HHS Public Access

Author manuscript

Reproduction. Author manuscript; available in PMC 2022 January 01.

Published in final edited form as:

Reproduction. 2021 January ; 161(1): 31–41. doi:10.1530/REP-20-0447.

Constitutive LH receptor activity impairs NO mediated penile smooth muscle relaxation

Deepak S. Hiremath[1], **Fernanda B.M. Priviero**[2], **R. Clinton Webb**[2], **CheMyong Ko**[3], **Prema Narayan**[1]

[1]Department of Physiology, Southern Illinois School of Medicine, Carbondale, IL, USA

[2]Cardiovascular Translational Research Center and Department of Cell Biology and Anatomy University of South Carolina, Columbia, SC, USA

[3]Department of Comparative Biosciences, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Abstract

Timely activation of the luteinizing hormone receptor (LHCGR) is critical for fertility. Activating mutations in LHCGR cause familial male-limited precocious puberty (FMPP) due to premature synthesis of testosterone. A mouse model of FMPP (KiLHR^{D582G}), expressing a constitutively activating mutation in LHCGR, was previously developed in our laboratory. KiLHR^{D582G} mice became progressively infertile due to sexual dysfunction and exhibited smooth muscle loss and chondrocyte accumulation in the penis. In this study we tested the hypothesis that KiLHR^{D582G} mice had erectile dysfunction due to impaired smooth muscle function. Apomorphine-induced erection studies determined that KiLHR^{D582G} mice had erectile dysfunction. Penile smooth muscle and endothelial function were assessed using penile cavernosal strips. Penile endothelial cell content was not changed in KiLHR^{D582G} mice. The maximal relaxation response to acetylcholine and the nitric oxide donor, sodium nitroprusside, was significantly reduced in KiLHR^{D582G} mice indicating an impairment in the nitric oxide (NO)- mediated signaling. Cyclic guanosine monophosphate (cGMP) levels were significantly reduced in KiLHR^{D582G} mice in response to acetylcholine, sodium nitroprusside and the soluble guanylate cyclase stimulator, BAY 41-2272. Expression of NOS1, NOS3 and PKRG1 were unchanged. The Rho-kinase signaling pathway for smooth muscle contraction was not altered. Together, these data indicate that KiLHR^{D582G} mice have erectile dysfunction due to impaired NO-mediated activation of soluble guanylate cyclase resulting in decreased levels of cGMP and penile smooth muscle relaxation. These studies in the KiLHR^{D582G} mice demonstrate that activating mutations in the mouse

Corresponding Author: Prema Narayan, Department of Physiology, Southern Illinois University, LSIII, 1135 Lincoln Drive, Carbondale, IL 62901. pnarayan@siu.edu.

Author contribution statement

DSH conceived the study, performed experiments, analyzed and interpreted the data and wrote the manuscript. FBMP and RCW provided expert advice on the myograph experiments and analysis of the myograph data. CK assisted with the myograph studies. PN conceived and supervised the study, analyzed and interpreted the data, wrote and finalized the manuscript. All authors provided critical feedback on the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

LHCGR cause erectile dysfunction due to impairment of the NO-mediated signaling pathway in the penile smooth muscle.

Keywords

LH receptor; constitutive activation; erectile dysfunction; NO signaling

Introduction

The luteinizing hormone receptor (LHCGR), a member of G-protein coupled receptor family, has a critical role in steroidogenesis and spermatogenesis in males (Ascoli et al., 2002, Narayan et al., 2019). Constitutively activating mutations in LHCGR resulting in single amino acid replacements and inherited in an autosomal dominant male-limited pattern cause a condition called familial male-limited precocious puberty (FMPP). The disorder is characterized by elevated testosterone levels, Leydig cell hyperplasia and precocious puberty in boys by 3-4 years of age (Themmen & Huhtaniemi, 2000, Ulloa-Aguirre et al., 2014). The most common mutation identified in FMPP is the mutation of aspartic acid at amino acid residue 578 to glycine (D578G). To understand the role of constitutively activated LHCGR in reproductive function, we previously developed a knock-in mouse model ($KiLHR^{D582G}$) by introducing an aspartic acid to glycine mutation at amino acid residue 582 (D582G) in mouse LHCGR, corresponding to the D578G in human LHCGR. $KiLHR^{D582G}$ mice exhibit similar phenotypes seen in FMPP patients such as precocious puberty, supraphysiological levels of testosterone and Leydig cell hyperplasia (McGee & Narayan, 2013).

Our previous studies showed that $KiLHR^{D582G}$ mice became progressively infertile by 6 months of age in spite of normal sperm count and motility (Hai et al., 2017, McGee & Narayan, 2013). Mating studies with superovulated females demonstrated that $KiLHR^{D582G}$ mice had viable sperm and normal accessory gland function. Sexual behavior studies revealed that $KiLHR^{D582G}$ male mice had normal mounting behavior but exhibited a longer latency to first intromission and a significantly shorter duration of intromission with an inability to ejaculate, suggesting erectile dysfunction (ED) (Hai et al., 2017). Morphological changes observed in the penile corpora cavernosa of $KiLHR^{D582G}$ mice included reduced smooth muscle content and chondrocyte accumulation without a change in collagen content (Hai et al., 2017). These changes are mediated by high levels of testosterone (Hiremath et al., 2020). However, the functional consequences of the morphological changes in the penis of the $KiLHR^{D582G}$ mice were not determined.

The penile corpora cavernosal smooth muscle lining the sinusoids plays a key role in penile erection (Dean & Lue, 2005, Traish, 2009). Various studies have demonstrated that a significant decrease in cavernosal smooth muscle causes impaired erectile response (Ahn et al., 2005, Mostafa et al., 2013, Lombo et al., 2016, Nehra et al., 1998). During sexual stimulation, the release of nitric oxide (NO) from the non-adrenergic/cholinergic nerves causes dilation of the cavernosal arteries and relaxation of the sinusoidal smooth muscle causing increased blood flow into the penis and resulting in further production of NO from

endothelial cells. Expansion of the sinusoids and the connective tissue matrix causes compression of the subtunical venules and reduces blood outflow (veno-occlusion) resulting in increased intracavernosal pressure and erection (Dean & Lue, 2005, Nunes & Webb, 2012). Thus, smooth muscle relaxation as well as the architecture of the extracellular matrix are important for erectile function.

The major pathway for smooth muscle relaxation is the NO-cyclic guanosine monophosphate (cGMP) pathway (Dean & Lue, 2005, Morelli et al., 2006, Nunes & Webb, 2012). NO released from the cavernous nerves and endothelial cells activates soluble guanylate cyclase (sGC) in the smooth muscle cells, increasing cGMP which activates protein kinase G (PRKG1). Activated PRKG1 decreases intracellular levels of calcium causing smooth muscle relaxation (Andersson, 2011, Dean & Lue, 2005). Noradrenaline from sympathetic nerves and endothelin and prostaglandin F2α from endothelial cells increase intracellular levels of calcium causing smooth muscle contraction (Dean & Lue, 2005, Morelli et al., 2006, Traish et al., 2000). In addition, the RhoA/Rho associated protein kinase (ROCK) pathway maintains contractile tone (Sopko et al., 2014). Dysregulation of these pathways have been shown to contribute to ED (Akingba & Burnett, 2001, Bivalacqua et al., 2004, Chiou et al., 2010, Toque et al., 2013).

We hypothesize that premature and chronic activation of LHCGR in the KiLHR^{D582G} mouse causes impaired smooth relaxation resulting in erectile dysfunction. To test this hypothesis, animal behavioral studies and investigation of smooth muscle relaxation and contraction pathways were performed. Our results show that the infertility caused by constitutive activation of mouse LHCGR is due to erectile dysfunction as a result of reduced NO-mediated production of cGMP resulting in decreased penile smooth muscle relaxation.

Materials and Methods

Animal Care

The generation of the KiLHR^{D582G} mouse model was described previously (McGee & Narayan, 2013). KiLHR^{D582G} mice (B6129S-Lhcgr$^{tm1.1Pnara}$/J) are available from the Jackson Laboratory as JAX#029311. KiLHR^{D582G} male mice were bred with B6129SF1/J hybrid female mice (Jackson Laboratory) to obtain heterozygous KiLHR^{D582G} male mice and wild-type (WT) littermates. Mice were maintained on 12-hour light and 12-hour dark cycle and fed a standard chow diet (Purina LabDiet 5008) and tap water. Male mice between 26–36 weeks of age were used for the studies. Mice were euthanized by CO_2 asphyxiation, followed by decapitation. Breeding of animals were done according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All animal studies were approved by the Institutional Animal Care and Use Committee at Southern Illinois University.

Apomorphine-induced erection studies

Each mouse was placed in a plexiglass chamber alone for a period of 30 mins to acclimatize to the new chamber. A single subcutaneous injection of saline was then administered, and the mouse was observed for 30 mins by video recording. An injection of R-(–)-apomorphine

hydrochloride hemihydrate (A4393, Sigma Aldrich, St. Louis, MO) at an optimal dose of 3.2 μg/kg dissolved in 0.1% ascorbic acid was administered subcutaneously to the same mouse and the mouse was recorded again for a period of 30 mins. Penile erections were identified in these recordings by the criteria described previously (Rampin et al., 2003). An erection was scored when the mouse stood up on its hindlimbs, bent its head towards the penis, held the penis and licked it with hip movements.

Real-time quantitative PCR

The penile body was separated from the glans penis and the cavernosa was isolated after removal of the urethra and nerve bundle under a dissecting microscope and snap frozen in liquid nitrogen. The cavernosal tissue samples were homogenized in TRIzol (Invitrogen, Carlsbad, CA) and RNA was extracted according to the manufacturer's instructions. Synthesis of cDNA was done using M-MLV Reverse Transcriptase (Promega, Madison, WI). RT-qPCR was performed using PowerUP SYBR Green Master mix (Applied Biosystems, Foster City, CA). Primers were designed to span an exon-exon junction to ensure amplification of only the cDNA. The sequences of the primers are shown in Table 1. Samples were analyzed in duplicate and normalized to ribosomal protein S2 (*Rps2)*. A template negative control and a calibrator sample, prepared by mixing equal amounts of cDNA from all the samples, were also analyzed. Data are expressed as relative to the calibrator sample using the $2^{-\Delta\Delta CT}$ method as previously described (McGee & Narayan, 2013).

