the presence of this kinase in cavernous smooth muscle cells and/or myofibroblasts. After incubation with DMSO, 10<math>\mu</math>M azaindole and 10<math>\mu</math>M Y-27632 relaxed precontracted tissues with 49.5 <math>\pm</math> 7.42% (p = 0.1470 when compared to vehicle) and 85.9 <math>\pm</math> 10.3% (p = 0.0016 when compared to vehicle), respectively. Additive effects on relaxation of human corpus cavernosum were seen after preincubation with 1<math>\mu</math>M vardenafil.</p> <p>Conclusion:</p> <p>The RKI Y-27632 causes a significant relaxation of corpus cavernosum in tissue strips of patients with severe erectile dysfunction. The additive effect of vardenafil and Y-27632 demonstrate that a combined inhibition of Rho-kinase and phosphodiesterase type 5 could be a promising orally administered treatment for severe ED.</p>

Title:

Additive effects of the Rho Kinase Inhibitor Y-27632 and vardenafil on relaxation of corpus cavernosum tissue of patients with erectile dysfunction and clinical phosphodiesterase type 5 inhibitor failure

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INTRODUCTION

Erectile dysfunction (ED) is a condition in which patients are unable to attain or maintain an erection sufficient for sexual intercourse. The first-line pharmacotherapeutic strategy for erectile dysfunction consists of phosphodiesterase type 5 inhibitors (PDE5-i) of which the overall efficacy rate is 60-70%.(1) Specific patients in which PDE5-i have a decreased efficacy are patients following radical prostatectomy, and those with diabetes, as these conditions are marked by a decreased nitric oxide (NO) release from the cavernous nerves and endothelium, and PDE5-i depend on an intact NO supply to display efficacy.(2) As these patients currently have to resort to intracavernous injections of vaso-active agents or penile prosthesis implantation, interest in alternative oral pharmacotherapeutical strategies has grown in recent years.(3,4)

One of the pathways that has been heavily investigated is the RhoA-Rho-kinase (ROCK) system. RhoA is a monomeric GTP-binding protein that activates Rho kinase.(5) The Rho-associated protein kinases 1 and 2 (ROCK1 and ROCK2) act on a large set of substrates and thereby induce multiple cellular responses. One of these substrates is myosin phosphatase target subunit 1 (MYPT1, a regulatory subunit of myosin light chain phosphatase), which - when phosphorylated - results in increased myosin light chain phosphorylation and which facilitates the interaction between myosin and actin. It hereby causes Ca2+-sensitization of the contractile proteins, enhancing smooth muscle contraction.(6) Therefore, the ROCK system seems to play an important role in maintaining cavernous smooth muscle tone in the flaccid state, and activation or overexpression of this cascade results in smooth muscle hypercontractility. Rho-kinase inhibitors (RKIs) could thus be useful for reversal of this mechanism to achieve relaxation of cavernosal smooth muscle, independent of nitric oxide supply.(4) Other targets include kinase C-potentiated phosphatase inhibitor of 17 kDa (CPI-17) and myosin II regulatory light chain (MLC), which like MYPT1 are also associated with smooth muscle contraction, as well as LIM-kinase 1 and 2 (LIMK1/2), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), formin homology 2 domain-containing 1 (FHOD1) and ezrin-radixin-moesin (ERM) associated with F-actin stabilization, inhibition of vascular smooth muscle cell proliferation, intermediate filament disruption and actin-membrane linkage and respectively.(7)

In different animal models of ED, an upregulation of the ROCK pathway in the corpus cavernosum was demonstrated while inhibition of this pathway with RKIs facilitated penile erection. Indeed, Jin et al. showed an upregulation of membrane-bound RhoA in aged rats with ED,(8) Bivalacqua et al described an upregulation of the ROCK pathway in diabetic rats with ED,(9) and Gratzke et al noted an upregulation of the ROCK pathway in rats which underwent bilateral cavernous nerve injury as a rat model for post-radical prostatectomy ED.(10)

While RKIs have been tested in early development phases in patients with ED, the profound upregulation of the ROCK system that was observed in small animal models for ED was never confirmed in humans. Therefore, the aim of this study was to test the expression of ROCK mRNA in corpus cavernosum of ED patients compared to healthy human corpus cavernosum, and to test the functional effects of two RKIs (azaindole and Y-27632), alone or in combination with vardenafil, on erectile tissue of patients with severe ED not responding to PDE5-i.

