Endothelial dysfunction occurs prior to clinical evidence of Polycystic Kidney Disease

Supplementary information

Karen M. Peterson[1], Federico Franchi[1], Darrel L. Loeffler[1], Peter J. Psaltis[1], Peter C. Harris[2], Lilach O. Lerman[2], Amir Lerman[1], and Martin Rodriguez-Porcel[1]

From the Divisions of Cardiovascular Diseases[1], and Nephrology and Hypertension[2]

Department of Internal Medicine, Mayo Clinic, Rochester, MN

Corresponding author:

Martin Rodriguez-Porcel, MD
200 First St SW
Rochester, MN 55905
Phone: 507-284-4442
Fax: 507-266-7929
e-mail: rodriguez.m@mayo.edu

Materials and Methods

Systemic hemodynamics and renal function: Blood pressure measurements: Conscious animals were trained to the tail cuff for 2 weeks prior to blood pressure measurements. Data presented are a mean of systolic BP readings obtained between 10.00 am and 12.00 am over at least 3 consecutive days, using the CODA blood pressure measurement system (Kent Scientific, Torrington, CT).[1]

Renal Blood Flow: Animals were anesthetized with isoflurane 2% and imaged with a High-Resolution Ultrasound (Vevo770, Visualsonics, Toronto, CA, 30MHz frequency probe) for the assessment of renal blood flow (RBF) as previously described. RBF was calculated as renal artery area x velocity time integral x heart rate, where renal artery area was calculated as $\pi x r^2$ and global renal perfusion calculated as RBF/renal volume (mL/min/100cc^3).[2]

Glomerular function: Creatinine was determined colorimetrically by Quantichrome Creatinine Assay Kit (BioAssay Systems, Hayward, CA) and proteins by the Bradford Protein Assay (BioRad, Hercules, CA), and expressed as protein/creatinine ratio.

Vascular Endothelial Function*:* Arterial rings were prepared as previously described.[3,4] Aortas were harvested from animals of both groups (5 controls and 5 PCK) and placed into cold modified Krebs-Ringer bicarbonate solution (control solution). Rings of tissue 2-3 mm long were dissected, transferred to organ chambers with 25 mL of control solution, and oxygenated with 94% O_2 and 6% carbon dioxide (CO_2). The tissue was suspended between two stirrups and connected to a strain gauge for continuous recording of isometric tension. The artery rings were equilibrated for 30 minutes at a resting tension. Tension was applied slowly over a span of 30 minutes to 1 g (5-6 wk animals) or 2 g (10-12 wk animals). Viability of the vessels was confirmed by a contractile response to 40 mmol/L potassium chloride (KCl) after application of desired tension. All chambers were then washed using the control solution.

After an equilibrium period of 30 minutes, rings were contracted with increasing doses of phenylephrine and their contraction calculated. A separate set of rings was pre-contracted with $3x10^{-7}$

mol/L phenylephrine (Pheonix Pharmaceuticals, Mountain View, CA), and then the response to the endothelium-dependent vasodilator acetylcholine (10^{-9}-10^{-4} mol/L, Sigma-Aldrich Corp., St. Louis, MO) was measured. In additional vascular rings from each group, a dose response to the endothelium-dependent vasodilator bradykinin (10^{-10}-10^{-5} mol/L, Sigma-Aldrich Corp., St. Louis, MO) was obtained. A separate set of rings was pre-incubated with the NO precursor L-arginine, prior to a dose-response to acetylcholine. A dose-response curve to sodium nitroprusside (SNP, 10^{-9}-10^{-4} mol/L) was used to test the non-endothelium vasorelaxation response. Complete relaxation of each ring was tested, at the end of each experiment, by exposure to 10^{-4} mol/L papaverine.

