Pkd1 Haploinsufficiency Increases Renal Damage and Induces Microcyst Formation following Ischemia/Reperfusion

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ABSTRACT

Mutations in PKD1 cause the majority of cases of autosomal dominant polycystic kidney disease (ADPKD). Because polycystin 1 modulates cell proliferation, cell differentiation, and apoptosis, its lower biologic activity observed in ADPKD might influence the degree of injury after renal ischemia/reperfusion. We induced renal ischemia/reperfusion in 10- to 12-wk-old male noncystic Pkd1−/− and wild-type mice. Compared with wild-type mice, heterozygous mice had higher fractional excretions of sodium and potassium and higher serum creatinine after 48 h. In addition, in heterozygous mice, also cortical damage, rates of apoptosis, and inflammatory infiltration into the interstitium at time points out to 14 d after injury all increased, as well as cell proliferation at 48 h and 7 d. The mRNA and protein expression of p21 was lower in heterozygous mice than wild-type mice at 48 h. After 6 wk, we observed dilated tubules, microcysts, and increased renal fibrosis in heterozygotes. The early mortality of heterozygotes was significantly higher than that of wild-type mice when we extended the duration of ischemia from 32 to 35 min. In conclusion, ischemia/reperfusion induces a more severe injury in kidneys of Pkd1-haploinsufficient mice, a process that apparently depends on a relative deficiency of p21 activity, tubular dilation, and microcyst formation. These data suggest the possibility that humans with ADPKD from PKD1 mutations may be at greater risk for damage from renal ischemia/reperfusion injury.


Autosomal dominant polycystic kidney disease (ADPKD) is the most common monogenic renal disease, with a prevalence of 1:400 to 1:1000. Mutations of the PKD1 gene are responsible for approximately 85% of the disease cases, whereas approximately 15% are caused by mutations in PKD2. Only half of patients reach the age of 58 yr without ESRD. ADPKD is a systemic disorder, however, including extrarenal manifestations typically represented by liver cysts, intracranial aneurysms, and heart valve alterations.

The PKD1 gene encodes polycystin 1, a large glycoprotein with an approximately 3000–amino acid extracellular portion that comprises domains that seem to be involved in protein–protein and protein–carbohydrate interactions. A number of studies support the involvement of the primary apical cilium in the pathogenesis of PKD by modulating signal transduction via intracellular Ca2+ transients. Polycystins 1 and 2 (PC1 and PC2) are thought to participate actively in this process. Ciliary mechanosensation has also been associated with STAT6-dependent changes in gene expression. In addition, the cellular effects of polycystins

Received April 29, 2008. Accepted August 6, 2009.
Published online ahead of print. Publication date available at www.jasn.org.
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seem to rely on interaction with the cytoskeleton and mediation of cell–cell adhesion.6,9

PC1 and PC2 activate a number of other pathways. PC1 may function as a G protein–coupled receptor.10 Its activation, following a process dependent on PC2, may also activate JAK2, leading to phosphorylation and activation of STAT1 and generation of STAT1 homodimers.11 These dimers bind to the p21 promoter in the nucleus, promoting its upregulation, reduction of Cdk2 activity, and cell arrest in G0/G1. It has also been shown that PC1 induces phosphatidylinositol 3 kinase–dependent Akt activation,12 whereas its C-terminus may interact with tuberin, regulating mammalian target of rapamycin activity.13 Moreover, PC1 is subjected to an autoproteolytic process in the G protein-coupled receptor proteolytic site domain, generating an extracellular N-terminal fragment,14 whereas its C-terminus seems to be cleaved, to be translocated to the nucleus, and to activate AP-1.15

Ischemia/reperfusion (IR) injury is a common cause of acute kidney injury (AKI), including patients with ADPKD. The cellular damage is secondary to a chain of biochemical and biologic abnormalities.16,17 An abnormal proliferative response of ADPKD cells to cAMP has been reported, apparently associated with defective intracellular Ca2+ homeostasis,18 and animal models of PKD have been associated with dysregulated cell-cycle activity.19 Piontek et al.,20 however, have shown that the effects of Pkd1 inactivation in mice are determined by a developmental switch on postnatal day 13. Interestingly, in this model, cellular proliferation was not significantly increased.

In this scenario, we hypothesized that a lower PC1 biologic activity might amplify the IR injury degree. Although the focal cyst formation in ADPKD is likely dependent on a two-hit mechanism,21 the functional effects of PC1 seem to rely on activity thresholds.22 PKD1-haploinsufficient kidney cells, therefore, might be unable to achieve the required PC1 activity level when exposed to IR. Although studies on the ischemia/PC2 relation have brought some potential contributions to this question,23,24 the relationship between PC1 and IR is basically unknown. By coordinating cell planar polarity in renal tubules, PC1 might exert a protective effect after an ischemic insult. In this study, this potential mechanism was investigated in Pkd1+/− mice obtained from an inbred mouse line with a Pkd1−/− haploinsufficient mice. Our findings of a more severe renal lesion in Pkd1-null heterozygotes suggest an increased risk for renal IR injury in Pkd1-haploinsufficient mice. Development of tubular dilation (TD) and microcysts (MCs) and increased renal fibrosis, in turn, suggest that the IR aggression has a higher long-term negative impact on Pkd1+/− kidneys.

RESULTS

Indirect Analyses of GFR

Because the histologic and immunohistochemical analyses were performed on three heterozygous (HT) and three wild-type (WT) groups, killed at 48 h (HT48h n = 10; WT48h n = 10), 7 d (HT7d n = 8; and WT7d n = 8), and 14 d after the 32-min IR insult (HT14d n = 8; and WT14d n = 8), the functional analyses were carried out preischemically and at 48 h in all animal groups; at 7 d in groups WT7d, WT14d, HT7d, and HT14d; and at 14 d only in the WT14d and HT14d groups. Two additional groups, Pkd1+/− (n = 11) and Pkd1+/− (n = 8), were subjected to a 35-min IR insult.