Functional studies in cavernosal strips

Penes from mice were dissected and placed in chilled physiological salt solution (PSS) of the following composition: 130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, 14.9 mM $NaHCO_3$, 5.6 mM glucose and 1.56 mM $CaCl_2$. The penile body was separated from the glans penis and the cavernosa was isolated after removal of the urethra and nerve bundle under a dissecting microscope. A slit was made along the shaft of the cavernosa to obtain two strips and one strip from each animal was snap frozen in liquid nitrogen for western blot analysis and the other strip was mounted on pins in a myograph chamber (Danish Myograph Technology, Aarhus, Denmark) coupled to a PowerLab data acquisition system (Software chart 5.0, AD instruments, Colorado Springs, USA). The strips were equilibrated in PSS at 37º C for 30 minutes bubbled with a mixture of 95% O_2 and 5% CO_2. Subsequently a resting tension of 5 mN was applied for 1h. To assess the contractile ability of the strips, PSS containing 120 mM potassium (KPSS) was added. Cumulative concentration-dependent contraction response was obtained by adding 10^{-9} to 10^{-4} M of the α1 adrenergic agonist, phenylephrine (PE, Sigma Aldrich, St. Louis, MO). Concentration dependent relaxation responses to 10^{-9} to 10^{-6} M acetylcholine (ACh, Cayman Chemical, Ann Arbor, MI), 10^{-9}-10^{-5} M of the NO donor, sodium nitroprusside (SNP, Sigma Aldrich, St. Louis, MO) and 10^{-9} to 10^{-4} M of the Rho kinase inhibitor, Y-27632 (Tocris, Minneapolis, MN) were obtained in tissue strips precontracted with 10^{-5} M PE. All stock solutions were prepared in deionized water except Y-27632 which was prepared in DMSO.

Western blot analysis

Frozen penile cavernosal strips from WT and KiLHR^{D582G} mice were obtained as described above. Each strip was then pulverized and sonicated in RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology, Dallas, TX). Sonication was performed on ice under the following conditions: six short bursts at power 4W for 10 seconds and four short bursts at power 4W for 5 seconds with a cooling period of 30 seconds between every burst. Samples were then centrifuged at 13,000 x g for 15 minutes and the supernatant was collected to estimate protein concentration using the Coomassie (Bradford) assay kit (Thermofisher Scientific, Waltham, MA). Forty micrograms of total protein were resolved on 8% sodium dodecyl sulfate polyacrylamide gels and the bands were transferred to Immobilin-FL PVDF membrane (Millipore-Sigma, Burlington, MA) at 100 volts for 1.5 hours. The membrane was then blocked with Odyssey blocking buffer diluted 1:1 in Tris-buffered saline (TBS) (LI-COR Biosciences, Lincoln, NE) and probed with either anti-rabbit monoclonal NO synthase3 (NOS3 or eNOS, 1:100, #32027, Cell Signaling Technology, Danvers, MA), polyclonal cGMP specific phosphodiesterase type 5 (PDE5, 1:100, #2395, Cell Signaling Technology), polyclonal NO synthase 1 (NOS1 or nNOS, 1:100, #4234, Cell Signaling Technology) or monoclonal protein kinase G (PRKG1,1:100, # 3248, Cell Signaling Technology) and anti-goat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000, AF5718, R&D systems, Minneapolis, MN) in 5% BSA in TBS/0.1% Tween-20 (TBST) overnight at 4º C. The membrane was washed three times with TBST for 5 minutes each followed by incubation with donkey anti-rabbit conjugated with Alexa Flour 790 (1:2000) and donkey anti-goat conjugated with Alexa Flour 680 (1:10000) secondary antibodies (Invitrogen, Carlsbad, CA) in 0.01% SDS in TBST for 1 hour at room temperature. The membrane was washed three times with TBST for 5 minutes each, followed by a final rinse in TBS and scanned using the Odyssey CLx infrared imaging system (LI-COR Biosciences, Lincoln, NE). The intensities of the specific bands were quantified using LI-COR Image Studio 3.1 software and normalized to the intensity of the loading control, GAPDH.

Immunohistochemistry

Penile body tissue samples were collected and fixed in modified Carnoy's solution (60% methanol, 30% ethanol and 10% acetic acid) for 4 hours at room temperature, rinsed briefly in phosphate buffered saline (PBS) and stored in 70% ethanol at 4° C before processing. Tissue samples were embedded in paraffin and 5 μm sections were prepared. Sections were blocked with 10% normal goat serum for 1 hour at room temperature was done followed by incubation with primary rat mouse pan-endothelial cell antigen (MECA-32) antibody overnight at 4° C (1:2 dilution, kindly provided by Dr. Sophia Ran, Southern Illinois University, School of Medicine). Sections were washed in TBST for 2 x 5 minutes and incubated in biotinylated secondary goat anti-rat antibody (1:200, Vector Laboratories, Burlingame, CA). Antigen-antibody complexes were visualized using Vectastain Elite ABC-DAB kit (Vector Laboratories) and counterstained with hematoxylin. Quantification of MECA-32 stained area in the corpora cavernosa was performed on four non-serial sections, each 25 μm apart, from each animal using ImageJ software (National Institutes of Health).

Determination of cGMP levels

Penile cavernosal tissues were dissected and equilibrated in PSS for 1 hour as described above. Tissue strips were precontracted with 10^{-5} M PE and relaxed with either 10^{-5} M SNP, 10^{-6} M ACh, or 10^{-5} M BAY 41-2272 and snap frozen immediately in liquid nitrogen after relaxation. For penile cGMP content, tissues were pulverized and homogenized in 5% trichloroacetic acid, centrifuged and extracted in water saturated ether. cGMP levels were determined by the ELISA kit (Cayman, Chemical, Ann Arbor, MI) according to manufacturer's instructions and were normalized to the weight of the cavernosal strip.

Data analysis

Relaxation responses to drugs were expressed as percentage of submaximal contraction induced by PE. Curves were fitted to data using non-linear regression using Prism 5 software program (Graphpad Software Inc.). Drug maximum responses and potencies were calculated as E_{max} (maximal response produced by the drug) and pEC_{50} (negative logarithm of molar concentration of drug that produced 50% of maximal response). Statistical significance of differences between WT and KiLHR^{D582G} mice was determined by unpaired Student's t-test using Prism 5 software. Outliers were identified using Grubbs test online (http://www.graphpad.com/quickcalcs/Grubbs1.cfm). $P < 0.05$ was considered statistically significant.

Results

KiLHR^{D582G} mice have erectile dysfunction

In our previous study (Hai et al., 2017), we demonstrated that KiLHR^{D582G} male mice exhibit progressive infertility and sexual behavior studies suggested KiLHR^{D582G} mice might have developed erectile dysfunction. To establish if the KiLHR^{D582G} mice develop erectile dysfunction, an erectile response to the dopamine receptor agonist, apomorphine, was determined. A systemic administration of apomorphine elicits penile erection in a number of species including mice and has been widely used as a way to determine erectile responsiveness (Simonsen et al., 2016, Matsumoto et al., 2005, Giuliano et al., 2002, Giuliano & Allard, 2001). Analysis of the video recording indicated that nine out of eleven (82%) WT mice injected with apomorphine exhibited erections, compared to one out of thirteen (8%) KiLHR^{D582G} mice (Table 2). Further, the number of erections per mouse was significantly lower in KiLHR^{D582G} mice compared with WT mice. No spontaneous erections were observed in either WT or KiLHR^{D582G} mice injected with saline during the 30-minute recording period. These results demonstrate that apomorphine was unable to elicit erections in KiLHR^{D582G} mice indicating that KiLHR^{D582G} mice had erectile dysfunction.

Expression of key components of the extracellular matrix are not altered in the corpora cavernosa of KiLHR^{D582G} mice.

The extracellular matrix (ECM) of the corpora cavernosa is mainly composed of collagen, elastic fibers and proteoglycans. Changes in these components could alter the structural integrity of the corpora cavernosa leading to erectile dysfunction (Costa et al., 2006, Luttrell et al., 2008, Sattar et al., 1994, Traish et al., 2003, Wang et al., 2015). We have previously

demonstrated that total collagen content, determined by the hydroxyproline assay, was unchanged in KiLHR^{D582G} mice (Hai et al., 2017). To determine if there were changes in specific components of the extracellular matrix, we measured the mRNA expression levels of the α1 and α2 subunits of collagen I (*Col1a1* and *Col1a2*) and collagen III (*Col3a1*), the major collagen isoforms in the mouse corpora cavernosa, elastin (*Eln*), the core component of elastic fibers and fibrillin (*Fbn1* and *Fbn2*), which provide a microfibril scaffold for elastin. Their expression levels in the corpora cavernosa were not significantly different between WT and KiLHR^{D582G} mice (Figure 1), suggesting that impairment in erectile function in the KiLHR^{D582G} mice was not due to alterations in the extracellular matrix.

The corpora cavernosa of KiLHR^{D582G} mice have normal endothelial cell content and function

Endothelial cells in the cavernosa produce NOS3 (eNOS), which plays an essential role in maintaining penile erection by generation of NO. Loss of endothelial cells can contribute to an impairment in penile erection (Burchardt et al., 2000, Liu et al., 2013). To determine if there was a reduction in endothelial cell content in corpora cavernosa of KiLHR^{D582G} mice, penile body sections from 26-week-old WT and KiLHR^{D582G} mice were stained with an antibody against an endothelial cell specific marker MECA-32 (Figure 2A). Immunohistochemical quantification by ImageJ analysis did not show a significant change in the stained area for MECA-32 in the cavernosa between WT and KiLHR^{D582G} mice (Figure 2B). To confirm these findings, quantitative real-time PCR and western blot analysis were performed to determine the levels of NOS3, another endothelial cell marker. The RNA and protein levels of NOS3 in the cavernosa of KiLHR^{D582G} mice were not significantly different compared to WT mice (Figures 2C–E). To assess endothelial cell function, endothelium-dependent relaxation to ACh was measured. The EC_{50} value for relaxation was not different between the WT and KiLHR^{D582G} tissues (Figure 2F and Table 3). However, maximal relaxation (E_{max}) to ACh was significantly reduced in the KiLHR^{D582G} mice compared with WT mice which could be due to reduced smooth muscle content and /or function. Together, these results indicate normal endothelial cell content and function in the cavernosa of KiLHR^{D582G} mice.