***Isolation and characterization of aortic vascular ECs*:** Rat ECs were isolated using explants technique previously described.[5-7] Rats were euthanized by CO_2, under a dissecting microscope the aorta quickly removed and dissected from surrounding adventitia in serum free DMEM. Vessels were then cut into 6 segments, opened longitudinally and placed lumen-side down in 6-well plates coated with rat tail collagen and minimal volume of Dulbeco's Minimal Essential Medium (DMEM) containing 20% Fetal Bovine Serum (FBS), penicillin/streptomycin and endothelial cell growth supplement (Sigma-Aldrich Corp., St. Louis, MO; 50µg/ml). Explants were then removed after colonies became visible at day 3-4. To make sure that the isolated cells are ECs, plated cells were characterized by immunofluorescence staining for vonWillebrand Factor (vWF) and their function tested by the incorporation of DiI-labeled acetylated low density lipoprotein (Ac-LDL).[8] For these studies we used ECs obtained from 4-5 different controls and 4-5 PCK animals at 5 and 10 weeks of age. With cells from each separate animal constituting a different sample.

RNA isolation and RT-PCR: Rat aortic ECs from wild type (WT) and PCK rats were grown in complete DMEM containing 10% FBS and 50 µg/ml endothelial cell growth supplementation (ECGS, Sigma-Aldrich Corp., St. Louis, MO) until confluent. Cells were then trypsinized, rinsed and pelleted for

ribonucleic acid (RNA) isolation. Fresh rat leg skeletal muscle and kidney were collected aseptically from CO_2 euthanized rats for negative and positive controls, respectively. RNA was isolated from samples via a Qiagen RNEasy Plus mini kit (Quiagen, Germantown, MD) according to manufacturer's instructions. Following isolation, 1 µg mRNA was treated with amplification grade DNase I, and subsequently converted to complementary deoxyribonucleic acid (cDNA) by reverse transcription with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) following manufacturer's recommended protocol in a thermocycler at 23 °C for 10 min, then 37 °C for 60 min, then denature at 95 °C for 5 min. PCR for PKHD1 was performed with 2 µl of cDNA product using native Taq DNA polymerase in a 50 µl reaction at an annealing temperature of 58.5°C. Polymerase chain reaction (PCR) for transferrin receptor was performed at an annealing temperature of 60°C. PKHD1 primer sequences used were CCTTTCAGCTCGTGTTCTCC (Forward) and GGACTCCCACCACAGACACT (Reverse) with a product size of 560bp. Transferrin Receptor primer sequences used were AGTCCAGTGTGGGAACAGG (Forward) and ACCATTTGGTTGAGCTGAGG (Reverse) with a product size of 509bp. PCR products were run on 2% agarose gel at 60V.

Western blotting: Western blotting was performed following standard protocols.[4,9-11] Aortic lysate and ECs from 5wk and 10wk old ECs from 4-5 control rats and 4-5 PCK rats were loaded separately onto 10% PAGE gels, electrophoresed for 90 min at 90V in Tris-glycine-sodium dodecyl sulfate (SDS) buffer, then transferred to polyvinylidene difluoride (PVDF) membranes in Tris-glycine-SDS-20% methanol at 12 V for one hour in a TE70X semi-dry transfer electrophoresis system (Hoefer, Holliston, MA). Membranes were blocked for one hour in Tris-Buffered Saline and Tween 20 (TBST-20) containing 5% milk (TBST:milk) followed by overnight incubation at 4ºC with the primary antibodies.

