Pkd2 haploinsufficiency alters intracellular calcium regulation in vascular smooth muscle cells

Qi Qian1,*, Larry W. Hunter2, Ming Li1, Miguel Marin-Padilla3, Y.S. Prakash2, Stefan Somlo4, Peter C. Harris1, Vicente E. Torres1 and Gary C. Sieck2

1Department of Medicine, Division of Nephrology, 2Department of Physiology and Biophysics and 3Department of Pathology, Mayo Clinic, Rochester, MN, USA and 4Department of Internal Medicine and Genetics, Yale University School of Medicine, New Haven, CT, USA

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Autosomal-dominant polycystic kidney disease is a multiorgan disease and its vascular manifestations are common and life-threatening. Despite this, little is known about their pathogenesis. Somatic mutations to the normal PKD allele in cystic epithelia and cyst development associated with the unstable Pkd2WS25 allele suggest a two-hit model of cystogenesis. However, it is unclear if this model can account for the cardiovascular pathology or if haploinsufficiency alone is disease-associated. In the present study, we found a decreased polycystin-2 (PC2, protein encoded by Pkd2 gene) expression in Pkd2+/−/C0 vessels, roughly half the wild-type level, and an enhanced level of intracranial vascular abnormalities in Pkd2+/−/C0 mice when induced to develop hypertension. Consistent with these observations, freshly dissociated Pkd2+/−/C0 vascular smooth muscle cells have significantly altered intracellular Ca2+ homeostasis. The resting [Ca2+]i is 17.1% lower in Pkd2+/− compared with wild-type cells (P = 0.0003) and the total sarcoplasmic reticulum Ca2+ store (emptied by caffeine plus thapsigargin) is decreased (P < 0.0001). The store operated Ca2+ (SOC) channel activity is also decreased in Pkd2+/− cells (P = 0.008). These results indicate that inactivation of just one Pkd2 allele is sufficient to significantly alter intracellular Ca2+ homeostasis, and that PC2 is necessary to maintain normal SOC activity and the SR Ca2+ store in VSMCs. Based on these findings, and the fact that [Ca2+]i signaling is essential to the regulation of contraction, production and secretion of extracellular matrix, cellular proliferation and apoptosis, we propose that the abnormal intracellular Ca2+ regulation associated with Pkd2 haploinsufficiency is directly related to the vascular phenotype.

INTRODUCTION

Autosomal-dominant polycystic kidney disease (ADPKD) is characterized by progressive renal cystic disease, often resulting in renal failure. The disease, however, is systemic with extrarenal cysts and cardiovascular abnormalities (1). The vascular manifestations of ADPKD are common, including saccular intracranial aneurysms (ICA) and dolichoectasias, aortic root dilatation, dissection of the thoracic aorta and cervicocephalic arteries, and coronary artery aneurysms (1,2). The prevalence of ICA and the incidence of aneurysmal rupture in ADPKD are five to 10 times higher than in the general population and aneurysmal rupture carries a combined severe morbidity–mortality rate of ~50% (3). Familial clustering of ICA has been observed in some ADPKD families (4,5). ADPKD is caused by mutations to either of two genes, PKD1 and PKD2 (6,7). The PKD1 and PKD2 encoded proteins, polycystin-1 and -2 (PC1 and PC2), are membrane-associated (8,9) and PC2 is a Ca2+-permeable channel that interacts with, and may be regulated by, PC1 (10–13). Somatic mutations to the normal PKD allele in cystic epithelia and cyst development associated with the unstable Pkd2WS25 allele suggest a two-hit model of cystogenesis (14,15). However, it is unclear if this model can account for the cardiovascular pathology (16), which is poorly understood, or if the heterozygous state is disease-associated. Both PKD1 and PKD2 mutations have been associated with ICAs (17–19). Expression of the polycystins in vascular smooth muscle cells (VSMCs) and vascular leakage
RESULTS