KiLHR^{D582G} mice have smooth muscle dysfunction in the corpora cavernosa

We next determined if smooth muscle function was altered in the corpora cavernosa of KiLHR^{D582G} mice. For this, concentration-dependent contraction to the α1 adrenergic agonist, PE and relaxation of PE -contracted tissue to the NO donor, SNP were measured in cavernosal strips. The maximal contractile and relaxation responses to PE and SNP, respectively were significantly reduced in the KiLHR^{D582G} mice compared with WT mice (Figure. 3A and B, Table 3), suggesting decreased smooth muscle function consistent with the reduction with smooth muscle content (Hai et al., 2017). The potency of the contractile response to PE was not different between the WT and KiLHR^{D582G} mice. However, the EC_{50} value for SNP was significantly higher (1.7 fold) in the KiLHR^{D582G} mice (Figure 3B and Table 3). Together, these data suggest impairment in the NO-mediated relaxation pathway.

KiLHR^{D582G} mice have normal levels of NO signaling pathway intermediates

As NO-cGMP mediated smooth muscle relaxation is the major erectile pathway in the corpora cavernosa, we examined the RNA and protein levels of enzymes that are required for production and degradation of cGMP or activated by cGMP. Neuronal nitric oxide synthase (NOS1) and NOS3 (examined above in Figure 2) are required for the production of NO from nonadrenergic-noncholinergic cavernous nerves and endothelial cells, respectively and NO is required for the production of cGMP. PDE-5 rapidly degrades cGMP. PRKG1 is a key downstream intermediate that is activated by cGMP and in turn phosphorylates various proteins and ion channels to decrease intracellular calcium and cause smooth muscle relaxation (Dean & Lue, 2005, Nunes & Webb, 2012). Determination of the RNA levels of these intermediates showed that there was no change in RNA levels of *Nos1* and *Prkg1* but *Pde5a* was significantly reduced in the cavernosa of KiLHR^{D582G} mice (Fig. 4A, C, E). Protein levels of NOS1 and PRKG1 were not different between the cavernosa of WT and KiLHR^{D582G} mice but PDE5 levels were significantly reduced in the cavernosa of KiLHR^{D582G} mice (Fig. 4B, D, F). PDE5 is the only intermediate examined that is specific to smooth muscle cells in the penis and no change in RNA levels were seen between WT and KiLHR^{D582G} mice when the expression was normalized to smooth muscle actin (data not shown).

The Rho kinase signaling pathway is not altered in KiLHR^{D582G} mice

To determine if upregulation of Rho kinase pathway contributed to impairment of smooth muscle relaxation, a concentration-dependent relaxation curve to the Rho kinase inhibitor, Y-27632 was performed (Figure 5A). The maximal relaxation response was significantly reduced in KiLHR^{D582G} mice, without a change in the potency (Table 3). Additionally, RNA levels of the Rho-kinase pathway signaling intermediates, *Rhoa, Rock1, Rock2*, were not altered in KiLHR^{D582G} mice compared with WT mice (Figure 5B–D). Together, these results suggest that the reduced relaxation response in the KiLHR^{D582G} mice is not due to a dysregulated Rho kinase pathway.

KiLHR^{D582G} mice exhibit reduced activation of soluble guanylyl cyclase (sGC)

Since the signaling intermediates of NO pathway were not altered and the maximal relaxation responses to ACh and SNP were lower, we examined the activity of sGC by measuring cGMP levels in response to ACh and SNP. The basal levels of cGMP were not different between the WT and KiLHR^{D582G} mice (Figure 6). The response to ACh and SNP was reduced in KiLHR^{D582G} mice compared to WT controls, suggesting that NO mediated activation of sGC was impaired in the KiLHR^{D582G} mice. BAY 41-2272, a NO independent stimulator of sGC, increased cGMP levels in WT and KiLHR^{D582G} mice over that obtained with SNP. However, the response was still lower in KiLHR^{D582G} mice compared to WT. Together, these data suggest that sGC activation is impaired in KiLHR^{D582G} mice.

Discussion

KiLHR^{D582G} mice with an activating mutation in LHCGR have ED primarily due to both a loss in cavernosal smooth muscle content (Hai et al., 2017) and function. The mechanism of smooth muscle dysfunction is due to an impairment in the NO- mediated pathway of smooth

muscle relaxation without a change in the Rho-kinase mediated contractile pathway. The D582G mutation in the KiLHR^{D582G} mice is analogous to the D578G mutation found in boys with FMPP. Young adult KiLHR^{D582G} mice are fertile for a short period of 3-4 months and develop ED as they age (Hai et al., 2017). The D578G mutation in LHCGR is germline, indicating that men harboring this mutation are not completely infertile (Shenker et al., 1993). However, there are no reports on the reproductive health of older FMPP patients. A significant finding from this study with the KiLHR^{D582G} mouse model is that it predicts that ED is a long-term reproductive pathology in FMPP.

One of the reproductive phenotypes exhibited by KiLHR^{D582G} male mice was the inability to successfully initiate and/or maintain penile intromission followed by ejaculation (Hai et al., 2017). These results suggested that KiLHR^{D582G} mice had erectile dysfunction; however, it could not be confirmed as visual assessment of penile erection during the sexual behavior studies was not possible (Hai et al., 2017). Herein, the apomorphine-induced erection studies provide evidence that KiLHR^{D582G} mice had impaired erectile function. Apomorphine is a dopamine agonist that is widely used to test erectile function in conscious animals and is the major neurotransmitter in the CNS shown to play a role in erectile function (Simonsen et al., 2016, Bernabé et al., 1999, Rampin et al., 2003). Systemic administration of apomorphine has been shown to elicit penile erections and is likely due to the involvement of D2 receptors at the central or spinal level (Simonsen et al., 2016, Matsumoto et al., 2005, Giuliano et al., 2002, Giuliano & Allard, 2001).

Normal erection requires the relaxation of the cavernosal smooth muscle allowing for expansion of the sinusoids and trapping of blood by compression of the subtunical venules to reduce blood outflow (Nehra et al., 1998, Traish, 2009). This veno-occlusive process requires compliance of the extracellular matrix comprised of collagen, elastic fibers and proteoglycans. This network of ECM and smooth muscle is essential for maintaining flaccidity and normal erectile response of the penis. Type I and III collagen are the major forms of collagen in the penile cavernosa responsible for high tensile strength during erection. Type I collagen is present predominantly while type III collagen is in minor proportions in the cavernosa (Moreland et al., 1995, Raviv et al., 1997). Elastic fibers in the penis are mainly composed of elastin deposited on a scaffolding of fibrillin and fibrillin-rich microfibrils and are located in sinusoids and veins in the cavernosa. Models of erectile dysfunction due to aging, diabetes or hypogonadism show a decrease in smooth muscle and increase in collagen content (Ferrini et al., 2007, Xie et al., 2007, Traish et al., 1999, Wang et al., 2015, Traish et al., 2003). Studies in mice and men with ED have shown a decrease in elastin and fibrillin (Costa et al., 2006, Sattar et al., 1994, Luttrell et al., 2008).

We have previously shown that KiLHR^{D582G} mice at 6 months of age exhibit changes in the penile architecture caused by a decrease in smooth muscle content, accumulation of chondrocytes but no change in total collagen content (Hai et al., 2017). Smooth muscle loss does not appear to be due to apoptosis (data not shown) and the mechanism of chondrocyte accumulation has not been determined. Although total collagen content was unchanged, individual collagen types and elastin content were not previously determined. In this study, we demonstrated that gene expression levels of collagen I, III, elastin and fibrillin are unaltered, indicating that impairment in erectile function was primarily due to smooth

muscle loss and not due to an altered ECM. However, it is possible that chondrocyte accumulation in the corpora cavernosa of the KiLHR^{D582G} mice reduces compliance of the penis causing veno-occlusive dysfunction.

A right balance between smooth muscle contraction and relaxation is needed for normal erectile function of the penis (Nunes & Webb, 2012, Morelli et al., 2006). Decreased relaxation or increased contractility of smooth muscle has been implicated in ED (Jiang & Chitaley, 2012, Chitaley, 2009, Chang et al., 2003, Gajbhiye et al., 2015). KiLHR^{D582G} mice exhibited a decrease in smooth muscle contraction in response to the α1 adrenergic agonist, PE. However, the efficacy of PE, determined by EC_{50} values, was not different, suggesting that activation of the α1 adrenergic receptor is not altered. Because α1 adrenergic receptors are specifically localized to smooth muscle cells in the cavernosa (Traish et al., 1999) and KiLHR^{D582G} mice have reduced cavernosal smooth muscle content (Hai et al., 2017), reduced contractile response to PE is most likely due to reduced smooth muscle content. The RhoA-Rho kinase pathway is another mediator of smooth muscle contraction and inhibits NO-mediated smooth muscle relaxation by decreasing NOS3 expression and inhibiting NOS3 activation (Sugimoto et al., 2007, Mita et al., 2005, Laufs & Liao, 1998). Our functional studies with the Rho kinase inhibitor Y-27632 showed no change in the potency of the relaxation response indicating that this pathway was not upregulated in the KiLHR^{D582G} mice. Normal RNA levels of the Rho kinase pathway signaling intermediates and NOS3 further demonstrate that decreased relaxation rather than increased contractility is likely to be the cause of ED in the KiLHR^{D582G} mice.