Primary antibodies used were: endothelial nitric oxide synthase (eNOS, Novus Biologicals, Littleton, CO; dilution 1:1000), eNOS phosphorylated at Serine 1177 (active, Cell Signaling Technology Inc, Danvers, MA, dilution 1:1000), eNOS phosphorylated at Threonine 495 (inactive, Cell

Signalling Technology Inc, Danvers, MA, dilution 1:1000), inducible NOS (iNOS, Upstate-Millipore, Billerica, MA, dilution: 1:1000). Because changes in the oxidant status has been shown to affect the bioavailability of NO, we also measured the expression of pro-oxidant enzymes NAD(P)H subfractions $p47^{phox}$ (Novus Biologicals, Littleton, CO; dilution 1:1000), $p67^{phox}$ (Upstate-Millipore, Billerica, MA, dilution 1:1000); and $gp91^{phox}$ (Abcam, Cambridge, MA, dilution 1:2000), and $p22^{phox}$ (Santa Cruz Biotechnology, Dallas, TX, 1:500). To measure cell proliferation we used proliferating cell nuclear antigen (PCNA, Cell Signaling Technology Inc, Danvers, MA; dilution 1: 1000), pan Akt, and Akt/Ser 473 (Cell Signalling Technology Inc, Danvers, MA, dilution 1: 2000). For apoptosis we used Caspase 3 (Cell Signaling Technology Inc, Danvers, MA; dilution 1: 1000).

Following incubation with primary antibody, membranes were washed in TBST and then incubated on rocker for one hour at room temperature with horseradish peroxidase conjugated secondary antibody diluted (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:2000) in TBST:milk. Then membranes were incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) per manufacturer's instructions and imaged by film exposure. Loading control antibody used was rabbit anti-GAPDH (Cell Signaling Inc., Danvers, MA; dilution 1:5000). Band densities were analyzed by ImageJ (National Institutes of Health, Bethesda, MD) and normalized to GAPDH as the loading control.

Cell viability: Based on the concept that dead cells will release lactate dehydrogenase (LDH), cell viability was measured with the LDH release assay (CytoTox 96 Non-radioactive assay, Promega, Madison, WI) using manufacturer's instructions.[12] Cells were grown in medium supplemented with heat-inactivated serum ($7x10^3$ cells per well, n=8) for 24 hours. After cell lysis by freeze-thaw, an aliquot of each lysate was transferred to the assay plate, substrate mix added to the samples in the assay plate and the Absorbance at 490 nm was measured using a plate reader. Data is expressed as percentage of total cellular LDH released into medium.

Cell proliferation: 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is converted to formazan only by the mitochondria in viable cells, so the MTT assay[13] was used as an indicator of cell proliferation. ECs were plated in 96-well plates ($3x10^3$) and 24 hours later cells were incubated with the MTT (Sigma-Aldrich, St. Louis, MO) reagent for 3 h, after which a solvent (0.4 N HCl isopropanol) was added, and plates were placed on a shaker at room temperature for 15 minutes. Absorbance was read in a spectrophotometer at a wavelength of 570 nm.

Angiogenic capacity: Angiogenic capacity was tested as previously described.[14-16] ECs were grown under normal growth conditions ($2.5x10^4$ cells) in DMEM (Gibco, Invitrogen, Carlsbad, CA) medium containing 50 µg/ml ECGS and seeded onto 250 µL of Matrigel in 24-well plates. Then, photographs of the wells (5/well) were obtained at 3 hours and the total length of tubes formed per image was calculated using the Metamorph program (Molecular Devices, Sunnyvale, CA).

Migration assay: The migration of ECs was assessed as previously described.[17] Cells were plated at $2.5x10^4$ and to prevent cell proliferation, thymidine (Sigma-Aldrich Corp., St. Louis, MO) was added to standard medium at a concentration of 3 mM. A defined area of the plate was cleared of ECs by scraping using a 20µl pipette tip, prior to the hypoxic (24 hours at 1% O_2) challenge. After 24 hours, cells were fixed in 4% paraformaldehyde and covered with PBS for imaging (Apotome Axiovert 200M, CarlZeiss MicroImaging Inc, Oberkochen, Germany; 10x magnification objective). A total of 8 images were obtained for each well from 3 replicate wells per treatment and the change in percent area covered by cells was calculated for each well.