**Pkd2<sup>+/−</sup> mice are more susceptible to intracranial vascular complications**

To determine whether animals heterozygous for a null Pkd2 mutation (truncation at exon 1) are more prone to develop intracranial vascular complications, 19 adult mice (10 wild-type and 9 Pkd2<sup>+/−</sup>) underwent unilateral carotid ligation and induction of hypertension (see methods for details). All animals tolerated the surgical procedure without immediate mortality, and blood pressures, measured at 2.5 months following the surgery, were similar in both groups (systolic blood pressure = 168±12 mmHg by the tail cuff method). However, three Pkd2<sup>+/−</sup> mice died at 1.5–2.5 months after the induction of hypertension (autopsies were not possible), while none of the wild-type mice died before sacrifice. All surviving animals were sacrificed at 3 months after the onset of hypertension. As shown in Figure 1 and Table 1, five of the six Pkd2<sup>+/−</sup> mice had detectable cerebral arterial lesions on gross examination. Only one wild-type animal had minimal middle cerebral artery dilatation with no change in the vessel wall thickness. These results indicate that the Pkd2<sup>+/−</sup> mice have a significantly increased risk of intracranial vascular complications compared with wild-type animals when placed under hemodynamic stress.

**Pkd2<sup>+/−</sup> vascular smooth muscle has decreased PC2 expression**

PC2 is expressed in VSMCs (21). To ascertain the level of PC2 expression in vessels from Pkd2<sup>+/−</sup> mice, quantitative western analysis was carried out using proteins isolated from freshly dissected aortic tunica media layer. PC2 was detected as a 110 kDa band using a polyclonal antibody YCB, as previously reported (9). Pkd2<sup>+/−</sup> vascular smooth muscles were found to express roughly half of the amount of PC2 compared to wild-type vessels, consistent with their hemizygous state (n = 3, Fig. 2A and B). From the same blots, analysis of calponin, a marker of the contractile phenotype, and smooth muscle α-actin confirmed the vascular smooth muscle phenotype (Fig. 2C). To confirm the decreased PC2 expression in Pkd2<sup>+/−</sup> vascular smooth muscle at the mRNA level, real-time PCR was carried out using tunica media layers from thoracic aortas isolated from wild-type and Pkd2<sup>+/−</sup> littermates. Consistent with the results from the quantitative western analysis, the Pkd2 mRNA levels in Pkd2<sup>+/−</sup> vessels (1.53±0.23, Pkd2 normalized to cyclophilin, n = 6) were about one-half of those measured in wild-type vessels (4.58±0.72, n = 6).

**Pkd2<sup>+/−</sup> VSMCs have decreased resting [Ca<sup>2+</sup>]<sub>i</sub> and the capacity of the SR Ca<sup>2+</sup> store**

PC2 has been located in the endoplasmic reticulum (ER) membrane and the overexpression of PC2 in PKD2-transfected LLCPK cells caused an increase in the vasopressin-induced Ca<sup>2+</sup> release from the ER (12). To determine whether a reduced expression of PC2 in VSMCs is associated with altered intracellular Ca<sup>2+</sup> regulation, the resting [Ca<sup>2+</sup>]<sub>i</sub> and the capacity of the sarcoplasmic reticulum (SR) Ca<sup>2+</sup> store in freshly dissociated Pkd2<sup>+/−</sup> and wild-type VSMCs were examined. Freshly dissociated cells were chosen because VSMCs in adult animals have an extremely low turnover rate and remain in the contractile phenotype. Fresh dissociation avoids the changes of cellular phenotype (from contractile to proliferative) inevitably associated with primary culture (25). The results represented here therefore most closely reflect their function in vivo.

![Figure 1. Juncions of the anterior, middle and posterior cerebral arteries from a wild-type (A) and two Pkd2<sup>+/−</sup> mice (B and C). Note the junctional dilatation (B) (arrow) and the luminal dilatation and the irregular thickening and thinning of the arterial wall (C, arrow) in the Pkd2<sup>+/−</sup> vessels. Anterior cerebral arteries from wild-type (D) and a Pkd2<sup>+/−</sup> (E) mice. Note the thinning of the wall, the dilatation of the lumen and the local hemorrhage in the Pkd2<sup>+/−</sup> vessel (arrows).](image-url)
The expression of PC2 in A calreticulin (RET) antibody was used to verify equal loading of the samples. The expression of calponin and a Pkd2 deficiency has a profound effect on the resting [Ca^{2+}] in high lysig signiificant changes in the SR Ca^{2+} homeostasis. The nuclear integrity of these cells was verified by their responsiveness to caffeine or cyclopiazonic acid stimulation after the resting [Ca^{2+}], measurement (data not shown).