The bilateral IR insult resulted in a significant increase in serum creatinine (SCr) in Pkd1+/− mice at 48 h and 7 d of follow-up, whereas only a NS trend of rise was observed in Pkd1+/− mice at 48 h (Figure 1A; Table 1). Pkd1+/− mice showed a SCr rise at 48 h from 0.33 ± 0.13 to 0.80 ± 0.40 mg/dl (P < 0.001), a value significantly higher than that observed in Pkd1+/− mice (0.60 ± 0.20 mg/dl; P < 0.005). The SCr level, in turn, returned to preischemic values by 14 d after the insult.

Blood urea nitrogen (BUN) rose at 48 h in Pkd1+/− and Pkd1+/− mice but did not differ between them at all three follow-up times (Figure 1B). A reduction in body weight was detected in both WTs and HTs after IR, but it returned to baseline values in both groups by 14 d after injury induction (Table 1). Thirty-five minutes of IR, in turn, led to significantly higher BUN levels in HTs than in WTs 48 h after the insult.

Figure 1. (A and B) Comparative analyses of SCr (A) and BUN (B) in Pkd1+/+ and Pkd1+/− male mice before insult and 48 h, 7 d, and 14 d after a 32 min of renal IR insult. *P < 0.001 versus preischemic (PI); †P < 0.05 versus PI; ‡P < 0.05 versus 48 h; §P < 0.005 versus Pkd1+/+. SCr was compared using two-way ANOVA, with the data presented as means ± SD, and BUN using the Friedman test, with the data expressed as median (minimal to maximal).
Table 1. Time course of $S_{Cr}$, BUN, and body weight in Pkd1+/− and Pkd1+/− male mice subjected to renal IR

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$S_{Cr}$ (mg/dl; Mean ± SD)</th>
<th>BUN (mg/dl; Median [Minimum to Maximum])</th>
<th>Body Weight (g; Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pkd1+/− (n = 26)</td>
<td>0.46 ± 0.17</td>
<td>22.7 (20.0 to 23.9)</td>
<td>21.15 ± 1.89</td>
</tr>
<tr>
<td>Pkd1+/- (n = 26)</td>
<td>0.33 ± 0.13</td>
<td>21.9 (19.5 to 23.2)</td>
<td>20.52 ± 2.74</td>
</tr>
<tr>
<td>48 h</td>
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<td></td>
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<tr>
<td>Pkd1+/− (n = 26)</td>
<td>0.60 ± 0.20</td>
<td>41.5 (36.0 to 53.2)*a</td>
<td>18.87 ± 1.41*</td>
</tr>
<tr>
<td>Pkd1+/- (n = 26)</td>
<td>0.80 ± 0.40b,c</td>
<td>45.4 (40.4 to 76.5)d</td>
<td>17.80 ± 2.10b</td>
</tr>
<tr>
<td>7 d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pkd1+/− (n = 16)</td>
<td>0.46 ± 0.17</td>
<td>30.4 (25.9 to 44.4)*a</td>
<td>19.34 ± 2.05*</td>
</tr>
<tr>
<td>Pkd1+/- (n = 16)</td>
<td>0.50 ± 0.15d,e</td>
<td>32.3 (29.3 to 45.9)d</td>
<td>18.81 ± 2.38a</td>
</tr>
<tr>
<td>14 d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pkd1+/− (n = 8)</td>
<td>0.62 ± 0.25</td>
<td>36.1 (32.1 to 40.5)*</td>
<td>20.10 ± 1.30</td>
</tr>
<tr>
<td>Pkd1+/- (n = 8)</td>
<td>0.50 ± 0.25</td>
<td>38.7 (35.1 to 43.2)d</td>
<td>19.40 ± 2.10</td>
</tr>
</tbody>
</table>

$S_{Cr}$ and body weight were compared using two-way ANOVA; BUN analysis was performed with the Friedmann test. PI, preischemic insult.

*P < 0.05 versus Pkd1+/−.

Higher $S_{Cr}$ (1.13 ± 0.07 versus 0.70 ± 0.03 mg/dl; P < 0.0003; Figure 2, A and B). The mice that were subjected to this time insult did not stay alive for the entire 14-d follow-up, but WTs survived remarkably longer than HTs (10 versus 3 d; P < 0.0001; Figure 2C).

Tubular Function Analyses

HT mice presented a significant rise in fractional excretion of Na+ (FENa) 48 h after the IR insult (0.56% [0.39 to 0.81%] to 2.10% [1.31 to 3.18%]; P < 0.05), an observation not detected in WT mice (Table 2; Supplemental Figure S1A). The 48-h value, in turn, was higher in Pkd1+/- than in Pkd1+/− mice (0.80% [0.66 to 1.00%]; P < 0.05). FE Na remained above baseline levels in HTs at 7 d, whereas at 14 d it no longer differed from the preischemic value. An increase in fractional excretion of K+ (FEK) was also verified in Pkd1+/− mice 48 h after IR (35.0% [19.4 to 61.7%] to 70.0% [45.3 to 95.4%]; P < 0.05) but was not observed in Pkd1+/- mice (Table 2; Supplemental Figure S1B). The values detected in HTs, in turn, were higher than in WTs both at 48 h (36.8% [29.1 to 47.0%] for WTs; P < 0.05) and at 7 d (59.0% [37.3 to 95.9%] versus 41.0% [26.3 to 48.4%]; P < 0.005).