Figure legends

Figure S1. Aortic vasoconstriction response to increasing doses of Phenylephrine in control and PCK 5-6 week old animals. Differences are not significant. The dose responses to phenylephrine clearly show that the vasculature in both groups was reactive to PE at comparable doses, although there was a small difference in the grams of tension achieved.

Figure S2. A, Endothelial cells (ECs) from both control and PCK express the endothelial marker vWF (left) and uptake of Acetylated LDL (right). **B**, Expression of fibrocystin and transferrin in control and PCK derived endothelial cells (ECs). vWF: vonWillebrand factor

Bibliography

1. Stroope A, Radtke B, Huang B, Masyuk T, Torres V, Ritman E, LaRusso N. Hepato-renal pathology in pkd2ws25/- mice, an animal model of autosomal dominant polycystic kidney disease. The American journal of pathology 2010;176:1282-91.
2. Xu R, Franchi F, Miller B, Crane JA, Peterson KM, Psaltis PJ, Harris PC, Lerman LO, Rodriguez-Porcel M. Polycystic kidneys have decreased vascular density: a microCT study. Microcirculation 2012;Nov 20.
3. Rodriguez-Porcel M, Lerman LO, Herrmann J, Sawamura T, Napoli C, Lerman A. Hypercholesterolemia and Hypertension Have Synergistic Deleterious Effects on Coronary Endothelial Function. Arteriosclerosis, thrombosis, and vascular biology 2003;23:885-91.
4. Rodriguez-Porcel M, Lerman LO, Holmes DR, Richardson D, Napoli C, Lerman A. Chronic antioxidant supplementation attenuates nuclear factor-kappaB activation and preserves endothelial function in hypercholesterolemic pigs. Cardiovascular research 2002;53:1010-8.
5. McGuire PG, Orkin RW. Isolation of rat aortic endothelial cells by primary explant techniques and their phenotypic modulation by defined substrata. Laboratory investigation; a journal of technical methods and pathology 1987;57:94-105.
6. Alexander MY, Brosnan MJ, Hamilton CA, Downie P, Devlin AM, Dowell F, Martin W, Prentice HM, O'Brien T, Dominiczak AF. Gene transfer of endothelial nitric oxide synthase improves nitric oxide-dependent endothelial function in a hypertensive rat model. Cardiovascular research 1999;43:798-807.
7. Suschek C, Rothe H, Fehsel K, Enczmann J, Kolb-Bachofen V. Induction of a macrophage-like nitric oxide synthase in cultured rat aortic endothelial cells. IL-1 beta-mediated induction regulated by tumor necrosis factor-alpha and IFN-gamma. J Immunol 1993;151:3283-91.

8. Bachar AR, Scheffer L, Schroeder AS, Nakamura HK, Cobb LJ, Oh YK, Lerman LO, Pagano RE, Cohen P, Lerman A. Humanin is expressed in human vascular walls and has a cytoprotective effect against oxidized LDL-induced oxidative stress. Cardiovascular research 2010;88:360-6.

9. Chade AR, Best PJ, Rodriguez-Porcel M, Herrmann J, Zhu X, Sawamura T, Napoli C, Lerman A, Lerman LO. Endothelin-1 receptor blockade prevents renal injury in experimental hypercholesterolemia. Kidney international 2003;64:962-9.

10. Chade AR, Rodriguez-Porcel M, Herrmann J, Zhu X, Grande JP, Napoli C, Lerman A, Lerman LO. Antioxidant intervention blunts renal injury in experimental renovascular disease. J Am Soc Nephrol 2004;15:958-66.

11. Chade AR, Zhu X, Lavi R, Krier JD, Pislaru S, Simari RD, Napoli C, Lerman A, Lerman LO. Endothelial progenitor cells restore renal function in chronic experimental renovascular disease. Circulation 2009;119:547-57.

12. Peterson KM, Aly A, Lerman A, Lerman LO, Rodriguez-Porcel M. Improved survival of mesenchymal stromal cell after hypoxia preconditioning: role of oxidative stress. Life sciences 2011;88:65-73.