To assess the effect of Pkd2 hemizygosity on the capacity of the SR Ca^{2+} store, VSMCs were stimulated with caffeine (5 mM) plus thapsigargin (1 μM) in zero Ca^{2+} perfusate. These concentrations are used routinely by many groups including ours to deplete the SR Ca^{2+} store in VSMCs (26,27). As shown in Figure 4A and B, the total SR Ca^{2+} store emptied by caffeine plus thapsigargin was significantly lower in the Pkd2^{+/−} (n = 72) than in the wild-type VSMCs (n = 48, P < 0.0001). These results indicate that the Pkd2 haploinsufficiency has a profound effect on the resting [Ca^{2+}], and the capacity of the SR Ca^{2+} store.

**Pkd2^{+/−} VSMCs have reduced SOC channel activity**

PC2 is known to directly associate with the transient receptor potential channel 1 (TRPC1) (28), a plasma membrane-spanning subunit of the SOC channel in VSMCs (29). Adequate store-operated calcium (SOC) channel activity is critical to the SR Ca^{2+} repletion and store (30,31). To determine whether the decreased SR Ca^{2+} store observed in Pkd2^{+/−} cells is due to reduced SOC activity, we first depleted the SR Ca^{2+} store by perfusing the cells with caffeine (5 mM) plus thapsigargin (1 μM) in zero Ca^{2+} Tyrodes containing nifedipine (1 μM). After the SR was depleted, Ca^{2+} (2 mM) was then added into the perfusate. SOC activity was detected as an increase in [Ca^{2+}], as shown in Figure 5A and B, Pkd2^{+/−} VSMCs (n = 15) exhibited a significant decrease in SOC activity compared to wild-type cells (n = 12, P = 0.008). These results indicate that the decreased PC2 expression is associated with abnormal SOC channel activity and this abnormality is probably responsible for the decreased capacity of the SR Ca^{2+} store in Pkd2^{+/−} VSMCs.

**DISCUSSION**

This study demonstrates that inactivation of just a single Pkd2 allele is associated with the expected dosage reduction of PC2 and significantly alters intracellular Ca^{2+} homeostasis. The level of PC2 also alters the SOC channel activity and the characteristics of the SR Ca^{2+} store in VSMCs.

PC2, in addition to being localized in the ER membrane, has recently been localized to the plasma membrane (32,33) and primary cilia (34). In this location, Ca^{2+} channel activity has been demonstrated in response to mechanosensation, which could be blocked using a specific PC2 antibody (34). In the present study, we showed that in VSMCs a reduction of PC2 expression resulted in decreased SOC channel activity, consistent with the reported finding that PC2 interacts with a SOC channel protein (28). This interaction could occur either via a conformational coupling between PC2 in the SR membrane and SOC channel protein in the adjacent plasma membrane (analogous to the coupling between inositol 1,4,5-triphosphate receptor in the ER/SR and TRPC in the plasma membrane) (35,36) and/or via an interaction with both located in the plasma membrane. Using primary cultured wild-type VSMCs, we found that PC2 is mainly localized in the SR membrane by immunohistochemistry and cell surface biotinylation (37), although a plasma membrane fraction of PC2 below the level of detection by these techniques could not be
excluding. Because SOC channel activity is critical to the Ca\textsuperscript{2+} repulsion of the SR (30,38), it is likely that the reduced SOC channel activity associated with the decreased level of PC2 results in the reduced capacity of the SR Ca\textsuperscript{2+} store.

Ca\textsuperscript{2+} signaling is essential to the regulation of contraction, production and secretion of extracellular matrix, cellular proliferation and apoptosis (39,40). Pkd2 haploinsufficiency and its associated abnormal intracellular Ca\textsuperscript{2+} regulation in VSMCs observed in this study are probably linked to the vascular phenotype in ADPKD. Elucidating the precise mechanism by which reduced PC2 levels resulted in these fundamental changes in Ca\textsuperscript{2+} homeostasis will help the understanding of the vascular lesions in ADPKD and the general role of PC2.