Histologic Analyses of Renal Injury

Histologic analyses performed in Pkd1+− and Pkd1+/- kidneys revealed a more prominent residual cortical damage in HTs than in WTs at 48 h, 7 d, and 14 d after IR. The Nikon index was 3.60 ± 0.22 in Pkd1+/− versus 2.40 ± 0.40 in Pkd1+/- mice (P < 0.05) 48 h after IR; 3.12 ± 0.22 in HTs versus 1.00 ± 0.26 in WTs (P < 0.0001) at 7 d; and 2.29 ± 0.56 versus 0.57 ± 0.29 (P < 0.05) at 14 d after the IR insult (Figure 3A). No significant injury signs or differences were detected between sham-operated (SO) WTs (0.22 ± 0.16) and HTs (0.13 ± 0.14). Whereas in WTs mild to moderate alterations were observed 48 h after IR and minimal to mild changes were seen at 7 and 14 d, HTs presented with moderate to severe damage 48 h after IR, moderate injury at 7 d, and mild to moderate damage 14 d after the insult. At 48 h, when cortical damage was the most severe in both groups, HT kidneys showed cavitation, dilatation, and hyaline casts predominantly in proximal tubules (PTs) and, to a lesser extent, in collecting tubules, whereas WT mice had their kidneys with PT dilations and microvacuolization, less extensive tubular involvement, and only a few hyaline casts (Figure 3B). The degree of tubule dilation, in turn, was more pronounced in HTs than WTs for similar time follow-ups.

PT and Endothelial Injury Analyses by Electron Microscopy

The immunoelectron microscopy analysis revealed more severe damage in HT than in WT mice at 48 h (Figure 4A). In WTs, mild alterations were observed after IR, including microvacuolization of some PT epithelial cells. HT kidneys, conversely, showed significant PT cell damage with intraluminal accumulation of apical membrane fragments and hyaline casts. Additional HT injury features are reported in Figure 4A. The WT mice showed subtle changes in endothelium, represented by peritubular capillary loss of part of the endothelial fenestration. HTs, however, showed more prominent damage, with significant loss of endothelial fenestration and accumulation of flocculent material (Figure 4B).

Cell Proliferation Analyses

The proliferating cell nuclear antigen (PCNA) studies showed a higher cellular proliferation rate in Pkd1+− than in Pkd1+/- mice 48 h and 7 d after IR, although the rates were markedly higher at 48 h (Figure 5). Cell proliferation was significantly reduced and no difference in baseline rates was detected between SO Pkd1+− (0.15%) and Pkd1+/- mice (0.12%).
analyses revealed indexes of PCNA-positive cells of 15.51% in HTs and 8.42% in WTs (P < 0.0001) at 48 h of follow-up and 3.05 versus 1.25%, respectively, 7 d after IR (P < 0.0001). At 14 d, however, the PCNA staining was substantially reduced and not different between HTs and WTs.

**Apoptosis Analyses**

Terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) analyses revealed a higher cell apoptotic rate in HT than in WT mice after IR at the three follow-up times, although the rates markedly declined with time (Figure 6). No positive staining was de-

**Figure 2.** (A and B) Comparative analyses of Scr (A) and BUN (B) in Pkd1+/− (n = 11) and Pkd1−/− (n = 8) male mice before insult and 48 h after a 35 min of renal IR insult. *P < 0.0001 versus PI; †P < 0.0005 versus PI; ‡P < 0.0003 versus Pkd1+/−; §P < 0.05 versus Pkd1+/−. Scr and BUN were compared using the nonpaired t test with Welch correction, with the data presented as means ± SD. The high mortality observed in the Pkd1−/− mice group beyond 48 h did not allow longer time evaluations. (C) Cumulative survival curves and comparative analysis between Pkd1+/− and Pkd1−/− male mice after 35 min of IR (P < 0.0001). Comparison performed using the Kaplan-Meier survival curve.

**Table 2.** Time course of FE_{Na,ur}, U_{osm}, S_{Na}, and S_{K} in Pkd1+/− and Pkd1−/− male mice subjected to renal IR

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FE_{Na,ur} (% Median to Maximum)</th>
<th>U_{osm} (mOsm/kg H2O Median to Maximum)</th>
<th>S_{Na} (mEq/L Median to Maximum)</th>
<th>S_{K} (mEq/L Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI</td>
<td>0.90 (0.69 to 1.27)</td>
<td>35.0 (19.4 to 61.7)</td>
<td>2.00 (3.13 to 3.18)</td>
<td>4.8 (1.0 to 0.8)</td>
</tr>
<tr>
<td>48 h</td>
<td>0.80 (0.66 to 1.00)</td>
<td>36.3 (4.29 to 47.0)</td>
<td>2.10 (3.13 to 3.18)</td>
<td>4.8 (1.0 to 0.8)</td>
</tr>
<tr>
<td>7 d</td>
<td>0.99 (0.80 to 1.27)</td>
<td>50.0 (4.29 to 47.0)</td>
<td>2.10 (3.13 to 3.18)</td>
<td>4.8 (1.0 to 0.8)</td>
</tr>
<tr>
<td>14 d</td>
<td>1.40 (0.88 to 2.30)</td>
<td>58.2 (3.27 to 70.3)</td>
<td>2.10 (3.13 to 3.18)</td>
<td>4.8 (1.0 to 0.8)</td>
</tr>
</tbody>
</table>

values were analyzed using two-way ANOVA; FE_{Na,ur}, U_{osm}, S_{Na}, and S_{K} were analyzed using the Friedman test.
The apoptotic rates were 62.02% in Pkd1+/+H11001/H11002 mice at 48 h (P < 0.0005), 3.99% in HTs versus 0.67% in WTs at 7 d (P < 0.01), and 1.37% in HTs versus 0.48% in WTs at 14 d of follow-up (P < 0.005).

Figure 3. (A) Comparative analysis of renal residual cortical damage evaluated by the Jablonski index in Pkd1+/+ and Pkd1+/− male mice 48 h, 7 d, and 14 d after renal IR (48 h n = 10 per group; 7 d n = 8 per group; 14 d n = 8 per group). #P < 0.05 versus Pkd1+/+; *P < 0.0001 versus Pkd1+/+. Nonpaired t test, with the data expressed as means ± SD. (B) Histologic representation of renal injury in Pkd1+/+ (a, c, e, and g) and Pkd1+/− (b, d, f, and h) mice. (a and b) SO. (c and d) Forty-eight hours after IR. (e and f) Seven days. (g and h) Fourteen days. Magnification, ×400.