13. Rodriguez-Porcel M, Gheysens O, Paulmurugan R, Chen IY, Peterson KM, Willmann JK, Wu JC, Zhu X, Lerman LO, Gambhir SS. Antioxidants improve early survival of cardiomyoblasts after transplantation to the myocardium. Mol Imaging Biol 2010;12:325-34.

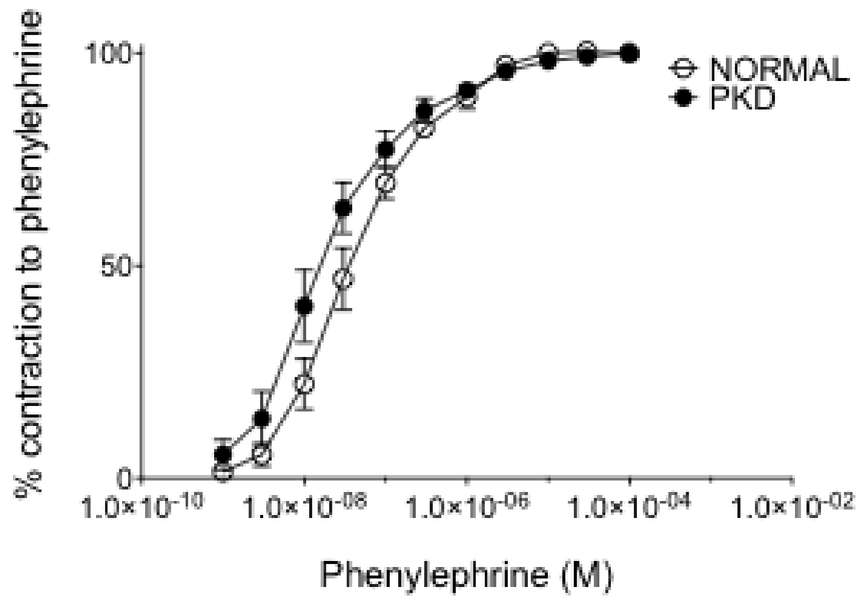
14. Annabi B, Lee YT, Turcotte S, Naud E, Desrosiers RR, Champagne M, Eliopoulos N, Galipeau J, Beliveau R. Hypoxia promotes murine bone-marrow-derived stromal cell migration and tube formation. Stem cells (Dayton, Ohio) 2003;21:337-47.

15. Zhu XY, Daghini E, Chade AR, Lavi R, Napoli C, Lerman A, Lerman LO. Disparate effects of simvastatin on angiogenesis during hypoxia and inflammation. Life sciences 2008;83:801-9.

16. Zhu XY, Urbieta Caceres VH, Favreau FD, Krier JD, Lerman A, Lerman LO. Enhanced endothelial progenitor cell angiogenic potency, present in early experimental renovascular hypertension, deteriorates with disease duration. Journal of hypertension 2011;29:1972-9.

17. Holroyd EW, Delacroix S, Larsen K, Harbuzariu A, Psaltis PJ, Wang L, Pan S, White TA, Witt TA, Kleppe LS, Mueske CS, Mukhopadhyay D, Simari RD. Tissue factor pathway inhibitor blocks angiogenesis via its carboxyl terminus. Arteriosclerosis, thrombosis, and vascular biology 2012;32:704-11.