PATIENTS & METHODS

Experimental design

Human corpus cavernosum samples were obtained from 21 patients with ED of various etiology who underwent IPP implantation because of failure of PDE5-i therapy (n = 7 for contractility experiments, n = 17 for qPCR). All patients were offered intracavernosal injections of prostaglandin E1, before considering IPP implantation. Control tissue was harvested by corpus

cavernosum needle biopsy taken at the distal third of the penile shaft (16 G. Tru-Cut Needle) from consenting age-matched donors without ED (International Index of Erectile Function Questionnaire - Erectile Function [IIEF-EF] > 25) who underwent transurethral resection of prostate adenomas in the setting of acute urinary retention (n = 5). Therefore, none of these patients was treated with 5 alpha-reductase inhibitors at the time of tissue harvesting. Patients and age-matched controls (P = 0.25 for age) consented to the use of penile tissues and the study protocol was approved by the ethical review board at Jessa Hospital, Hasselt, Belgium and the Biobank Limburg, Hasselt, Belgium. Therefore, the study complies with international regulations for protection of patients. Control tissues from needle biopsies were snap frozen and used for quantitative polymerase chain reaction (qPCR) analysis due to the size limit of the tissue. Patient characteristics and risk factors for ED are summarized in Table 1.

#### qPCR

Real-time qPCR was used to identify expression of various components in the RhoA-Rho-kinase (ROCK) system in human corpus cavernosum of patients and age-matched healthy controls. Total RNA was isolated using RNeasy mini columns (Qiagen S.A., Courtaboeuf, France) and further purified by Dnase digestion. The mRNA expression of ROCK1, ROCK2 and  $\beta$ -actin was measured by real-time quantitative PCR (TaqMan-PCR) using an ABI Prism 7700 sequence detection instrument (Applied Biosystems, Inc., Carlsbad, CA, USA). In brief: 1  $\mu$ g of total RNA was transcribed into cDNA with Superscript II RT cDNA synthesis kit (Gibco, Inc., Carlsbad, CA, USA). Normalization of the cycle threshold (CT) data was done to the reference gene  $\beta$ -actin.

#### Immunofluorescence microscopy

Immunohistochemistry staining against ROCK subtypes 1 and 2 and  $\alpha$  smooth muscle actin ( $\alpha$ SMA) was performed on corpus cavernosum of an ED patient. Samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Paraffin sections were dewaxed in xylene and rehydrated with a graded ethanol series. Slides were washed in Tris-buffered saline (TBS). For epitope retrieval, slides were exposed in citrate buffer (pH 6) and heated to about 90°C for 60 minutes. After washing in TBS, the slides were blocked for 60 minutes using BSA 1%, NGS 3%, Triton X-100 0.3% in PBS (Sigma-Aldrich, St. Louis, MO, USA), followed by incubation overnight on 4°C in a humidified chamber with 1/200 Rb ROCK1 (Abcam, Cambridge, UK), 1/200 Rb anti-ROCK2 (Abcam, Cambridge, UK), 1/200 Ms anti-smooth muscle actin (Dako, Copenhagen, Denmark). Then, slides were rinsed in PBS, incubated for one hour on room temperature with secondary antibodies 1/500 Alexa Fluor 594 donkey anti-mouse, 1/500 Alexa Fluor 488 donkey anti-rabbit (Invitrogen, Carlsbad, CA, USA) as secondary antibodies. Control staining in the absence of primary antibodies was used to evaluate nonspecific staining by secondary antibodies. Nuclear staining was performed using 4',6-diamidino-2-phenylindole or DAPI (Invitrogen, Carlsbad, CA, USA). Slides were evaluated and photographed using a Olympus microscope (Olympus, Tokyo, Japan).