Figure 4. Immunoelectron microscopy analysis in Pkd1+/+ and Pkd1+/− male mice 48 h after renal IR. (A) Representative images of PTs in Pkd1+/+ (a and b) and Pkd1+/− (c and d) mice. WT controls show mild alterations whereas HTs show significant tubular cell damage with intraluminal hyaline casts (*) and shedding of lining cells with increased amounts of protein in the lumen of PTs. Some HT tubular cells showed rarefaction of the cytoplasm with intracellular vacuoles, swollen organelles, apoptosis, and loss of brush border (arrows), whereas others presented nuclear condensation, cytoplasmic swelling, and loss of nuclei in up to one third of the tubular profile. (B) Representative images of peritubular capillaries in Pkd1+/+ (a and b) and Pkd1+/− (c and d) mice. HTs show a significantly more severe loss of peritubular capillary endothelial fenestration, as well as accumulation of flocculent material. Magnifications: ×10,000 in A and B (b); ×20,000 in B (a, c, and d).
The verified differences in TUNEL-positive cells reflect predominantly the epithelial compartment, although the quantification process also included interstitium.

**p21 Expression Analyses**

The p21 immunohistochemical experiments demonstrated a lower staining rate in cells of Pkd1+/−/− kidneys 48 h after IR (Figure 7, A and B). The rate of nuclear p21-positive cells was 0.58% in HT and 3.18% in WT mice (P < 0.0005). A decreased p21 cytoplasmic expression was also detected in Pkd1+/−/− kidneys compared with the Pkd1+/+ controls (0.12 versus 0.62%; P < 0.05). Although at much lower levels, the nuclear p21 staining at 7 d was still slightly higher in WTs (0.35 versus 0.39%, respectively). The nuclear and cytoplasmic expression did not differ between Pkd1+/−/− and Pkd1+/+ SO mice (0.04 and 0.09 versus 0.05 and 0.09%, respectively).

Real-time reverse transcriptase–PCR (RT-PCR) evaluation of p21 mRNA expression revealed a pattern similar to that yielded by immunostaining. At 48 h of follow-up, p21 expression was 0.73 ± 0.29 arbitrary units (AU) in Pkd1+/−/− kidneys and 2.30 ± 1.55 AU in Pkd1+/+ controls (P < 0.03; Figure 7C).

**Macrophage Infiltration Analyses**

Although negative and positive controls for the anti–Mac-2 antibody were adequate, our initial immunohistochemical analyses revealed that it cross-reacts with tubular epithelial cells in mouse kidneys, in addition to its specific staining for macrophages. Despite this observation, also reported by other investigators, its staining pattern in renal interstitium was appropriately restricted to macrophages. Our results showed a more prominent infiltration in HTs and WTs at all follow-up times after the insult (Figure 8), whereas no difference was detected between Pkd1+/−/− and Pkd1+/+ SO mice (0.33 ± 0.06 positive cells/field [PC/F]) and Pkd1+/+ (0.34 ± 0.04 PC/F) SO mice. HTs showed a more intense Mac-2 staining than WTs at 48 h (4.43 ± 0.81 versus 2.06 ± 0.32 PC/F; P < 0.01), at 7 d (5.07 ± 0.43 versus 2.61 ± 0.54 PC/F; P < 0.05), and at 14 d (1.80 ± 0.36 versus 0.71 ± 0.15 PC/F; P < 0.01).

**Na-K-ATPase Distribution Analyses**

Immunohistochemical analysis for Na-K-ATPase revealed no difference between SO WTs and HTs, showing a predominant basolateral and cytoplasmic staining in collecting and distal tubules, with significantly lower signal in PTs. At 48 h after IR, this pattern was preserved in WTs but with markedly decreased signal intensity (Supplemental Figure S2). HTs, conversely, kept the same signal profile in cytoplasm and basolateral membrane, with slightly increased signal in medullary collecting ducts, and a few tubules showing specific staining in the luminal membrane. At day 7, the membrane distribution pattern remained the same in WTs, with the signal intensity increasing to a level close to the SO mice, whereas HTs returned to the baseline profile.
Follow-up, Col1a1 and Col1a2 expressions were, respectively, 2.75 ± 0.86 and 4.11 ± 2.21 AU in Pkd1+/− kidneys and 0.92 ± 0.27 and 0.94 ± 0.27 AU in Pkd1+/+ kidneys (P < 0.02 and P < 0.0002, respectively; Figure 9C).

**TD and MC/Cyst Formation Analyses**

Serial sections of the seven Pkd1+/− and seven Pkd1+/+ left kidneys removed 6 wk after the IR insult revealed significant TD and MC formation in HTs from cortex to inner medulla but not in WTs (Figure 10). No differences in TD and MC/cyst formation were observed between Pkd1+/− and Pkd1+/+ SO mice (TDI 6.0 [3.0 to 7.0] dilated tubules [DT] in Pkd1+/− and 1.0 [0.0 to 1.0] DT in Pkd1+/+ [NS]; TDII 3.0 [0.0 to 4.0] and 0.0 [0.0 to 0.0] DT, respectively [NS]; MC 0.0 [0.0 to 0.0] in Pkd1+/− and Pkd1+/++; and cysts 0.0 [0.0 to 0.0] in both groups). HTs showed increased TDII and MC formation 6 wk after IR (TDI 4.5 [1.0 to 7.0] DT [NS] versus SO; TDII 6.5 [5.0 to 8.0] DT [P < 0.05] versus SO; MC 3.5 [2.0 to 5.0; P < 0.02] versus SO; and cysts 0.0 [0.0 to 0.0; NS] versus SO). Two Pkd1+/− mouse kidneys showed two cysts each. WTs, conversely, showed no increase in these parameters at 6 wk (TDI 1.5 [1.0 to 2.0] DT; TDII 1.5 [0.0 to 2.0] DT; MC 0.0 [0.0 to 0.0]; and cysts 0.0 [0.0 to 0.0]). The TDII and MC formation indexes, in turn, were significantly higher in Pkd1+/− than Pkd1+/+ mice (P < 0.02 and P < 0.02, respectively).