Endothelial cells lining the sinusoids and nonadrenergic-noncholinergic (NANC) cavernous nerves mediate smooth muscle relaxation by generation and release of NO (Dean & Lue, 2005, Nunes & Webb, 2012). Various animal models of aging, hypercholesterolemia and diabetes have demonstrated a significant impairment in endothelium-dependent relaxation due to a reduction in endothelial cell content, and NOS3 expression and activity in the penile cavernosa (Yeşilli et al., 2001, Gholami et al., 2003, Akingba & Burnett, 2001, Way & Reid, 1999, Cartledge et al., 2001, Azadzoi & Tejada, 1991, Behr-Roussel et al., 2002, Burchardt et al., 2000, Liu et al., 2013). Maximal endothelium-dependent (ACh-mediated) smooth muscle relaxation was reduced in KiLHR^{D582} mice. Evaluation of cavernosal smooth muscle relaxation in response to the NO donor, SNP, also showed a decrease in the maximal response in KiLHR^{D582G} mice. Because endothelial cell content and NOS3 expression levels were not altered in KiLHR^{D582G} mice, the reduced E_{max} in response to both SNP and ACh is likely a reflection of decreased smooth muscle content and function in KiLHR^{D582G} mice rather than endothelial cell loss. We were unable to perform electric field stimulation (EFS) studies to evaluate relaxation of smooth muscle by NO produced by the non-adrenergic, non-cholinergic cavernous nerves. Although levels of NOS1 and NOS3 were unchanged in KiLHR^{D582G} mice, we cannot rule out the possibility that their activities are reduced resulting in decreased endothelial and neuronal NO production that could also contribute to the reduced relaxation response in KiLHR^{D582G} mice.

NO-mediated activation of sGC causing an increase in cGMP and cGMP mediated activation of PRKG1 is the major mechanism for smooth muscle relaxation of the corpora cavernosa (Nunes & Webb, 2012, Burnett, 2006, Bivalacqua et al., 2007, Hedlund et al.,

2000). cGMP levels were significantly decreased in response to ACh and SNP in KiLHR^{D582G} mice compared to WT mice suggesting impaired activation of sGC. BAY 41–2272, which directly activates sGC independent of NO availability, significantly increased cGMP levels over that produced by ACh or SNP alone in both WT and KiLHR^{D582G} mice. However, the levels in the KiLHR^{D582G} mice were not rescued to the levels seen in the WT mice. Together these data suggest that the decrease in cGMP in KiLHR^{D582G} mice is due to impaired activation of sGC.

Another potential mechanism that can also contribute to the reduced cGMP levels is its increased hydrolysis by PDE5A. However, results from this study showed lower levels of PDE5A, which is likely due to reduced smooth muscle content in the cavernosa of KiLHR^{D582G} mice. In support of our finding, several studies have shown that expression of PDE5A is specific to smooth muscle cells and reduced expression of PDE5A is a consequence of reduction in smooth muscle content (Traish et al., 2003, Traish et al., 1999, Traish et al., 2005, Yang et al., 2009). Confirming this, a significant change in RNA levels of *Pde5a* was not observed when it was normalized to smooth muscle actin RNA.

At present, we do not know if this decrease in cGMP results in decreased PRKG1 activity in KiLHR^{D582G} mice. Surprisingly, RNA and protein levels of the enzyme in the corpora cavernosa were unchanged. We expected a decrease in the PRKG1 levels as the enzyme is expressed in the cavernosal smooth muscle cells which are decreased in the KiLHR^{D582G} mice (Hai et al., 2017). However, reports suggest that PRKG1 is also expressed in the smooth muscle of the cavernosal arteries and perhaps also the endothelial cells lining the arteries and sinusoids (Hedlund et al., 2000, Waldkirch et al., 2008). As a result, a decrease in PRKG1 due to smooth muscle loss could potentially be masked by normal levels of expression in these other cell types.

The well documented pathologies of Leydig cell hyperplasia and adenomas seen in the testes of FMPP patients and in the KiLHR^{D582G} mice are due to the direct action of the constitutively activated LHCGR in the Leydig cells (Boot et al., 2011, Hai et al., 2017, Liu et al., 1999, McGee & Narayan, 2013, Shenker et al., 1993). In this study, we have demonstrated that constitutive activation of the LHCGR results in extragonadal pathologies. We propose that the extragonadal effects on penile smooth muscle function are indirectly mediated by testosterone rather than a direct action of LHCGR as there is no evidence for functional LHCGR in the corpus cavernosum (Hai et al., 2017). Support for this hypothesis is provided by our previous study that showed that high levels of testosterone decrease smooth muscle content in the penile corpora cavernosa and that a narrow range of optimal testosterone levels regulate penile morphology (Hiremath et al., 2020). Both low levels of testosterone, as seen in the hypogonadism, and supraphysiological levels of testosterone, as seen in FMPP, decrease penile smooth muscle content (Hiremath et al., 2020, Okumu et al., 2014, Traish et al., 1999, Wang et al., 2015). It is thought that the primary mechanism by which testosterone regulates erectile physiology is by regulating the expression and activity of NOS based on animal models of castration and testosterone replacement. Castration results in decreased NOS mRNA or protein expression and activity which are restored with testosterone replacement (Marin et al., 1999, Park et al., 1999, Penson et al., 1996, Seo et al., 1999, Zvara et al., 1995). We did not see a change in the expression of NOS1 or NOS3 in the

KiLHR^{D582G} mice compared to controls suggesting that while physiological levels of testosterone are required for NOS expression, supraphysiological levels of testosterone have no effect. However, as mentioned above, NOS activities were not determined.

In summary, the lack of apomorphine-induced erection and decreased smooth muscle relaxation provide evidence that older KiLHR^{D582G} mice have erectile dysfunction. Impaired NO-mediated activation of sGC resulting in decreased levels of cGMP is responsible for the decreased smooth muscle relaxation. The mechanism by which sGC activation is reduced in KiLHR^{D582G} mice requires further investigation.

Acknowledgements

We thank Stacey McGee for assistance with the processing and embedding of tissues and Dr. Johanna Hannan for helpful suggestions on the apomorphine studies.

Funding

This work was supported in part by the Research Seed Grant from SIU School of Medicine (PN) and an award from the DiaComp Pilot/Feasibility Program, NIDDK (RCW).

References

Ahn G, Sohn Y, Kang K, Ahn B, Kwon J, Kang S, Lee B & Hwang W 2005 The effect of PDE5 inhibition on the erectile function in streptozotocin-induced diabetic rats. International Journal of Impotence Research 17 134–141. [PubMed: 15578039]

Akingba AG & Burnett AL 2001 Endothelial nitric oxide synthase protein expression, localization, and activity in the penis of the alloxan-induced diabetic rat. Molecular Urology 5 189–197. [PubMed: 11790282]

Andersson K-E 2011 Mechanisms of penile erection and basis for pharmacological treatment of erectile dysfunction. Pharmacological Reviews 63 811–859. [PubMed: 21880989]

Ascoli M, Fanelli F & Segaloff DL 2002 The lutropin/choriogonadotropin receptor, a 2002 perspective. Endocrine Reviews 23 141–174. [PubMed: 11943741]

Azadzoi KM & Tejada ISD 1991 Hypercholesterolemia impairs endothelium-dependent relaxation of rabbit corpus cavernosum smooth muscle. The Journal of Urology 146 238–240. [PubMed: 2056597]

Behr-Roussel D, Bernabe J, Compagnie S, Rupin A, Verbeuren TJ, Alexandre L & Giuliano F 2002 Distinct mechanisms implicated in atherosclerosis-induced erectile dysfunction in rabbits. Atherosclerosis 162 355–362. [PubMed: 11996955]

Bernabé J, Rampin O, Sachs BD & Giuliano F 1999 Intracavernous pressure during erection in rats: an integrative approach based on telemetric recording. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 276 R441–R449.

Bivalacqua TJ, Champion HC, Usta MF, Cellek S, Chitaley K, Webb RC, Lewis RL, Mills TM, Hellstrom WJ & Kadowitz PJ 2004 RhoA/Rho-kinase suppresses endothelial nitric oxide synthase in the penis: a mechanism for diabetes-associated erectile dysfunction. Proceedings of the National Academy of Sciences 101 9121–9126.

Bivalacqua TJ, Liu T, Musicki B, Champion HC & Burnett AL 2007 Endothelial nitric oxide synthase keeps erection regulatory function balance in the penis. European Urology 51 1732–1740. [PubMed: 17113219]

Boot AM, Lumbroso S, Verhoef-Post M, Richter-Unruh A, Looijenga LH, Funaro A, Beishuizen A, Van Marle A, Drop SL & Themmen AP 2011 Mutation analysis of the LH receptor gene in Leydig cell adenoma and hyperplasia and functional and biochemical studies of activating mutations of the LH receptor gene. Journal of Clinical Endocrinology and Metabolism 96 E1197–205. [PubMed: 21490077]

Burchardt T, Burchardt M, Karden J, Buttyan R, Shabsigh A, De La Taille A, Ng PY, Anastasiadis AG & Shabsigh R 2000 Reduction of endothelial and smooth muscle density in the corpora cavernosa of the streptozotocin induced diabetic rat. The Journal of Urology 164 1807–11. [PubMed: 11025774]

Burnett AL 2006 The role of nitric oxide in erectile dysfunction: implications for medical therapy. The Journal of Clinical Hypertension 8 53–62.

Cartledge J, Eardley I & Morrison J 2001 Nitric oxide-mediated corpus cavernosal smooth muscle relaxation is impaired in ageing and diabetes. BJU International 87 402–407. [PubMed: 11251537]

Chang S, Hypolite J, Changolkar A, Wein A, Chacko S & Disanto M 2003 Increased contractility of diabetic rabbit corpora smooth muscle in response to endothelin is mediated via Rho-kinase β. International Journal of Impotence Research 15 53–62. [PubMed: 12605241]

Chiou WF, Liu HK & Juan CW 2010 Abnormal protein expression in the corpus cavernosum impairs erectile function in type 2 diabetes. BJU International 105 674–680. [PubMed: 19751257]

Chitaley K 2009 Type 1 and Type 2 diabetic-erectile dysfunction: Same diagnosis (ICD-9), different disease? The Journal of Sexual Medicine 6 262–268. [PubMed: 19267848]

Costa WS, Carrerete FB, Horta WG & Sampaio FJ 2006 Comparative analysis of the penis corpora cavernosa in controls and patients with erectile dysfunction. BJU International 97 567–569. [PubMed: 16469027]

Dean RC & Lue TF 2005 Physiology of penile erection and pathophysiology of erectile dysfunction. Urological Clinics of North America 32 379–95, v.