#### Isometric tension measurement in *in vitro* studies

Immediately after harvesting the samples during IPP implantation, they were immersed in ice-cold Krebs-Henseleit solution (in mmol/L: NaCl 112, KCl 5.9, CaCl<sub>2</sub> 2.0 MgCl<sub>2</sub> 1.2, NaH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, glucose 11.5) and transported. The samples were divided into six equally sized longitudinal strips of approximately 2 mm x 5 mm. These strips were then transferred into organ baths of 45 mL Krebs-Henseleit solution at 37°C equilibrated with 95% O<sub>2</sub>, 5% CO<sub>2</sub>. Strips were mounted between two clips, which were in turn attached at one side to a fixed glass bar and on a force transducer. The height of the force transducer was adjustable to be able to give a precise preload to the mounted tissue strips. The force transducer was connected to an amplifier and a laptop with chart recording software (LabChart). The mounting was followed by a 60-minute equilibration period, in which a preload tension was fixed at 3g. Every 10 minutes the tension was adjusted to maintain the preload of 3g. After the equilibration period, the strips

were precontracted with 10  $\mu\text{mol} / \text{L}$  phenylephrine. Ten minutes after precontraction, the baths were washed-out two times. This was repeated once more whereafter the tissue was precontracted a third time with 10  $\mu\text{mol} / \text{L}$  phenylephrine. The maximal tension after this precontraction was defined as 100% tension. After stabilization of the tension traces, 1  $\mu\text{M}$  vardenafil or the vehicle (dimethyl sulfoxide or DMSO) was added. Subsequently, increasing concentrations of azaindole or Y-27632 were added (range  $10^{-9} \text{ M} - 10^{-5} \text{ M}$ ).

Statistical analysis

All values are expressed as mean  $\pm$  standard error of the mean (SEM). Statistical differences were determined by a Student's *t*-test or Mann-Whitney test depending on normality of the data. A *P* value of  $<0.05$  was considered to indicate a statistically significant difference.

**RESULTS**

qPCR

We wanted to investigate the regulation of the RhoA-Rho-kinase (ROCK) system in patients with ED and failure of PDE5-i therapy. As a comparison, we harvested needle biopsies from control subjects without ED. Quantitative evaluation shows that the expression of ROCK1 was unchanged ( $P>0.05$ ), while ROCK2 (relative expression:  $7570\pm3013$  vs  $2924\pm432$   $P<0.05$ ) was significantly upregulated in ED patients. These data are shown in Figure 1 and 2. The expression of  $\alpha\text{SMA}$  was increased in corpus cavernosum from ED patients (relative expression  $504243\pm15265$  vs  $385483\pm25456$   $P<0.05$ ). Overall cellular content was comparable as illustrated by similar CT values for  $\beta$ -actin (data not shown).

Immunofluorescence microscopy

With immunofluorescence microscopy we noted that ROCK protein subtype 1 and subtype 2 colocalized with  $\alpha\text{SMA}$ , confirming the presence of this kinase in cavernous smooth muscle cells lining the sinusoids of the penis (Figure 1 and 2). Immunofluorescence could not be conducted on control tissue because of small sample sizes.

Isometric tension measurement in *in vitro* studies

The relaxant capacity of RKIs and vardenafil on human corpus cavernosum was tested in organ bath experiments (Figure 3 and 4). After preincubation with DMSO, 10  $\mu\text{M}$  azaindole and 10  $\mu\text{M}$  Y-27632 relaxed precontracted tissues with  $49.5 \pm 7.42\%$  ( $p = 0.1470$  when compared to vehicle) and  $85.9 \pm 10.3\%$  ( $p = 0.0016$  when compared to vehicle), respectively.

Added effects on relaxation of human corpus cavernosum were seen after preincubation with 1  $\mu\text{M}$  vardenafil, which relaxed precontracted tissues with  $24.4 \pm 19.3\%$ . Indeed, after preincubation with 1  $\mu\text{M}$  vardenafil, 10  $\mu\text{M}$  azaindole and 10  $\mu\text{M}$  Y-27632 relaxed precontracted tissues with an additional  $40.8 \pm 5.52\%$  ( $p = 0.1998$ ) and  $73.6 \pm 18.7\%$  ( $p = 0.0480$ ) respectively, when compared to vehicle.