Figure S1



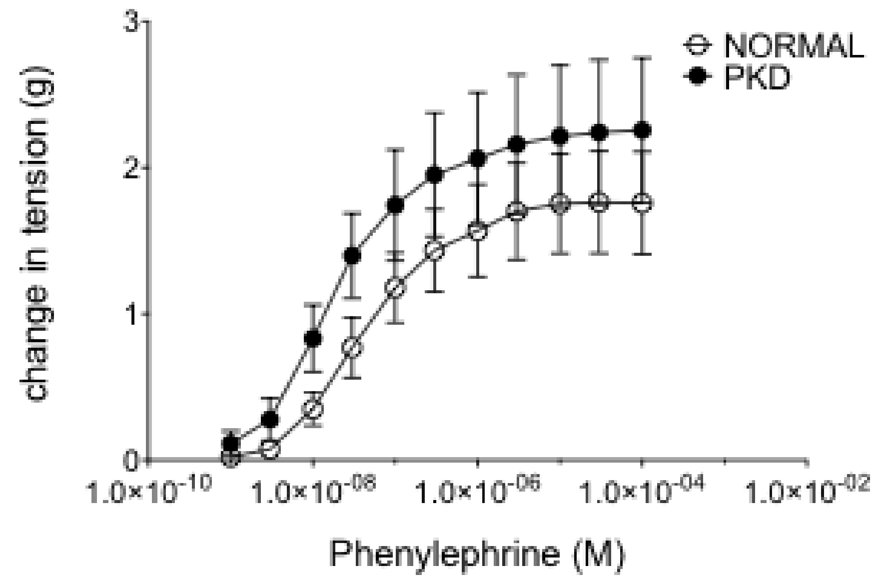
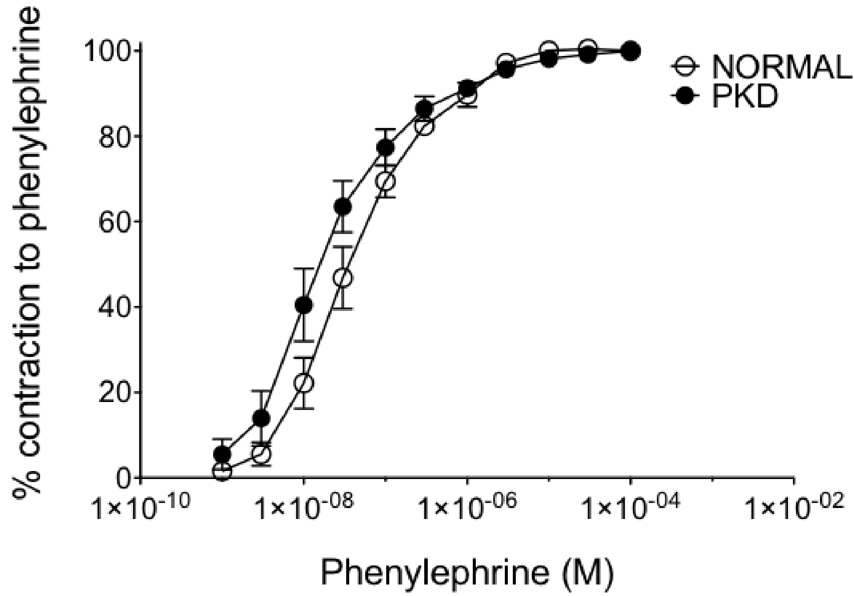