**MATERIALS AND METHODS**

**Pkd2\textsuperscript{+/−} mice and genotyping**

The Pkd2 gene targeting strategy and the generation of Pkd2\textsuperscript{+/−} mice with a true null Pkd2 allele has been reported (15). In this study Pkd2\textsuperscript{+/−} mice were crossed with C57BL/6 mice to generate wild-type and Pkd2\textsuperscript{+/−} animals. DNA was isolated from a small section of tail using the QIAamp tissue kit (Qiagen) following the protocol by the manufacturer. Genomic DNA was analyzed by PCR with two pairs of specific primers that amplify the wild-type and mutant alleles as previously reported (15).

**Induction of intracranial vascular lesions**

Six- to 9-month-old, littermate wild-type (five male, five female) and Pkd2\textsuperscript{+/−} (five male, four female) mice underwent a right carotid artery ligation, left nephrectomy and subcutaneous implantation of a deoxycorticosterone acetate (DOCA) tablet (50 mg, 3 months sustained release, Innovative Research of America, Sarasota, FL, USA). Postoperatively, the animals were fed regular chow and given 1% sodium chloride solution to drink. All surviving animals were sacrificed 3 months later. Their brains and circles of Willis were dissected for examination. Contrary to rats, wild-type mice do not develop prominent intracranial vascular lesions with this protocol (41,42).

**Western analysis**

The aortic tunica media smooth muscle layer from wild-type and Pkd2\textsuperscript{+/−} mice was minced into small pieces and homogenized using an Eberbach 114 V homogenizer in buffer containing (in mM) 150 NaCl, 5 EDTA, 50 Tris–HCl, pH 7.4, 1% Triton X-100, and an EDTA-free protease inhibitor mix at 4°C for 10 min. Post nuclear supernatants were obtained by spinning the homogenized whole cell lysates (1500×g×10 min) and protein contents determined by a microtiter Lowry assay (Bio-Rad, Hercules, CA, USA). Five to 20 μg of protein were denatured in the sample buffer (125 mM Tris pH 6.8, 5% β-mercaptoethanol, 6% SDS, 20% glycerol and 0.2% bromophenol blue) at 65°C and subjected to SDS–PAGE on Tris–acetate 3–8% gradient gels (Invitrogen). Fractionated protein was electrotransferred to a PVDF membrane (Invitrogen) and detected with the appropriate antibodies using ECL chemiluminescence (Santa Cruz). The PC2 antibody (YCB9) was used to detect PC2 (9). All the primary and secondary antibodies were diluted to 1:2500. For the quantitative western analysis, the proteins from the wild-type and Pkd2\textsuperscript{+/−} smooth muscles were prepared and fractionated in parallel under an identical experimental protocol and transferred onto a single blot. Hence, the ECL exposure times are identical.

**Quantitative real-time PCR measurements**

Total RNA was extracted from the tunica media of thoracic aortas using TRIzol reagent (Invitrogen). A total of 12 aortas, from six wild-type and six Pkd2\textsuperscript{+/−} littermates, were used for this experiment. A 1.5 μg aliquot of total RNA was reverse-

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**Figure 4.** Caffeine plus thapsigargin induced SR Ca\textsuperscript{2+} release in wild-type (n = 48) and Pkd2\textsuperscript{+/−} (n = 72) VSMCs in zero Ca\textsuperscript{2+} Tyrodes perfusate. (A) Peak F/F<sub>0</sub> (the ratio of the maximum increment [Ca\textsuperscript{2+}] dependent fluorescent intensity over pre-stimulus fluorescent intensity) following caffeine (5 mM) plus thapsigargin (1 μM) stimulation (wild-type 2.50 ± 0.61, Pkd2\textsuperscript{+/−} 1.79 ± 0.07, P < 0.00001). The results are expressed as means ± SEM. (B) Representative tracings of [Ca\textsuperscript{2+}] responses to caffeine plus thapsigargin in wild-type and Pkd2\textsuperscript{+/−} VSMCs.