**DISCUSSION**

Although abnormalities have been reported in Pkd1– and Pkd2-haploinsufficient models, the understanding and effects of this condition are still limited. On the basis of the biologic properties of PC1, we reasoned that this condition might lead to increased susceptibility to IR. IR injury is a major cause of AKI, involving tubular cell death, endothelial dysfunction, and inflammation. It is interesting that quiescent kidney cells enter the cell cycle after postischemic AKI, being followed by the induction of cell-cycle inhibitors, particularly p21. The increased expression of this protein seems to be involved in the adequate coordination of cell cycle, required for maximization of renal epithelial cell recovery from injury and reduction of the damage extension.

We analyzed the relationship between Pkd1 haploinsufficiency and the risk for renal IR using an inbred 129Sv mouse line with a Pkd1 null mutation. Because the HT mice virtually do not develop renal cysts by 12 wk of age, they constitute an appropriate model of Pkd1 haploinsufficiency. The higher S_Cr

**Figure 6.** (A) Comparative analysis of TUNEL staining in kidneys of Pkd1+/+ and Pkd1+/− male mice at 48 h, 7 d, and 14 d of follow-up after renal IR (48 h n = 10 per group; 7d n = 8 per group; 14 d n = 8 per group). *P < 0.0005 versus Pkd1+/+; **P < 0.01 versus Pkd1+/+; ***P < 0.005 versus Pkd1+/+.

(B) Representative images of TUNEL staining in Pkd1+/− (a, c, e, g, i, k, m, and o) and Pkd1+/− (b, d, f, h, j, l, n, and p) mice (a, b, c, and d) SO. (e, f, g, and h) Forty-eight hours after IR. (i, j, k, and l) Seven days. (m, n, o, and p) Fourteen days. Magnifications: ×400 in a, b, e, f, i, j, m, and n; ×1000 in c, d, g, h, k, l, o, and p.
increase in Pkd1$^{+/−}$ than in Pkd1$^{+/+}$ mice 48 h after the induction of IR indicates a more pronounced GFR reduction in HTs than in WTs and suggest that the Pkd1-haploinsufficient state increases the degree of IR lesion. BUN did not differ between the two groups for the 32-min IR, but when this time was raised to 35 min, both BUN and S$_{Cr}$ were significantly higher in HTs than in WTs at 48 h. It must be noted, in addition, that although the 35-min–related injury was followed by death of all mice, WTs survived significantly longer than HTs, also supporting increased susceptibility to IR injury in Pkd1$^{+/−}$ mice. The increase in FC$_{Na}$ and FE$_{K}$ in the HT mice 48 h and 7 d after IR, not verified in the WT mice, indicates a higher level of tubular injury in the Pkd1-haploinsufficient mice. The observation that Pkd1$^{+/−}$ kidneys show Na-K-ATPase expression in the luminal membrane in some tubules, in turn, suggests that its misregulation may play a role in these functional findings.

A more severe residual cortical damage in HTs than in WTs was shown at the three evaluated time points after renal IR. Whereas significant lesions were detected at 48 h in proximal and collecting tubules in Pkd1$^{+/−}$-kidneys, only low-degree abnormal findings were found in Pkd1$^{+/+}$ PTs. The involvement of collecting tubules in the IR injury of Pkd1-haploinsufficient mice is not surprising, because PC1 has been shown to be particularly expressed in the distal nephron and collecting tubules.$^{32,33}$ Pkd1 haploinsufficiency, therefore, magnifies the morphologic injury determined by IR up to 14 d after the insult. This conclusion is also supported by the immunoelectron microscopy analysis, which revealed more severe proximal tubular and endothelial injuries in HTs.

A higher cellular proliferation rate was found 48 h and 7 d after renal IR in Pkd1$^{+/−}$ than in Pkd1$^{+/+}$ mouse kidneys. The lack of difference observed at 14 d was an expected finding, because at this point, a marked reduction in cell proliferation is described in kidneys submitted to IR.$^{34}$ Pkd1 haploinsufficiency, therefore, seems to hamper the appropriate cell-cycle coordination required after IR. The higher rate of apoptosis revealed by TUNEL assays in Pkd1$^{+/−}$ compared with Pkd1$^{+/+}$ mice, in turn, suggests that the increased post-IR apoptosis associated with Pkd1 haploinsufficiency may contribute to the higher degree tubular injury.$^{16}$ The significant decline in apoptotic rate observed by 7 d, conversely, is consistent with its marked fall observed after the first days after IR.$^{35}$

The initial increase of p21 expression at 48 h after IR and its significant decline at 7 d in WTs are consistent with another mouse model of renal IR injury,$^{36}$ revealing a normal p21 up-regulation shortly after this insult, followed by a return to pre-injury levels within the next days. This course in WT kidneys, associated with nonincreased p21 expression at 48 h in HTs, indicates that Pkd1-haploinsufficient mice are unable to up-regulate p21 early after the IR insult. This failure to increase p21 expression, moreover, is associated with evidence of disturbed cell-cycle coordination, as outlined by inappropriately elevated cell proliferation and apoptosis. A proper cell-cycle