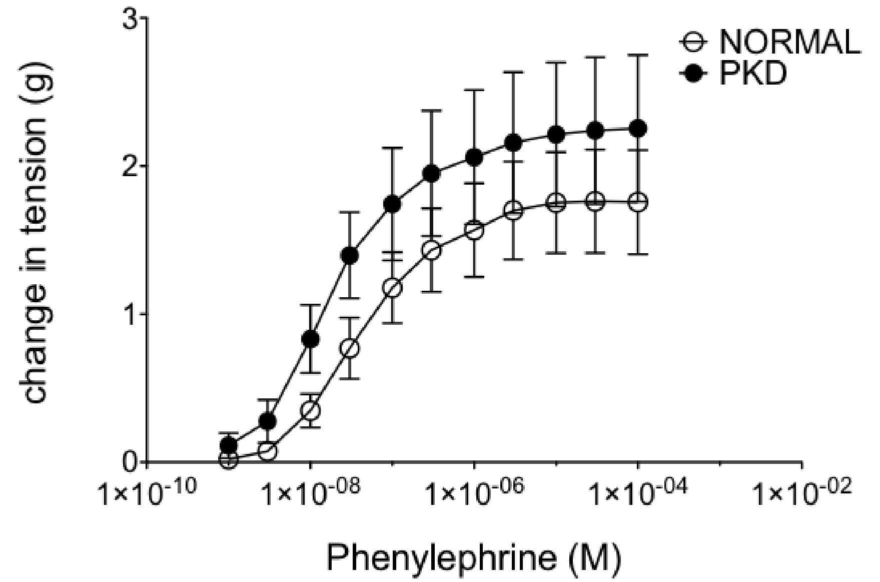
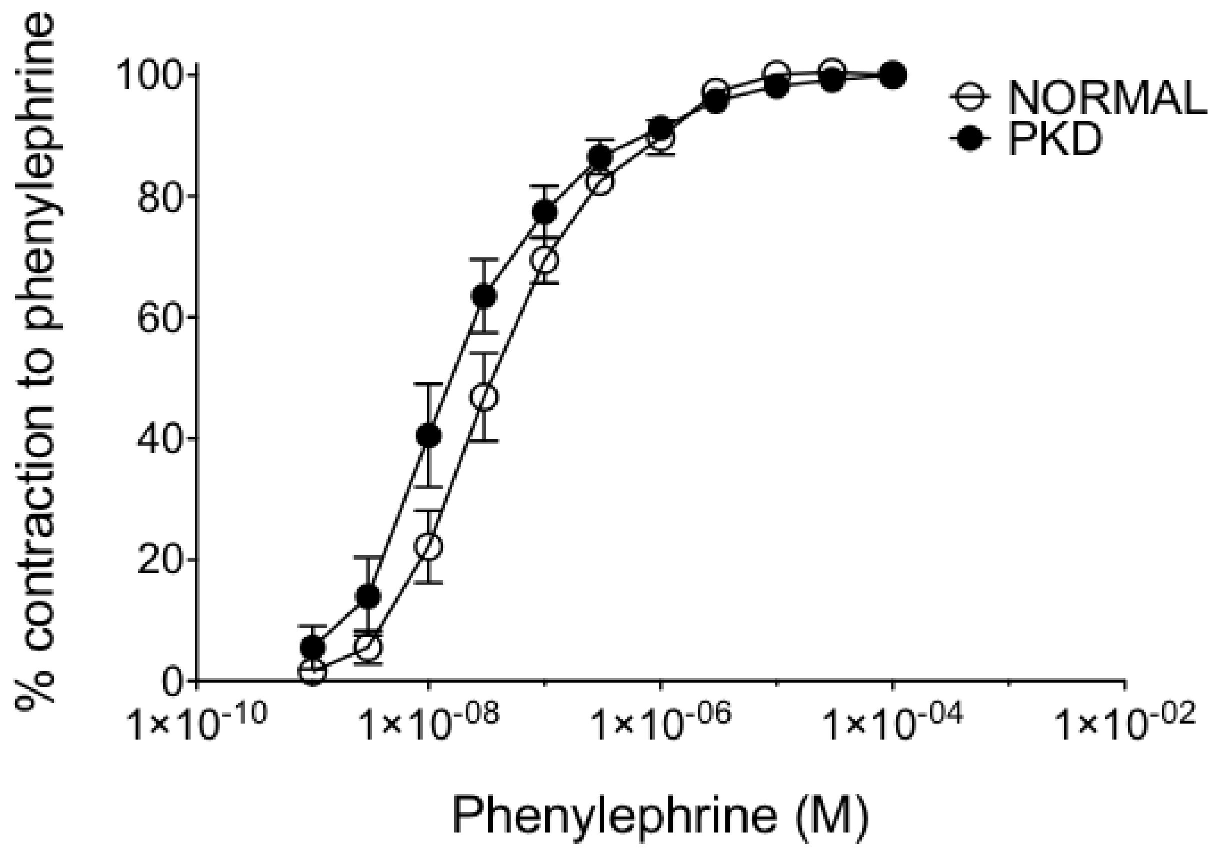