**Figure 5.** Ca\textsuperscript{2+} uptake via store operated channels (SOCs) after SR Ca\textsuperscript{2+} depletion in wild-type (n = 12) and Pkd2\textsuperscript{+/−} (n = 15) VSMCs. (A) Maximal F/F<sub>0</sub> after reintroducing 2 mM extracellular Ca\textsuperscript{2+} following the depletion of SR by caffeine plus thapsigargin (wild-type 1.27 ± 0.05 vs Pkd2\textsuperscript{+/−} 1.10 ± 0.04, P = 0.008). The L-type channels were blocked by nifedipine (1 μM). The results are expressed as means ± SEM. (B) Representative tracings of Ca\textsuperscript{2+} intake in wild-type (blue) and Pkd2\textsuperscript{+/−} (red) VSMCs.
transcribed using SuperScript First-Strand Synthesis System (Invitrogen) at 37°C for 1 h. The amount of Pkd2 mRNA per vessel was quantified using the real-time PCR SYBR Green assay. The 50 µl PCR reaction contained 2 µl of diluted (10-fold) cDNA, 1× SYBR Green JumpStart Tag ReadyMix (Sigma), and 200 nM cyclophilin specific primers (5'-GCTGTC-TCTTTTCCGCCG-3', 5'-CCGTGATGTCTGAAACACG-3') and Pkd2 primers (5'-TGGTTCTAGTTGACGCGTA-3', 5'-GACATAGCGGATCTGGTTTAC-3'). PCR reactions were performed in the DNA Engine Opticon System (MJ Research) with 40 cycles at 95°C for 40 s and 60°C for 1 min. All data were represented as relative to the Cyclophilin level.

**Dissociation of VSMCs from the thoracic aorta**

The age- and sex-matched (6–8 weeks) wild-type and Pkd2+/− mice were anesthetized with a mixture of ketamine (110 mg/kg) and xylazine (10 mg/kg; i.m.); the aorta was removed and placed in ice-cold, oxygenated Hanks’ balanced salt solution (HBSS), buffered with 10 mM HEPES (pH 7.4). A total of 19 mice (10 Pkd2+/− and nine wild-type littermates with C57Blk6 background) were used for all the calcium experiments. Isolated aortic tunica media layers were finely minced after scraping off the intima and adventitia layers. VSMCs were isolated using a commercial kit (Papain dissociation system, Worthington Biochemical) modified from the protocol suggested by the manufacturer. Briefly, the minced smooth muscle was suspended in Earle’s balanced salt solution (EBSS) containing papain (10 U/ml) and Dnase (1000 U/ml) and incubated at 37°C × 10 min. Elastase (0.05 mg/ml) and collagenase (0.5 mg/ml) were added and the mixture was gently triturated and bubbled with oxygen. The dissociated VSMCs were collected 8 and 10 min later and plated onto the collagen-coated glass cover-slip for study.

**Immunofluorescent staining and confocal microscopy**

Cells seeded on collagen coated glass slides were fixed with 2.5% freshly made paraformaldehyde (pH 7.6) and permeabilized when indicated with methanol and acetone (at 3:1 ratio) for 10 min. After washing with PBS (3×), primary antibodies (in PBS with 10% goat serum) were added and incubated for 45 min. The slides were washed (3× PBS) and FITC or TRD-conjugated secondary antibodies added (Santa Cruz). Controls included cells handled in the same fashion except stained with only primary or only secondary antibodies. The cells were examined using an confocal immunofluorescent microscope.

**Global [Ca2+]i measurements and recording techniques**

VSMCs, plated on cover-slip, were loaded with the cytoplasmic Ca2+ indicator fluo 3-AM (5 µM, Molecular Probes) for 45 min at 37°C in EBSS. After rinsing in normal Tyrodes medium containing (in mM): 145 NaCl, 4 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES (pH 7.4). The cover-slip was mounted on the stage of a Nikon Diaphot inverted microscope and perfused with normal Tyrodes at 4 ml/min at room temperature. The calibration and the techniques of the [Ca2+]i measurement using real-time confocal imaging has been described in detail previously (43). For the caffeine and thapsigargin stimulation, the cells were first perfused with zero Ca2+ Tyrodes for 2 min before the introduction of caffeine (5 mM) and thapsigargin (1 µM) in zero Ca2+ Tyrodes at 4 ml/min.

**Statistical analysis**

All values are presented as mean ± SEM as determined by Student’s t-test. A P-value of <0.05 was considered statistically significant.

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