**DISCUSSION**

Erectile dysfunction (ED) is highly prevalent, and 5-20% of the population have moderate to severe ED. Currently, the treatment is based on PDE5 inhibition.(11) Although many patients benefit from these drugs, drop-out rates of  $> 50\%$  have been reported, with lack of efficacy as the most important reason.(12) Possibly, the persistent need for endogenous NO production plays an important role in the failure of PDE5 inhibitors, especially in disease states in which NO bioavailability is impaired. Therefore, the interest in strategies to regulate the cavernosal



smooth muscle tone without a need of endogenous NO production has grown significantly in recent years.(2)

Rho-kinases (ROCK) are members of the serine/threonine kinases and function as downstream effectors of the small GTP-binding protein RhoA. There are two isoforms of Rho-kinase, namely ROCK1 and ROCK2. ROCK1 has a ubiquitous tissue distribution while ROCK2 is mostly present in neural and muscular tissues, and has been extensively characterized in vascular smooth muscle cells.(13) Their crucial role in a variety of cellular functions such as proliferation, apoptosis, contraction, fibrosis, endothelial cell dysfunction and cardiomyocyte dysfunction has been frequently reported. Rho-kinases have therefore been implicated in the pathophysiology of hypertension, stroke, arteriosclerosis, pulmonary arterial hypertension, vasospastic angina, diabetes, cardiac ischemia/reperfusion injury and heart failure, which rendered them important pharmacotherapeutical targets.(13) Interestingly, the Rho-kinase inhibitor fasudil has demonstrated to be efficient in preventing coronary vasospasm,(14) in the treatment of microvascular angina,(15) and in ameliorating the exercise tolerance of patients with stable effort angina.(16) Also, fasudil and Y-27632 have been shown to be useful in the reversal of established fibrosis and have been suggested as a powerful treatment in the reversal of pulmonary arterial hypertension.(17) The ROCK1/2 pathway might therefore be a promising target for reversal of erectile dysfunction.

Unlike other smooth-muscle types in which the contractile state varies more frequently, penile smooth muscle remains tonically contracted to maintain the flaccid state of the penis, unless NO is released and these effects are outweighed by the pro-relaxant including the cyclic guanosine monophosphate (cGMP)/protein kinase (PKG) pathway cGMP-PKG pathway.(3) If NO bioavailability is reduced by certain diseases such as diabetes and neural injury (vide supra), corpus cavernosum smooth muscle is rendered in a hypercontractile state. This state becomes even more pronounced when the ROCK pathway is activated or overexpressed as has been shown in various animal models for ED. Surprisingly, in humans it has never been shown that corpus cavernosum ROCK upregulation is prevalent in subjects suffering from ED. Herein we provide the first evidence that specifically ROCK2 is overexpressed in corpus cavernosum smooth muscle in the presence of severe ED.

Therefore, targeting the ROCK pathway in the search of new therapeutic targets and strategies for ED is a logical step and in different animal models of ED and in in-vitro studies, it has been shown that inhibition of ROCK results in relaxation of corporeal smooth muscle and potentiation of erection.(8-10) In their seminal paper, Chitale et al. showed that Rho-kinase antagonism by injection of Y-27632 stimulates rat penile erection independently of nitric oxide in healthy rats and strongly potentiated nerve-stimulation induced erectile activity.(18) Later, administration of the Rho-kinase inhibitor Y-27632 or antagonisation of the ROCK system by gene therapy improved erectile function in diseased (diabetes or cavernous nerve injury) or aged rats. Furthermore, the effect of Y-27632 on erectile responses was found to be dose dependent.(8) Besides acute effects, chronic administration of RKIs has been shown to preserve erectile function after cavernous nerve injury by reduction of apoptosis of cavernous smooth muscle,(19) and in diabetic animals similarly by upregulating anti-apoptotic pathways in the penile innervation.(9) Also, additive effects of RKIs and PDE5-i have been reported in several animal models. Rajeskaran et al. demonstrated there was a synergistic effect on the erectile response in old rats after intracavernosal administration of Y-27632 and a PDE5-i, compared to the administration of Y-27632 alone.(20) Furthermore, Wilkes et al. investigated the effect of Rho kinase inhibition in hypertensive rats. The erectile response improved significantly and immediately after intracavernosal injection of Y-27632. An intracavernosal injection of PDE5-i followed by Y-27632 resulted in a synergistic improvement of the erectile response.(21)