Ferrini MG, Kovanecz I, Sanchez S, Vernet D, Davila HH, Rajfer J & Gonzalez-Cadavid NF 2007 Long-term continuous treatment with sildenafil ameliorates aging-related erectile dysfunction and the underlying corporal fibrosis in the rat. Biology of Reproduction 76 915–23. [PubMed: 17287493]

Gajbhiye SV, Jadhav KS, Marathe PA & Pawar DB 2015 Animal models of erectile dysfunction. Indian journal of urology: IJU: journal of the Urological Society of India 31 15. [PubMed: 25624570]

Gholami SS, Rogers R, Chang J, Ho H-C, Grazziottin T, Lin C-S & Lue TF 2003 The effect of vascular endothelial growth factor and adeno-associated virus mediated brain derived neurotrophic factor on neurogenic and vasculogenic erectile dysfunction induced by hyperlipidemia. The Journal of Urology 169 1577–1581. [PubMed: 12629419]

Giuliano F & Allard J 2001 Dopamine and sexual function. International Journal of Impotence Research 13 S18–S28. [PubMed: 11477488]

Giuliano F, Allard J, Rampin O, Droupy S, Benoit G, Alexandre L & Bernabe J 2002 Proerectile effect of systemic apomorphine: existence of a spinal site of action. The Journal of Urology 167 402–406. [PubMed: 11743364]

Hai L, Hiremath DS, Paquet M & Narayan P 2017 Constitutive luteinizing hormone receptor signaling causes sexual dysfunction and Leydig cell adenomas in male mice. Biology of Reproduction 96 1007–1018. [PubMed: 28339861]

Hedlund P, Aszódi A, Pfeifer A, Alm P, Hofmann F, Ahmad M, Fässler R & Andersson K-E 2000 Erectile dysfunction in cyclic GMP-dependent kinase I-deficient mice. Proceedings of the National Academy of Sciences 97 2349–2354.

Hiremath DS, Geerling EC, Hai L & Narayan P 2020 High levels of androgens cause chondrocyte accumulation and loss of smooth muscle in the mouse penile body. Biology of Reproduction 102 1225–1233. [PubMed: 32068232]

Jiang X & Chitaley K 2012 The promise of inhibition of smooth muscle tone as a treatment for erectile dysfunction: where are we now? International Journal of Impotence Research 24 49–60. [PubMed: 21975566]

Laufs U & Liao JK 1998 Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. Journal of Biological Chemistry 273 24266–24271.

Liu G, Duranteau L, Carel J-C, Monroe J, Doyle DA & Shenker A 1999 Leydig-cell tumors caused by an activating mutation of the gene encoding the luteinizing hormone receptor. New England Journal of Medicine 341 1731–1736. [PubMed: 10580072]

Liu G, Sun X, Bian J, Wu R, Guan X, Ouyang B, Huang Y, Xiao H, Luo D, Atala A et al. 2013 Correction of diabetic erectile dysfunction with adipose derived stem cells modified with the vascular endothelial growth factor gene in a rodent diabetic model. PloS One 8 e72790. [PubMed: 24023647]

Lombo C, Morgado C, Tavares I & Neves D 2016 Effects of prolonged ingestion of epigallocatechin gallate on diabetes type 1-induced vascular modifications in the erectile tissue of rats. International Journal of Impotence Research 28 133–138. [PubMed: 27169491]

Luttrell IP, Swee M, Starcher B, Parks WC & Chitaley K 2008 Erectile dysfunction in the type II diabetic db/db mouse: impaired venoocclusion with altered cavernosal vasoreactivity and matrix. American Journal of Physiology-Heart and Circulatory Physiology 294 H2204–H2211. [PubMed: 18326798]

Marin R, Escrig A, Abreu P & Mas M 1999 Androgen-dependent nitric oxide release in rat penis correlates with levels of constitutive nitric oxide synthase isoenzymes. Biology of Reproduction 61 1012–1016. [PubMed: 10491638]

Matsumoto K, Yoshida M, Andersson KE & Hedlund P 2005 Effects in vitro and in vivo by apomorphine in the rat corpus cavernosum. British Journal of Pharmacology 146 259–267. [PubMed: 16025145]

McGee SR & Narayan P 2013 Precocious puberty and Leydig cell hyperplasia in male mice with a gain of function mutation in the LH receptor gene. Endocrinology 154 3900–3913. [PubMed: 23861372]

Mita S-I, Kobayashi N, Yoshida K, Nakano S & Matsuoka H 2005 Cardioprotective mechanisms of Rho-kinase inhibition associated with eNOS and oxidative stress-LOX-1 pathway in Dahl salt-sensitive hypertensive rats. Journal of Hypertension 23 87–96. [PubMed: 15643129]

Moreland R, Traish A, Mcmillin M, Smith B, Goldstein I & De Tejada Saenz I 1995 PGE1 suppresses the induction of collagen synthesis by transforming growth factor-beta 1 in human corpus cavernosum smooth muscle. The Journal of Urology 153 826–834. [PubMed: 7861547]

Morelli A, Filippi S, Vignozzi L, Mancina R & Maggi M 2006 Physiology of erectile function: an update on intracellular molecular processes. EAU-EBU Update Series 4 96–108.

Mostafa ME, Senbel AM & Mostafa T 2013 Effect of chronic low-dose tadalafil on penile cavernous tissues in diabetic rats. Urology 81 1253–1260. [PubMed: 23726447]

Narayan P, Ulloa-Aguirre A & Dias JA 2019 Gonadotropin hormones and their receptors. In: Yen and Jaffe's Reproductive Endocrinology. 8th ed 25–57. Strauss JL & Barbieri RL (eds.) Philadelphia, PA: Elsevier.

Nehra A, Azadzoi KM, Moreland RB, Pabby A, Siroky MB, Krane RJ, Goldstein I & Udelson D 1998 Cavernosal expandability is an erectile tissue mechanical property which predicts trabecular histology in an animal model of vasculogenic erectile dysfunction. The Journal of Urology 159 2229–2236. [PubMed: 9598575]

Nunes KP & Webb RC 2012 Mechanisms in Erectile Function and Dysfunction: An Overview. In: Erectile Dysfunction-Disease-Associated Mechanisms and Novel Insights into Therapy. IntechOpen.

Okumu LA, Braden TD, Vail K, Simon L & Goyal HO 2014 Low androgen induced penile maldevelopment involves altered gene expression of biomarkers of smooth muscle differentiation and a key enzyme regulating cavernous smooth muscle cell tone. The Journal of Urology 192 267–273. [PubMed: 24316094]

Park K, Kim S, Kim K & Paick J 1999 Effects of androgens on the expression of nitric oxide synthase mRNAs in rat corpus cavernosum. BJU International 83 327–333. [PubMed: 10233504]

Penson DF, Ng C, Cai L, Rajfer J & Gonzalez-Cadavid NF 1996 Androgen and pituitary control of penile nitric oxide synthase and erectile function in the rat. Biology of Reproduction 55 567–74. [PubMed: 8862773]

Rampin O, Jérôme N & Suaudeau C 2003 Proerectile effects of apomorphine in mice. Life Sciences 72 2329–2336. [PubMed: 12639699]

Raviv G, Kiss R, Vanegas JP, Petein M, Danguy A, Schulman C & Wespes E 1997 Objective measurement of the different collagen types in the corpus cavernosum of potent and impotent men:

an immunohistochemical staining with computerized-image analysis. World Journal of Urology 15 50–55. [PubMed: 9066095]

Sattar AA, Wespes E & Schulman C 1994 Computerized measurement of penile elastic fibres in potent and impotent men. European Urology 25 142–144. [PubMed: 8137855]

Seo SI, Kim SW & Paick JS 1999 The effects of androgen on penile reflex, erectile response to electrical stimulation and penile NOS activity in the rat. Asian Journal of Andrology 1 169–74. [PubMed: 11225889]

Shenker A, Laue L, Kosugi S, Merendino JJ Jr, Minegishi T & Cutler GB Jr 1993 A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. Nature 365 652. [PubMed: 7692306]

Simonsen U, Comerma-Steffensen S & Andersson KE 2016 Modulation of dopaminergic pathways to treat erectile dysfunction. Basic and Clinical Pharmacology and Toxicology 119 63–74. [PubMed: 27541930]

Sopko NA, Hannan JL & Bivalacqua TJ 2014 Understanding and targeting the Rho kinase pathway in erectile dysfunction. Nature Reviews Urology 11 622. [PubMed: 25311680]

Sugimoto M, Nakayama M, Goto TM, Amano M, Komori K & Kaibuchi K 2007 Rho-kinase phosphorylates eNOS at threonine 495 in endothelial cells. Biochemical and Biophysical Research Communications 361 462–467. [PubMed: 17651694]

Themmen APN & Huhtaniemi IT 2000 Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function. Endocrine Reviews 21 551–583. [PubMed: 11041448]

Toque HA, Nunes KP, Yao L, Liao JK, Webb RC, Caldwell RB & Caldwell RW 2013 Activated Rho kinase mediates diabetes-induced elevation of vascular arginase activation and contributes to impaired corpora cavernosa relaxation: Possible involvement of p38 MAPK activation. The Journal of Sexual Medicine 10 1502–1515. [PubMed: 23566117]

Traish A, Kim N, Moreland R & Goldstein I 2000 Role of alpha adrenergic receptors in erectile function. International Journal of Impotence Research 12 S48.