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**Figure 7.** (A) Comparative analysis of p21 staining in kidneys of Pkd1$^{+/−}$ and Pkd1$^{+/+}$ male mice after 48 h and 7 d of renal IR (48 h n = 10 per group; 7 d n = 8 per group). $^P < 0.0005$ versus Pkd1$^{+/+}$; $^P < 0.05$ versus Pkd1$^{+/−}$. $^2$ proportion test, with the data expressed as median (lower quartile to upper quartile). (B) Representative images of p21 staining in Pkd1$^{+/−}$ (a, c, e, g, i, and k) and Pkd1$^{+/+}$ (b, d, f, h, j, and l) mice. (a, b, c, and d) SO. (e, f, g, and h) Forty-eight hours after IR. (i, j, k, and l) Seven days. (m) Negative control in kidney. (n) Positive control in breast cancer. (C) Comparative analysis of p21 mRNA levels in Pkd1$^{+/−}$ and Pkd1$^{+/+}$ kidneys of male mice 48 h after renal IR (n = 8 per group), performed by real-time RT-PCR. $^P < 0.03$ versus Pkd1$^{+/+}$. $^t$ test with Welch correction. Magnifications: $\times 400$ in B (a, b, e, f, i, j, m, and n); $\times 1000$ in B (c, d, g, h, k, and l).
regulation after AKI has been shown, in fact, to be a key determinant of an adequate tissue recovery response. In this setting, it is expected that p21 upregulation exerts a renal protective effect against IR by adequately regulating cell cycle after injury. The defective tubular response and increased susceptibility to ischemia in Pkd1-haploinsufficient mice, therefore, seem to be determined by this lack of upregulation.

Our findings are consistent and supported by previous results that show increased p21 transcriptional levels in murine kidneys after IR, as well as increased renal function impairment, mortality, and cell-cycle activity after renal IR in p21−/− mice compared with their p21+/+ counterparts. Additional data suggest that the post-IR p21 upregulation is p53 independent. The higher susceptibility to IR injury observed in the p21−/− animals, therefore, seems to result from inappropriately regulated cell-cycle activity in kidney cells. Interestingly, this group has also shown that cell-cycle inhibitors protected renal cells from cisplatin-induced apoptosis. In addition, in support of our findings, other recent studies showed that mouse renal epithelial Pkd1−/− cells express p21 at lower levels than Pkd1+/+ cells and demonstrated a diminishment of p21 expression in human ADPKD cystic kidneys as compared with normal ones. We propose, therefore, that the lack of p21 upregulation after IR in Pkd1−/− mice is responsible, at least partially, for the increased initial injury and the latter changes in recovery observed in these animals.

Altered renal hemodynamics, defective vascular response, or dysregulated planar cell polarity, however, may also contribute to the higher degree of IR injury and associated findings seen in Pkd1−/− mice. Given that cell-cycle inhibitors can rescue neural tube abnormalities in zebrafish lacking Vangl2, a molecule involved in planar cell polarity signaling, a potential link between Pkd1 haploinsufficiency and IR-related defects in planar cell polarity might result from p21 relative deficiency. A defective tubular response to injury in HTs might also result from disruption of PC1 likely roles in cell–cell and/or cell–matrix interactions and inappropriate adjustment to cellular stress, because PC1 is a regulator of endoplasmic reticulum Ca2+ release. Last, that IR results in early shortening and later lengthening of cilia after the insult in mice suggests that renal cilia may act in the repair process.

Our data support that Pkd1 haploinsufficiency also affects the post-IR renal inflammatory component, amplifying such response. They also suggest that this is a slightly later event than the other IR responses associated with Pkd1 haploinsufficiency. Reduced levels of p21 might be also responsible for such amplification, because p21 gene transfer has been shown to downregulate the expression of several inflammatory mediators. On the basis of these observations and that renal disease progression seems to be affected by fibrosis in ADPKD, we analyzed a follow-up time of 6 wk after IR. In this setting, we showed more extensive interstitial expansion and higher Col1a1 and Col1a2 expression in Pkd1−/− than Pkd1+/+ mice, findings consistent with a higher level of kidney fibrosis in HTs. These results suggest that IR insults may favor disease progression in ADPKD.

Figure 8. (A) Comparative analysis of kidney Mac-2 staining in Pkd1+/+ and Pkd1+/− male mice after 48 h, 7 d, and 14 d of renal IR (48 h n = 10 per group; 7 d n = 8 per group; 14 d n = 8 per group). **P < 0.01 versus Pkd1+/−; *P < 0.05 versus Pkd1+/−. Nonpaired t test, with the data expressed as means ± SD. (B) Representative images of Mac-2 staining in Pkd1+/+ (a, c, e, and g) and Pkd1+/− (b, d, f, and h) mice. (a and b) SO. (c and d) Forty-eight hours after IR. (e and f) Seven days. (g and h) Fourteen days. Magnification, ×400.
acceleration in Pkd1-haploinsufficient kidneys by inducing renal fibrosis.

HT kidneys showed significant TD and development of MCs 6 wk after IR, a finding not detected in their WT counterparts. Bigger cysts were only occasionally observed in HTs after IR but never in WTs. One study showed that inactivation of Pkd1 in mice after postnatal day 13 determines remarkably milder cystic formation than earlier inactivation. Additional data show that kidney-specific Pkd1 inactivation leads to

Figure 9. (A) Comparative analysis of relative area of interstitial expansion in Pkd1+/+ (n = 7) and Pkd1+/− (n = 7) male mice 6 wk after renal IR. *P < 0.005 versus SO; **P < 0.01 versus Pkd1+/+.

Nonpaired Mann-Whitney test, with the values expressed as median (lower quartile to upper quartile). (B) Histologic representation in Pkd1+/+ (a, c, and e) and Pkd1+/− (b, d, and f) mice. (a and b) SO. (c, d, e, and f) Six weeks after IR. Magnification, ×400 in a, b, c, and d; ×200 in e and f.