Surprisingly, the possible biological and therapeutic importance of this pathway in humans has been investigated and described only sparsely. In this study, we found that the expression of ROCK1 in corpus cavernosum tissue of ED patients was unchanged when compared to healthy



controls, while ROCK2 expression was significantly upregulated. Immunohistochemistry revealed that ROCK colocalized with  $\alpha$ SMA, which confirms the presence of this kinase in human cavernous smooth muscle cells. Furthermore, we could confirm the previous findings of Guagnini et al. that Rho-kinase inhibitors relax corpus cavernosum strips of patients with ED, not responding to PDE5-inhibitors.(22) After incubation with DMSO, azaindole and Y-27632 relaxed the precontracted human tissues. Finally, we describe for the first time additive effects of a combination of PDE5-i with RKIs in humans. Indeed, the combination of Y-27632 and vardenafil was significantly more effective in relaxing precontracted human corpus cavernosum strips than vardenafil alone. This finding indicates that RKIs increase responsiveness to PDE5-i and thus may become a valid treatment option to rescue difficult-to-treat ED patients with an orally available medication. The expression on ROCK2 in the vascular smooth muscle may indicate that RKIs cause decreases in vascular tone and thus in systemic blood pressure, lowering the dose of RKI and adding a PDE5i potentially reduces systemic side effects and enhances local effects resulting from preferential expression of PDE5a in the cavernous smooth muscle.(3) It must be noted that promising *in vitro* results do not always result in the therapeutic use of drugs. For example, the concentration needed to achieve *in vivo* effects may be so high that adverse effects occur before the desired effect takes place. There is, however, some experience in humans with the use of RKIs which were well tolerated. Knipe et al. revised past experience with the systemic use of fasudil in a recent article. Fasudil is a RKI that has been used in humans to reduce cerebral vasospasm and stable angina. For both indications, fasudil was found to be effective and well tolerated.(17) Thus, even though clinical experience with RKIs is limited, they are a rational target for therapeutic use in ED.

The present study has some limitations. Due to ethical restrictions, it was not possible to harvest corpus cavernosum samples in control patients without ED that would allow us to do organ bath experiments. For the same reason, no quantitative differences in protein level could be evaluated. Also, direct assessment of the ROCK2 protein concentration and kinase activity was not performed, as qPCR only assesses mRNA expression. Further studies are therefore needed to confirm an upregulation of ROCK2 protein and kinase activity. Ultimately, selective ROCK2 inhibition was not tested. Indeed, the ROCK2-selective kinase inhibitor KD025 showed good efficacy and was found to have a favorable safety profile when tested as a therapy for cerebral ischemia in mice.(23) Future studies are needed to assess if ROCK2-selective kinase inhibitors are applicable in the treatment of ED, as less side-effects are to be expected with selective inhibition.

However, our findings indicate that RKIs may become a valid treatment option to help difficult-to-treat ED patients with an orally available medication.

We can conclude that the Rho-kinase inhibitor Y-27632 causes a significant relaxation of corpus cavernosum in tissue strips of patients with severe erectile dysfunction. The additive effect of vardenafil and Y-27632 demonstrate that a combined inhibition of Rho-kinase and PDE5 could be a promising orally administered treatment for severe ED.