Traish AM 2009 Androgens play a pivotal role in maintaining penile tissue architecture and erection: a review. Journal of Andrology 30 363–369. [PubMed: 18802199]

Traish AM, Munarriz R, O'connell L, Choi S, Kim SW, Kim NN, Huang YH & Goldstein I 2003 Effects of medical or surgical castration on erectile function in an animal model. Journal of Andrology 24 381–387. [PubMed: 12721214]

Traish AM, Park K, Dhir V, Kim NN, Moreland RB & Goldstein I 1999 Effects of castration and androgen replacement on erectile function in a rabbit model. Endocrinology 140 1861–1868. [PubMed: 10098525]

Traish AM, Toselli P, Jeong SJ & Kim NN 2005 Adipocyte accumulation in penile corpus cavernosum of the orchiectomized rabbit: A potential mechanism for veno-occlusive dysfunction in androgen deficiency. Journal of Andrology 26 242–248. [PubMed: 15713830]

Ulloa-Aguirre A, Reiter E, Bousfield G, Dias JA & Huhtaniemi I 2014 Constitutive activity in gonadotropin receptors. Advances in Pharmacology 70 37–80. [PubMed: 24931192]

Waldkirch E, Uckert S, Sigl K, Imkamp F, Langnaese K, Richter K, Jonas U, Sohn M, Stief C, Wolf G et al. 2008 Expression and distribution of cyclic GMP-dependent protein kinase-1 isoforms in human penile erectile tissue. J Sex Med 5 536–43. [PubMed: 18194177]

Wang X-J, Xu T-Y, Xia L-L, Zhong S, Zhang X-H, Zhu Z-W, Chen D-R, Liu Y, Fan Y & Xu C 2015 Castration impairs erectile organ structure and function by inhibiting autophagy and promoting apoptosis of corpus cavernosum smooth muscle cells in rats. International Urology and Nephrology 47 1105–1115. [PubMed: 25997594]

Way KJ & Reid JJ 1999 The effects of diabetes on nitric oxide-mediated responses in rat corpus cavernosum. European Journal of Pharmacology 376 73–82. [PubMed: 10440092]

Xie D, Odronic SI, Wu F, Pippen A, Donatucci CF & Annex BH 2007 Mouse model of erectile dysfunction due to diet-induced diabetes mellitus. Urology 70 196–201. [PubMed: 17656247]

Yang R, Huang Y-C, Lin G, Wang G, Hung S, Dai Y-T, Sun Z-Y, Lue TF & Lin C-S 2009 Lack of direct androgen regulation of PDE5 expression. Biochemical and Biophysical Research Communications 380 758–762. [PubMed: 19338748]

Yeşilli Ç, Yaman Ö & Anafarta K 2001 Effect of experimental hypercholesterolemia on cavernosal structures. Urology 57 1184–1188. [PubMed: 11377344]

Zvara P, Sioufi R, Schipper H, Begin L & Brock G 1995 Nitric oxide mediated erectile activity is a testosterone dependent event: a rat erection model. International Journal of Impotence Research 7 209–219. [PubMed: 8770664]

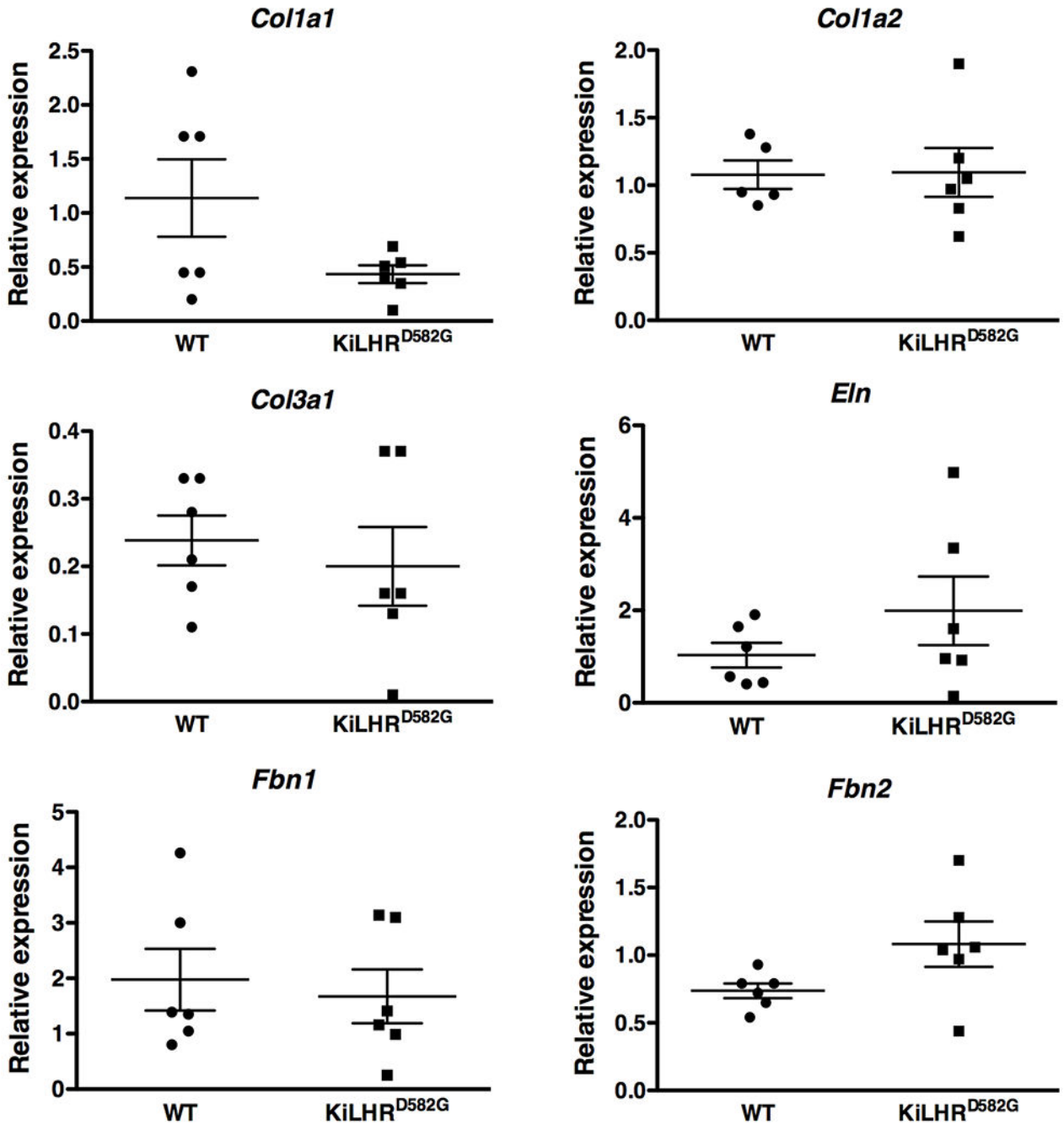

Figure 1:

Normal collagen, elastin and fibrillin RNA levels in the cavernosa of KiLHR^{D582G} mice. Relative expression of *Col1a1*, *Col1a2*, *Col3a1*, *Eln*, *Fbn1* and *Fbn2* represents levels of RNA normalized to internal control *Rps2* and relative to calibrator sample prepared by mixing equal amounts of cDNA from all samples. Data are expressed as mean ± SEM (n=5-6).

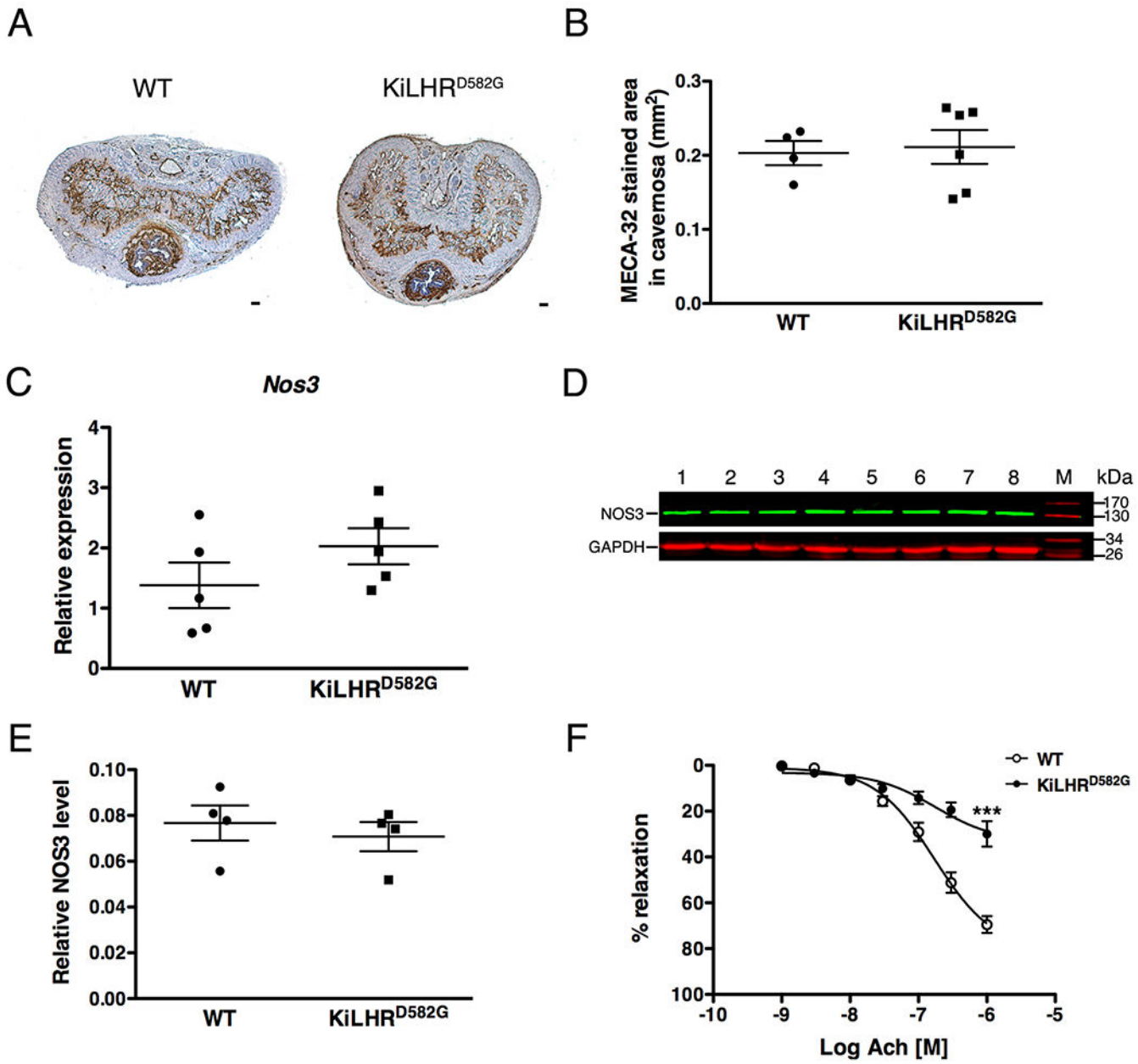

Figure 2:

Normal endothelium content in the cavernosa of KiLHR^{D582G} mice. (A) Representative photomicrographs of immunohistochemical staining of MECA-32 in WT (n=4) and KiLHR^{D582G} (n=6) mice. Scale bars, 100 μm. (B) Quantification of MECA-32 stained area in the corpora cavernosa of WT and KiLHR^{D582G} mice determined by Image-J analysis. (C) Quantification of *Nos3* by RT-qPCR (n=5). Relative expression represents levels of RNA normalized to internal control *Rps2* and relative to calibrator. (D) Western blot image for NOS3 protein levels. GAPDH was used as the loading control. M, Molecular weight markers, lanes 1,3,5,7 – WT and lanes 2,4,6,8 - KiLHR^{D582G} (n=4). Expected size of NOS3 is 140 kDa. (E) Levels of NOS3 relative to GAPDH was determined by quantification of

intensities of bands on western blots. (F) Concentration dependent relaxation response induced by ACh (n=6). Data are expressed as mean ± SEM. *** $P < 0.001$ compared to WT.

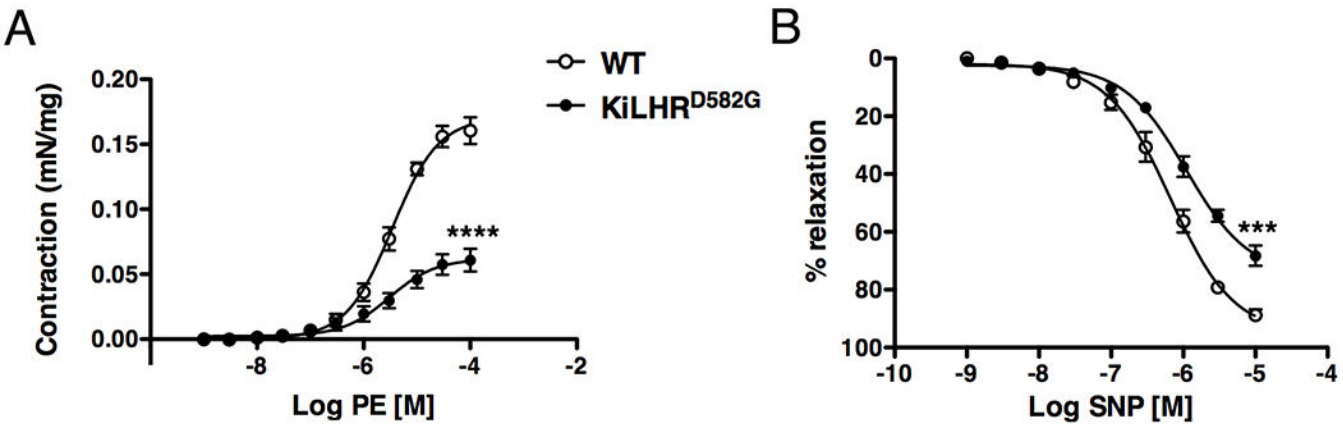

Figure 3:

Smooth muscle dysfunction in the cavernosal segments of WT and KiLHR^{D582G} mice. (A) Concentration dependent contractile response induced by PE (B) Concentration dependent relaxation response induced by SNP. Data are expressed as mean ± SEM (n=6). *** P < 0.001, **** P< 0.0001 compared to WT.

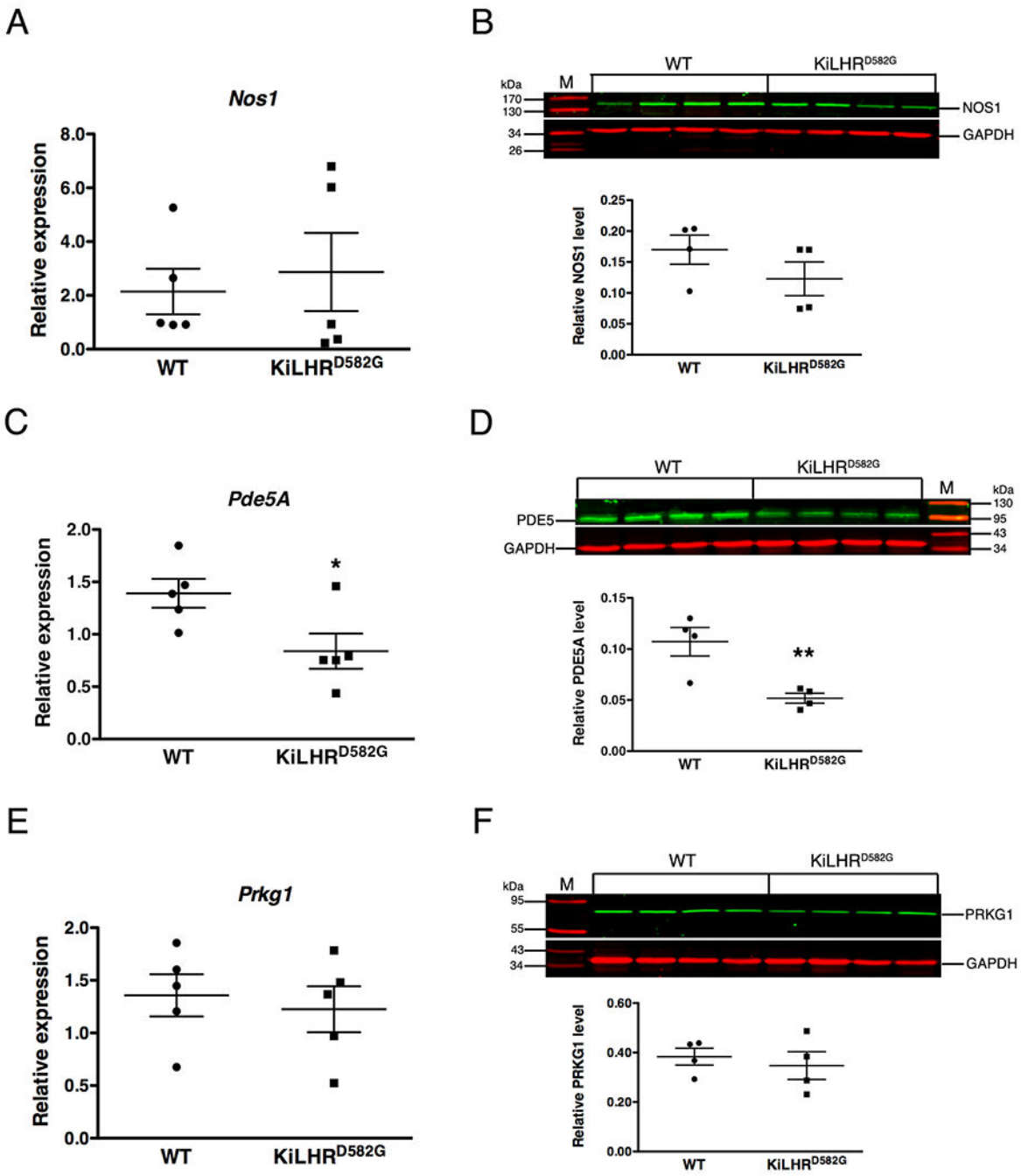

Figure 4:

Normal RNA and protein levels of signaling intermediates in the NO signaling pathway for smooth muscle relaxation in the cavernosa of KiLHR^{D582G} mice. Quantification of *Nos1* (A), *Pde5a* (C) *and Prkg1* (E) by RT-qPCR (n=5). Representative western blot images for NOS1 (B), PDE5 (D), PRKG1 (F) protein levels. GAPDH was used as the loading control. Quantification of the level of each protein relative to GAPDH was determined by quantification of intensities of bands on western blots (n=4). M, Molecular weight markers.

Expected sizes of NOS1, PDE5, PRKG1 and GAPDH are160, 100, 78, 37 kDa respectively. Data are expressed as mean ± SEM. *P<0.05, **P<0.01 compared to WT.