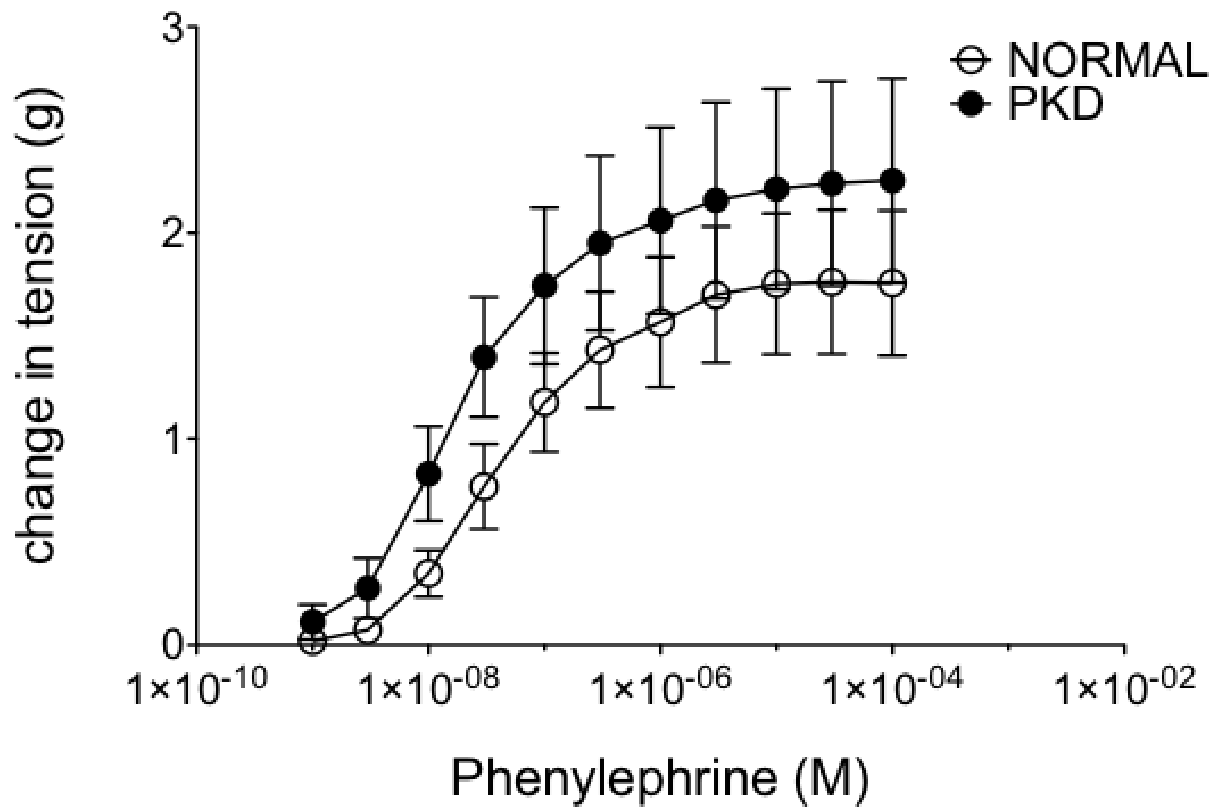
Figure S1







% contraction to phenylephrine
100
80
60
40
20
0
1×10-10
1×10-08
1×10-06
1×10-04
1×10-02
Phenylephrine (M)
NORMAL
PKD



NORMAL
PKD
change in tension (g)
3
2
1
0
1×10-10
1×10-08
1×10-06
1×10-04
1×10-02
Phenylephrine (M)

Figure S2