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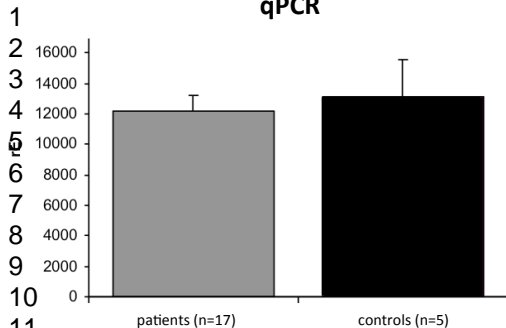
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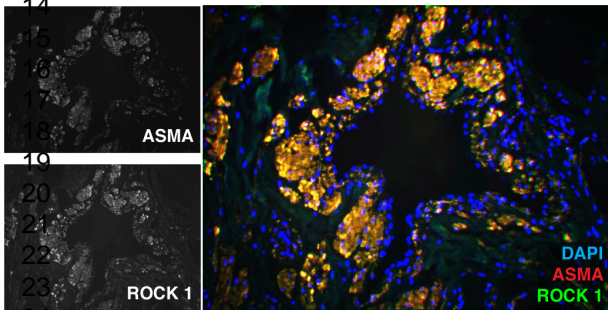
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	patients qPCR (n=17)	controls qPCR (n=5)	patients contractility (n=7)
Age (years)	57.6 ± 2.2	60.3 ± 2.3	56.4 ± 2.8
<b>Risk factors for ED</b>			
Smoking (%)	12 (71)	1 (20)	2 (29)
Hypertension (%)	8 (47)	0	3 (42)
Diabetes mellitus (%)	5 (29)	1 (20)	2 (29)
Duration of ED (months)	75.5 ± 18.2	1 (20)	96 ± 30.8
Total testosterone (ng/mL)	3.6 ± 0.4		3.8 ± 0.5
Doppler peak flow (cm/s)	21.1 ± 1.7		16.8 ± 2.3
IIEF-EF score	6.6 ± 0.8	All ≥ 25	10 ± 1.6

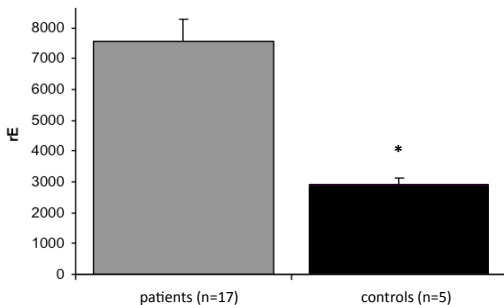
## qPCR



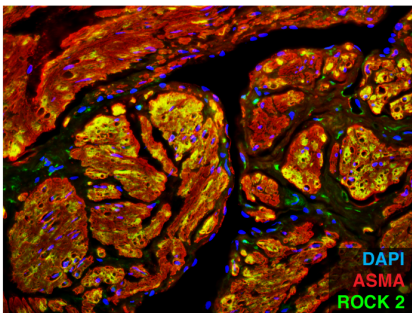
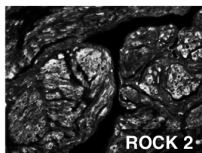
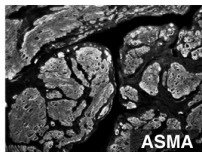
## Immunofluorescence



## qPCR

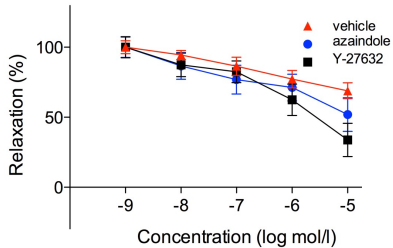
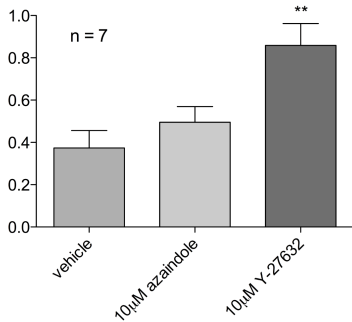