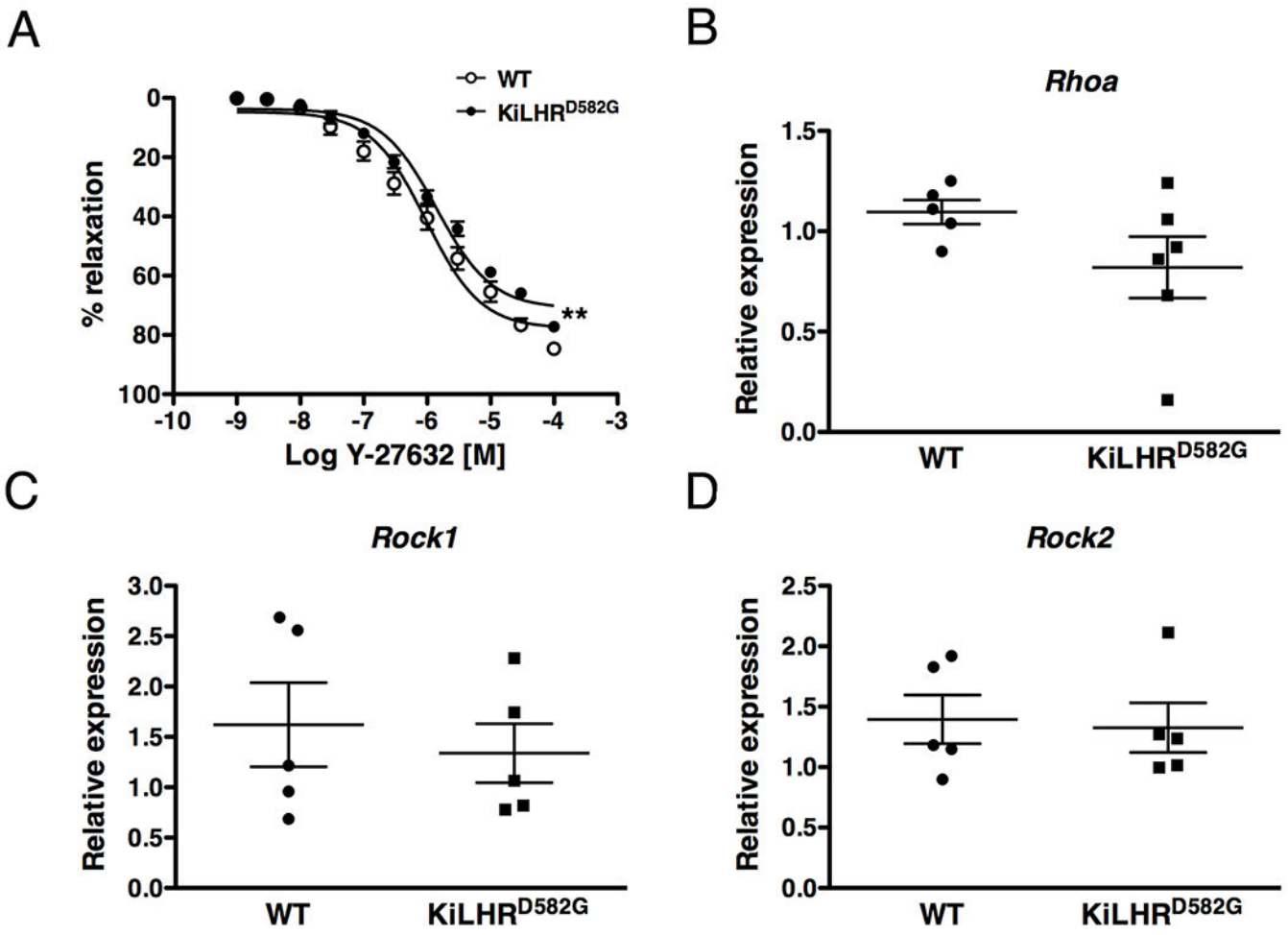

Figure 5:

Normal gene expression levels of signaling intermediates of the Rho-kinase smooth muscle contractile pathway in the cavernosa of KiLHR^{D582G} mice. (A) Relaxation response induced by Y-27632, a Rho-Kinase inhibitor (n=6). Quantification of *Rhoa* (B), *Rock1* (C), *Rock2* (D) RNA by RT-qPCR (n=5). Relative expression represents levels of RNA normalized to the internal control *Rps2* and relative to the calibrator sample prepared by mixing equal amounts of cDNA from all samples. Data are expressed as mean ± SEM. ** $P < 0.01$ compared to WT.

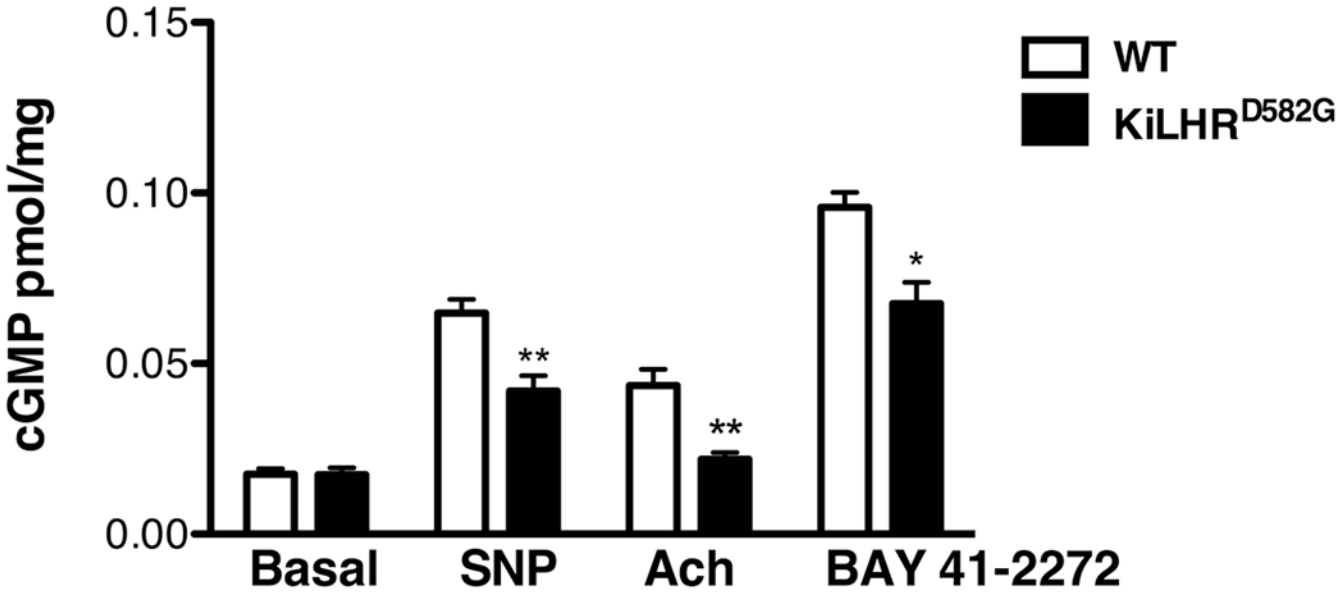

Figure 6:

cGMP content in the corpora cavernosa of WT and KiLHR^{D582G} mice. Tissues were precontracted with PE (10^{-5}M) and stimulated with SNP (10^{-5} M), ACh (10^{-6} M) or BAY 41-2272 (10^{-5} M). Data are expressed as mean ± SEM. (n=5 each for basal and SNP and n=4 each for ACh and BAY41-2272). *P<0.05, **P<0.01 compared to WT.

Table 1

Primers used in quantitative RT-PCR.

Gene	Primer sequence		RefSeq number
	Forward	Reverse	
Col1a1	GCG GTA ACG ATG GTG CTG TT	GCT TCA CCC TTA GCA CCA ACT	NM_007742.4
Col1a2	CAT TAT CTG TGA AGA CCC AGA CTGC	CTT TCT GCC CCT TTG GCC CTA	NM_007743.3
Col3a1	ACA GAG GAG AAA CTG GCC CT	ACC TTT GTC ACC TCG TGG AC	NM_009930.2
Eln	ATC AAA GCA CCA AAG CTG CC	CTG CAC CAG CTA CTC CAT AGG	NM_007925.4
Fbn1	ATC CTT GTG AGT TGT GCC CC	CGT CCA TAT CAA CTG CTG AGTC	NM_007993.2
Fbn2	CGG AGC CAA ATC AAT TCA GCA	CCC GTT CTG GCA TCC ATT CT	NM_010181.2
Nos1	CAC AGA TGA GGT TTT CAG CTC C	CGT GTG TGT CCC CGT TTA GT	NM_008712.3
Nos3	ATG CTG CTA GAA ATC GGG GG	CAC ACA GCC ACA TCC TCA AG	NM_008713.4
Pde5a	CAC AGG GAA GAG GTT GTT GG	ACC ACA GAA TGC CAG GTA GG	NM_153422.2
Prkg1	AAG AGC CCA CAG TCG AAG GA	CCG TAT TCC ACG GGG TAC AT	NM_011160.3
Rhoa	CTC CGT CGG TTC TCT CCA TA	ATG CAA GGC TCA AGG CGAG	NM_016802.5
Rock1	TGT TGG CAA TCA GCT ACC TT	CTT TCC TGC AAG CTT TTA TCC A	NM_009071.2
Rock2	GTT CAG TTG GTT CGT CAT AAG GC	GGC CAT AAT ATC TCT TTC TTC CCAG	NM_009072.2
Rps2	CCA GGT TCA AGG CTT TCGT	GCA CAG GGA CGA TGG AAA	NM_008503

Table 2

Effects of apomorphine on penile erection. Data are presented as mean ± SEM.

Genotype/ Treatment		No. of responders	No. of erections/mouse
WT (*n*=11)			
	Saline	0/11	0
	Apomorphine	9/11	1.18 ± 0.26
KiLHR^{D582G} (*n*=13)			
	Saline	0/13	0
	Apomorphine	1/13	0.15 ± 0.15**

** P <0.01 compared to WT apomorphine treatment.

Table 3

Summary of functional studies in cavernosal strips. Values are expressed as mean ± SEM (n=6).

Drug	WT			KiLHR^{D582G}	
	E_{max}	pEC_{50}		E_{max}	pEC_{50}
Phenylephrine	0.16 ± 0.01 mN/mg	5.46 ± 0.054		0.06 ± 0.01 mN/mg****	5.52 ± 0.14
Sodium nitroprusside	88.8 ± 1.9 %	6.17 ± 0.052		68.3 ± 3.4 %***	5.95 ± 0.057*
Acetylcholine	69.5 ± 3.6 %	6.76 ± 0.088		30.0 ± 5.5 %***	6.75 ± 0.24
Y-27632	84.6 ± 1.4 %	6.01 ± 0.072		77.2 ± 1.2 %**	5.85 ± 0.055

* P < 0.05,

** P < 0.01,

*** P < 0.001,

**** P < 0.0001 compared to WT.

E_{max}, Maximum response elicited by the drug; pEC_{50}, negative logarithm of molar concentration of drug that produced 50% of maximal response.