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Original Article

Polycystin-2 expression is increased following experimental ischaemic renal injury

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Abstract

Background. Mutations in *PKD2* account for 15% of patients with autosomal dominant polycystic kidney disease. Expression of the *PKD2* protein, polycystin-2, is developmentally regulated, suggesting a major role for this protein during nephrogenesis. However, the regulation of polycystin-2 expression in the adult kidney has not been previously explored.

Methods. We have utilized an established model of renal ischaemic injury to study polycystin-2 expression in adult rat kidney for up to 120 h following ischaemia.

Results. Our results indicate that polycystin-2 expression is increased in the post-ischaemic kidney by up to 5-fold, with a peak in expression at 48 h reperfusion. This time course mirrored the increase in cell proliferation observed. In the non-ischaemic kidney, polycystin-2 expression was highest in distal nephron segments but faint proximal tubular staining was also observed. No expression was seen in glomeruli. In the ischaemic kidney, polycystin-2 expression was greatly increased but the increase in expression was not restricted to segments with the highest number of proliferating cells. Moreover, polycystin-2 was detectable mainly intracellularly following ischaemia. Consistent with this, polycystin-2 was completely sensitive to endoglycosidase H during renal recovery, suggesting that it remains largely retained within the endoplasmic reticulum under these conditions.

Conclusions. Our results provide the first evidence that polycystin-2 is increased following renal ischaemia, but show that this increase is not restricted to actively proliferating cells. The increase in polycystin-2 may relate instead to the process of cellular repair or differentiation following injury.

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited human renal disease affecting ~10% of patients worldwide on renal replacement therapy. The cardinal feature of ADPKD is the formation of multiple renal and non-renal cysts from epithelial structures. Mutations in two genes, *PKD1* (85%) and *PKD2* (15%) account for nearly all patients with ADPKD. Recent studies suggest that the *PKD1* protein, polycystin-1, may have a multifunctional role in regulating cell adhesion, differentiation, proliferation and cation transport. Polycystin-2, the *PKD2* protein, has been shown to reconstitute non-selective Ca^{2+} channel activity either independently or in association with polycystin-1 [1,2].

Pkd1- and *Pkd2*-deficient mice develop severe renal cystic disease and die *in utero* [3,4]. Epithelial formation appears to proceed normally in these animals until embryonic day 15.5 (E15.5) when cysts start to appear just as the tubules begin to elongate. During nephrogenesis, the expression of both proteins is increased particularly in mature tubular epithelium. These findings suggest that polycystin-1 and polycystin-2 play a major role in epithelial morphogenesis during kidney development.

Immunolocalization studies have shown both overlapping and distinct expression patterns for polycystin-1 and polycystin-2 in the kidney and other tissues [5,6]. In addition, other studies have suggested that polycystin-2 expression may be much higher than that of polycystin-1 in adult kidney [7,8]; the functional significance of this finding is unclear. Nevertheless, the factors that regulate polycystin-2 expression in adult kidney have not been previously investigated.

The rat ischaemia/reperfusion model is a well-established model of reversible experimental renal injury characterized by tubular cell death, injury and regeneration. The recovery process has been described as one that recapitulates kidney development 'in reverse' [9]. We have tested the hypothesis that polycystin-2 plays a role in the process of renal repair and regeneration in adult rat kidney following ischaemic injury.

Subjects and methods

Animals and experimental model

Mature male Sprague–Dawley rats (200–250 g, $n=3$ control group, $n=3$ per ischaemia reperfusion time point) were housed at 21°C, 12 h light/dark cycle, and allowed free access to food and water. Under Fluothane™ (Zeneca, UK) anaesthesia, the left renal artery and vein was exposed using a flank incision, and clamped for 45 min. Following ischaemia, the clamped kidney was reperfused for 2, 6, 24, 48 and 120 h. After the stated time of reperfusion the ischaemic (left, I) and non-ischaemic (right, NI) kidney were removed and tissue from each kidney either snap frozen in liquid nitrogen or fixed with 10% neutral buffered formalin. Fixed tissue was embedded in paraffin and 5 mm sections cut for normal histology and immunohistochemistry.

Affinity purification

The generation of a specific antisera, p30, to a fusion protein containing the C-terminal 258 amino acids of human polycystin-2, has been described in a previous publication [5]. This antibody (afp30) was further affinity purified to the same fusion protein coupled to cyanogen bromide (CNBr)-activated sepharose beads (Sigma, UK) according to the manufacturer's instructions. As with p30, afp30 was found to recognize both native and recombinant human polycystin-2 by western blotting, immunoprecipitation and dual immunofluorescence [10].