Figure 10. (A) Comparative analysis of TD, MC formation index, and cyst formation index in Pkd1+/+ (n = 7) and Pkd1+/− (n = 7) male mice 6 wk after renal IR. *P < 0.02 versus SO; †P < 0.05 versus SO; ‡P < 0.02 versus Pkd1+/+. Nonpaired Mann-Whitney test, with the values expressed as median (lower quartile to upper quartile). (B) Histologic representation in Pkd1+/+ (a, c, and e) and Pkd1+/− (b, d, and f) mice. (a and b) SO. (c, d, e, and f) Six weeks after IR. Magnification, ×400 in a, b, c, and d; ×200 in e and f.

performed by real-time RT-PCR. †P < 0.02 versus Pkd1+/+; ¥P < 0.0002 versus Pkd1+/+. t test with Welch correction. Magnification, ×400 in B.
slowly progressive cystic disease in adult mice, suggesting that Pkd1 deficiency does not initiate enough cell proliferation for cyst formation and that additional stimuli are probably required for cystogenesis. Takakura et al. confirmed these findings and showed that renal injury constitutes a “third hit” in Pkd1 1KO mice, leading to rapid cyst formation in adulthood. Our findings support and are supported by those previous data and extend this principle, placing IR as a likely additional hit in a Pkd1−/− model. It must be noted, however, that although one could expect that the MCs formed in our model after IR would arise from cells with germine and somatic mutations in the Pkd1 alleles, this remains to be demonstrated. Patel et al. recently showed that ischemic kidney injury induced cyst formation in adult Kif3a mutant mice. Although these investigators claimed that cilia abnormalities play a key role in this process, it is interesting to note that our Pkd1-haploinsufficiency model seems not to be associated with ciliary defects. A critical point must be raised, however, in this work, HT’s and WT’s developed distinct levels of renal injury, whereas in the study by Patel et al., the degree of injury was not documented. On the basis of our findings, one could consider that their mutants might be also more susceptible to renal IR. If this was the case, then in our and their situations, it would be expected that the cystic phenotypes result from PC1-related dysfunctions in tubular luminal regulation, but one could not completely exclude the possibility that the phenotypes are associated with a higher degree of post-IR tubular injury.

Our data suggest that Pkd1-null heterozygotes—mice and possibly humans—are associated with a higher risk for renal IR injury, a process that seems to depend on a relative deficiency of p21 activity, and with IR-induced TD and MC formation. These findings place PC1 as a likely renal protective molecule and may lead to preventive procedures against possible kidney injury in patients with ADPKD and to the development of protective agents to be used in such patients in at-risk situations. Our results also show that IR leads to increased renal fibrosis in Pkd1 haploinsufficiency and support the concept that renal IR may function as an additional hit for cystogenesis, suggesting that it may contribute to acceleration of kidney disease in ADPKD.

CONCISE METHODS

Animal Model
The generation of our 129Sv mouse line with a Pkd1 null allele was based on a construct that had part of exon 2 and the entire exon 3 replaced with a reporter gene (lacZ) cloned in frame to the remainder of exon 2 and followed by the neomycin resistance gene (neoR). The lacZ-neor 5’ segment includes transcriptional termination sequences that prevent expression of 3’ Pkd1 exons. The mice were genotyped using a three-primer PCR strategy. This study was directed to HT mice for the Pkd1−/− mutation with 10 to 12 wk of life, because, at this age, they present virtually no renal cysts, representing a pure Pkd1-haploinsufficiency model. WT littermates were used as controls. The research was conducted in accordance with international standards of animal care and experimentation.

IR Model
All experiments were carried out in male mice to avoid potential gender-related experimental heterogeneity. Mice were weighed and subjected thereafter to the IR protocol. After anesthesia with sodium pentobarbital (65 μg/g body wt) injected intraperitoneally, an incision was made in the central abdomen, the two renal pedicles were exposed, and microvascular clamps were applied to them. After 32 min of renal ischemia, clamps were removed, blood flow returned to the kidneys, and the incision was closed. During the procedure, mice were appropriately hydrated, and their body temperature was kept at approximately 37°C using an adjustable heating pad. When reperfusion was incomplete after clamp release, the experiment was terminated and the mouse was killed. The mice received 400-μl aliquots of saline solution after the procedure, infused intraperitoneally. The mice were returned to their cages after surgery and allowed free access to food and water. Additional groups of SO HT (n = 8) and WT (n = 8) mice were also included as negative controls for the immunohistochemical analyses. Two additional HT (n = 8) and WT (n = 11) mouse groups were subjected to the same protocol, except for an IR insult of 35 min.

Biochemical Determinations
One day before each biochemical determination, the mice were housed in metabolic cages without food or water. Blood samples were drawn by retro-orbital bleeding before IR and 48 h, 7 d, and 14 d after ischemic insult. Blood samples were allowed to clot and were centrifuged to obtain serum. BUN was measured according to Crocker’s protocol (Celm, Barueri, Brazil), whereas SCr and urine creatinine (UCr) values were determined using a colorimetric assay (Labtest, Lagoa Santa, Brazil). Mice with baseline SCr >0.6 mg/dl and BUN >25 mg/dl were excluded from the study. FE_{Na} was calculated using the equation FE_{Na} = (U_{Na} \times S_{Cr})/(S_{Na} \times U_{Cr}) \times 100, where U_{Na} is urine Na+. FE_{K} was obtained using a similar approach. U_{osm} was measured using a Vapro 5500 vapor osmometer (Wescor, Logan, UT).

Morphologic Assessment
Kidneys were harvested from SO mice and 48 h, 7 d, and 14 d after the insult. The mice were anesthetized with intraperitoneal sodium pentobarbital (80 μg/g body wt) and subjected to thoracotomy, a catheter insertion in the left ventricle, and a right atrial cut to drain the blood. They were then perfused with saline solution at a 100-mmHg pressure until complete exsanguination. Immediate perfusion with Millonig formalin, modified by Carlson, followed this step. Kidneys were fixed in situ with 4% buffered paraformaldehyde, paraffin embedded, sectioned at 4 μm, and stained using hematoxylin and eosin (H&E). All morphologic analyses were performed blindly by
an experienced renal pathologist. This analysis included the evaluation of the degree of renal tubular necrosis after IR using a grading scale proposed by Jablonski et al. The Jablonski index comprises the potential presence of brush border loss, tubular cast formation, TD, thickened basement membrane, inflammation, number of apoptotic cells, number of mitotic cells, signs of tubular regeneration, PT damage, and distal nephron damage. This analysis allows grading of the extent of the cortical necrosis from 0 to 4, where 0 reflects no necrosis; 1 includes mitoses and necrosis of individual cells; 2 means necrosis of all cells in adjacent proximal convoluted tubules, with survival of surrounding tubules; 3 comprises necrosis confined to the distal third of the proximal convoluted tubule with a band of necrosis extending across the inner cortex; and 4 reflects necrosis affecting all three segments of the proximal convoluted tubule. These analyses were performed in renal cortex, including two sections per mouse and 10 fields per section, totalizing 20 fields per mouse, in groups of at least eight mice.