## Immunofluorescence





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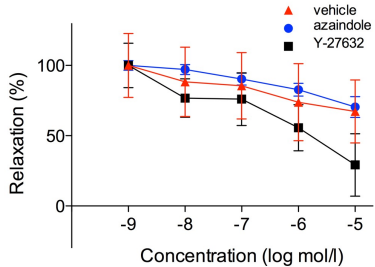
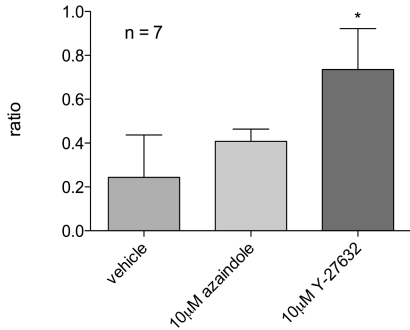


Figure and Table legend

Table 1: Characteristics of patients undergoing penile-implant surgery and healthy controls. Left column: tissue of patients used for qPCR experiments. Middle column: tissue of healthy control used for qPCR experiments. Right column: tissue of patients used for contractility experiments. (ED: erectile dysfunction, IIEF-EF: International Index of Erectile Function Questionnaire – Erectile Function)

Figure 1: Expression of Rock1 in human corpus cavernosum. Upper panel: Rock1 mRNA expression (relative to the expression of  $\beta$ -actin) of human corpus cavernosum tissue of patients with erectile dysfunction and clinical phosphodiesterase type 5 inhibitor failure compared with healthy controls. Lower panel: Immunofluorescence of human corpus cavernosum of a patient with erectile dysfunction and clinical phosphodiesterase type 5 inhibitor failure displaying expression of ROCK1 (ASMA:  $\alpha$  smooth muscle actin, ROCK1: RhoA-Rho-kinase subtype 1, DAPI: 4',6-diamidino-2-phenylindole)

Figure 2: Expression of Rock2 in human corpus cavernosum. Upper panel: Rock2 mRNA expression (relative to the expression of  $\beta$ -actin) of human corpus cavernosum tissue of patients with erectile dysfunction and clinical phosphodiesterase type 5 inhibitor failure compared with healthy controls. Lower panel: Immunofluorescence of human corpus cavernosum of a patient with erectile dysfunction and clinical phosphodiesterase type 5 inhibitor failure displaying expression of ROCK2 (ASMA:  $\alpha$  smooth muscle actin, Rock2: RhoA-Rho-kinase subtype 2, DAPI: 4',6-diamidino-2-phenylindole, \*:  $P < 0.05$ )

Figure 3: Effects of the Rho-kinase inhibitors azaindole and Y-27632 on the relaxation of isolated human corpus cavernosum compared to adding vehicle (DMSO)

Left panel: effects on relaxation of adding vehicle (DMSO) or the Rho-kinase inhibitors azaindole and Y-27632 to a concentration of  $10\mu\text{M}$ . The ratio is the relationship between the tension of the precontracted tissue measured immediately before or fifteen minutes after adding the compounds. Right panel: dose-reponse curve of the vehicle and the Rho-kinase inhibitors azaindole and Y-27632. Dosages  $\geq -5 \log \text{mol/L}$  cause unspecific inhibition and were therefore not tested (\*\*:  $P < 0.01$ , DMSO: dimethyl sulfoxide)

Figure 4: Effects of the Rho-kinase inhibitors azaindole and Y-27632 on the relaxation of isolated human corpus cavernosum compared to adding vehicle (DMSO), after preincubation with  $1\mu\text{M}$  vardenafil

Left panel: effects on relaxation of adding vehicle (DMSO) or the Rho-kinase inhibitors azaindole and Y-27632 to a concentration of  $10\mu\text{M}$ , after preincubation with  $1\mu\text{M}$  vardenafil. The ratio is the relationship between the tension of the precontracted tissue measured immediately before or fifteen minutes after adding the compounds. Right panel: dose-reponse curve of the vehicle and the Rho-kinase inhibitors azaindole and Y-27632, after preincubation with  $1\mu\text{M}$  vardenafil.

Dosages  $\geq -5 \log \text{ mol/L}$  cause unspecific inhibition and were therefore not tested (\*:  $P < 0.05$ , DMSO: dimethyl sulfoxide)

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