Immunoblotting

Total cellular membrane fractions from rat kidney were prepared and processed for western blotting using a modification of a method previously described for human kidney [11]. In brief, tissue homogenates were prepared from frozen rat kidneys as before and membranes isolated in a similar manner except that a sucrose cushion step was omitted [11]. In preliminary studies, p30 and afp30 were found to recognize native rat polycystin-2. Band intensities were quantified using a Bio-Rad GS-690 scanning densitometer using Molecular Analyst version 4 software and adjusted to that of the α1 subunit of Na^+-K^+-ATPase as a control for protein loading and integrity.

Glycosylation analysis

Endoglycosidase H (EndoH) is an enzyme that specifically cleaves simple high-mannose type oligosaccharides (inserted in the ER) from the protein backbone, and resistance to EndoH is typically acquired as the protein traffics through the Golgi undergoing further post-translational modification of attached carbohydrate residues to form more complex sugars [10]. Membrane fractions from kidney or cultured cells were solubilized in 1% SDS, 10 mM Tris–HCl (pH 7.5) supplemented with a Complete™ mini protease inhibitor cocktail (Boehringer Mannheim). Lysates corresponding to 10–50 mg of total protein were treated with Endo H following the manufacturer's protocols (Boehringer Mannheim). The reactions were performed for 2 h at 37°C with 500 U of Endo H in the appropriate buffer in 40 ml of total reaction volume. After incubation, an equal volume of 2×SDS sample buffer was added to each reaction, and incubated at 50°C for 30 min prior to SDS–PAGE and western blot analysis as previously described [10].

Immunohistochemistry and immunofluorescence

Immunohistochemistry for rat polycystin-2 and proliferating cell nuclear antigen (PCNA) were performed as previously described [5]. An affinity-purified monospecific antibody raised to the C-terminal epitope of human polycystin-2 (afp30) and a commercial mAb (PC10) raised to proliferating cell nuclear antigen (PCNA) (Dako, UK) were used as primary antibodies. Controls included sections stained with the primary antibody omitted or using a non-immune rabbit IgG fraction. The proliferative index (PI, %) was assessed by counting PCNA positive cells in 12 fields (×200) of a section of ischaemic and non-ischaemic cortex or medulla at each time point. Each field contained at least 60 cells.

Cell culture

COS-1 cells were cultured in DMEM/F12 medium (Gibco, Life Technologies, CA) supplemented with 10% fetal calf serum (FCS) at 5% CO_2 in a 37°C incubator. Cells were first plated in 100 mm dishes (immunoblotting) or glass coverslips (PCNA). After 24 h, they were rendered quiescent by a further 48 h incubation in serum-free DMEM/F12 supplemented with apo-transferrin (5 μg/ml) before serum containing medium was replaced. Cells were harvested at 0, 24 and 48 h and solubilized in 1% SDS for immunoblotting or fixed with neutral buffered formalin (10%) for PCNA staining.

Statistical analysis

All data are expressed as mean ± SEM. Differences between control and experimental groups were assessed using the Student's *t*-test and the null hypothesis was rejected at $P<0.05$.

Results

Polycystin-2 expression in normal rat kidney

p30 recognized a band of ~110 kDa corresponding to monomeric polycystin-2 in membranes prepared from normal rat kidney cortex and medulla. Expression of polycystin-2 appeared to be approximately 2-fold higher in medulla compared with cortex by densitometry (Figure 1A).