**Electron Microscopy**

Samples were fixed in 4% paraformaldehyde, transferred to 2% aqueous glutaraldehyde for 2 h, stained with 2% aqueous osmium tetroxide, dehydrated in a graded ethanol series, and embedded in L.R. white resin (London Resin Company, Ltd., Berkshire, England). Ultrathin sections were cut, placed on nickel grids, and counterstained with aqueous 4% uranyl acetate and Reynolds lead citrate. The specimens were examined in a Jeol 1010 transmission electron microscope.

**Immunohistochemistry**

A monoclonal IgG2 antibody (Ab) to PCNA (Dako, Carpen- teria, CA), a monoclonal IgG2b Ab to p21 (Santa Cruz Bio- technology, Santa Cruz, CA), a monoclonal IgG2a Ab to Mac-2 (Cedarlane, Ontario, Canada), and a monoclonal IgG1 Ab to the α1-subunit of Na-K-ATPase (Affinity BioReagents, Golden, CO) were used. Control sections for p21, PCNA, and Mac-2 analyses, incubated with PBS instead of primary Abs, showed negative or negligible staining in PTs. In the p21 and PCNA analyses, the evaluation of each mouse included eight fields representing renal cortex and two in renal medulla. In the Mac-2 study, the evaluation of each mouse included analysis of renal interstitium in 10 fields located in the cortex.

Staining for apoptosis was obtained using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany). Positive controls were pretreated with 1 U/ml DNase, whereas negative controls were incubated without terminal deoxynucleotidyl transferase. Nuclei were counterstained with 3,3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO). The analysis of each section included 10 distinct fields. The quantifications of p21-, PCNA-, and TUNEL-positive nuclei, as well as Mac-2–positive cells, were performed under light microscopy at ×400 magnification. The corresponding results for p21, PCNA, and TUNEL are expressed as PC/total cells ratios and for Mac-2 as PC/F given the significantly lower cell count.

**Interststitial Expansion Index**

Three-micrometer paraffin-embedded kidney sections from specimens collected 6 wk after IR were mounted on slides and submitted to Masson’s trichrome and H&E staining. The fractional interstitial area of the renal cortex was determined by morphometry with a video camera connected to an image analyzer (QWin; Leica, Wetzlar, Germany). Ten cortical grid fields (each with 37,000 μm² in area) of each right kidney were evaluated in a blinded manner. The interstitial areas were manually encircled on a video screen, and the percentage value was determined by computerized morphometry.

**TD/Cystic Index**

Kidney sections from organs collected 6 wk after IR were mounted on slides and stained with H&E. Ten cortical and medullary grid fields (each with 37,000 μm² in area) of each left kidney were evaluated. The TD/cyst quantification was performed using a screen with dots distant one from another by 13,625 μm. The measures were obtained by counting the number of dots located in the tubular lumen. The degree of TD was defined by the number of dots in the lumen, following previously suggested criteria: (1) TD1, dilation >13,625 μm but including only one dot; (2) TD2, two dots; (3) MC, three to nine dots; and (4) cyst, ≥10 dots.
Statistical Analysis
The data were preanalyzed by the K-S test. When parametric, they were compared using two-way ANOVA, with the lower standard difference posttest. When nonparametric, the Friedmann test was used for longitudinal analyses, with the Student-Newman-Keuls posttest, whereas transversal analyses were performed using the Kruskal-Wallis test, with the Student-Newman-Keuls posttest. Because the tables were built on the basis of transversal analyses, the data were presented according to the test used in the corresponding analysis. The residual cortex damage analysis (Jablonski index) and the Mac-2 analysis were carried out using the nonpaired t test. The p21, Colla1, and Colla2 real-time RT-PCR evaluation and the 35-min-IR BUN and S_c analyses, in turn, were performed with the nonpaired Mann-Whitney test. The 35-min survival comparison was analyzed using the Kaplan-Meier survival curve. The PCNA, TUNEL, and p21 immunostaining analyses were carried out using the χ² proportion test. The interstitial expansion index and the TD/cystic index analyses were performed using the nonpaired Mann-Whitney test. The tests were applied using Prism 4.00 (GraphPad Software, San Diego, CA) and Sigma-Stat (SPSS, Chicago, IL). We accepted an α error =5% to reject the null hypothesis.

ACKNOWLEDGMENTS
This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (grants 2006/52037-1 to L.F.O. and 2006/52038-2 to A.P.B. and L.F.O.) and by Laboratórios de Investigação do Estado de São Paulo (grants 2006/52037-1 to L.F.O. and 2006/52038-2 to A.P.B.). We thank Thales de Brito, MD, PhD, for technical suggestions, Isaac de Castro, PhD, for valuable help in the statistical analyses, Denise Malheiro, MD, for help in electron microscopy analyses, Rildo Volpini, PhD, for technical help, and Elia Caldini, PhD, for technical suggestions. We also thank Mirian Boim, PhD, and Carine Arnoni for helpful assistance with real-time RT-PCR.

DISCLOSURES
None.

REFERENCES
22. Boletta A, Qian F, Onuchic LF, Bhunia AK, Phakhdeekitcharoen B, Hanaoka K, Guggino W, Monaco L, Germino GG: Polycystin-1, the


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