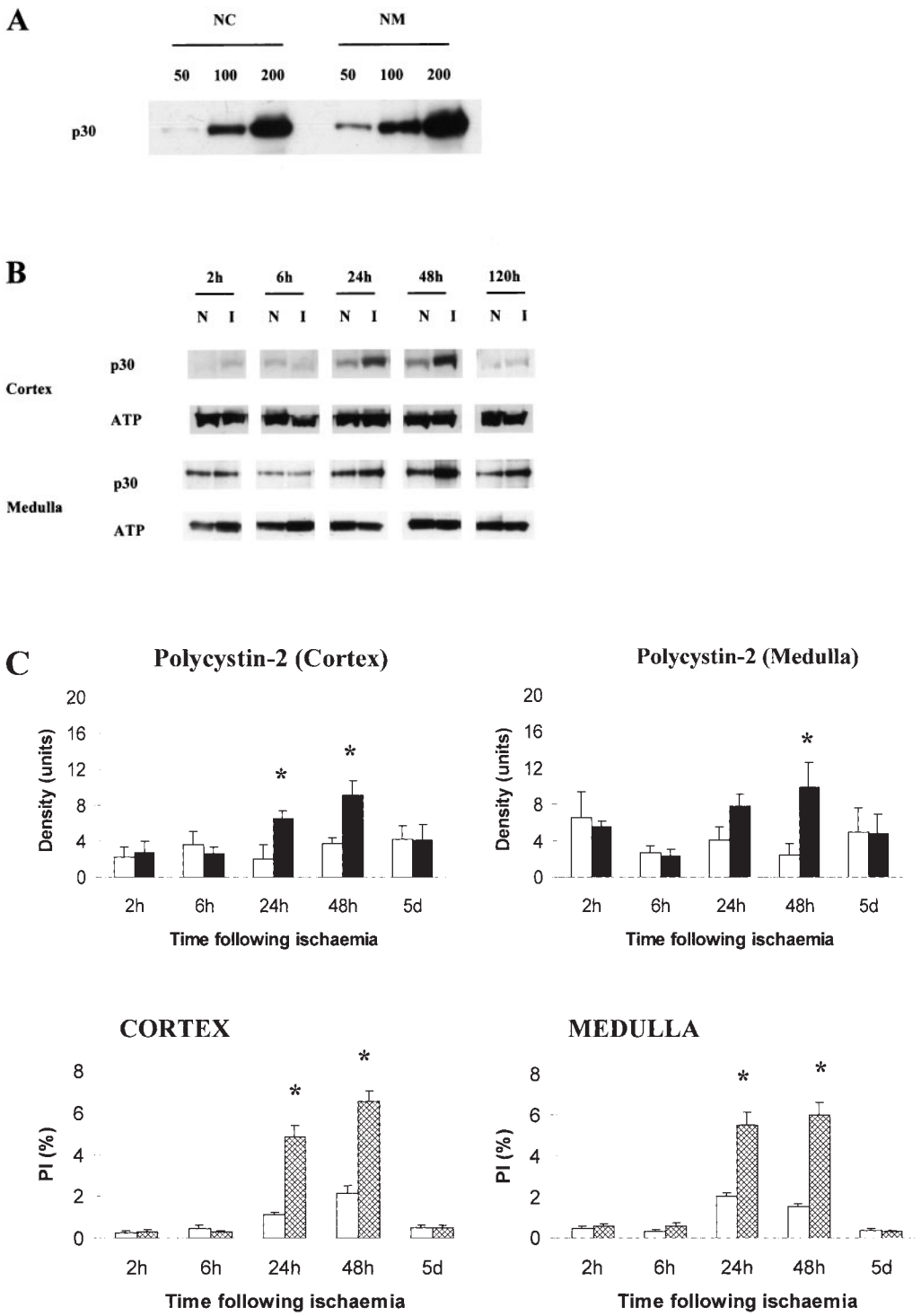

Fig. 1. Polycystin-2 expression in normal and ischaemic rat kidney. p30 was used to detect polycystin-2 expression by immunoblotting. (**A**) Normal cortex (NC) and normal medulla (NM) from a representative animal ($n=3$). Kidney membranes were loaded at 50, 100 and 200 μg per lane. Pre-immune sera showed no signal (not shown). (**B**) Non-ischaemic (N) and ischaemic (I) cortex and medulla from a representative animal ($n=3$). Kidney membranes were loaded at 100 μg per lane (cortex) or 50 μg per lane (medulla). The α1 subunit of Na^+-K^+-ATPase (ATP) was used as a control for protein loading and integrity. (**C**) The mean changes in p30 expression for all three animals as measured by densitometry (adjusted for α1 subunit of Na^+-K^+-ATPase expression) are shown in the graphs (upper left and upper right). The white columns represent non-ischaemic kidneys and the black bars represent ischaemic kidneys. Changes in PCNA expression expressed as the PI (%) over the same duration from one representative animal are shown in the lower left (cortex) and lower right (medulla) graphs. The white bars represent values from non-ischaemic kidney and the hatched bars, values from ischaemic kidneys. $^*P<0.05$ when compared to the non-ischaemic control at the same time point. Bars indicate SEM.

Polycystin-2 expression in post-ischaemic rat kidney

Polycystin-2 levels were increased by up to 5-fold in both cortex and medulla in the post-ischaemic kidney at 48 h, returning to baseline levels by 120 h (Figure 1B). This rise was significant for cortex at 24 h and just non-significant for medulla at 24 h ($P = 0.07$) but was significant for both cortex and medulla at 48 h. In contrast, levels of the α1 subunit of Na^+-K^+-ATPase showed no detectable change following renal ischaemia at all time points measured.

Localization of polycystin-2 in the normal and post-ischaemic kidney

The expression of polycystin-2 was examined by immunohistochemistry in normal, non-ischaemic and post-ischaemic kidney tissue using afp30 (Figure 2). As previously found in normal human kidney, polycystin-2 expression in normal rat kidney cortex was detectable mainly in the distal convoluted tubules and cortical collecting ducts [5]. Weak expression was found in proximal tubules but glomeruli were negative. In normal rat medulla, weak expression was detected in the collecting ducts and thick ascending limbs. Following ischaemia, expression was increased in all nephron segments. Moreover, the localization of polycystin-2 remained predominantly intracellular with no clear visualization of surface membrane expression.

The level of polycystin-2 immunoreactivity in the post-ischaemic kidney mirrored that found on immunoblotting with peak expression observed at 48 h post-ischaemia. The proliferative index as measured by PCNA-positive cells was significantly increased in the ischaemic (I) compared to the non-ischaemic (NI)

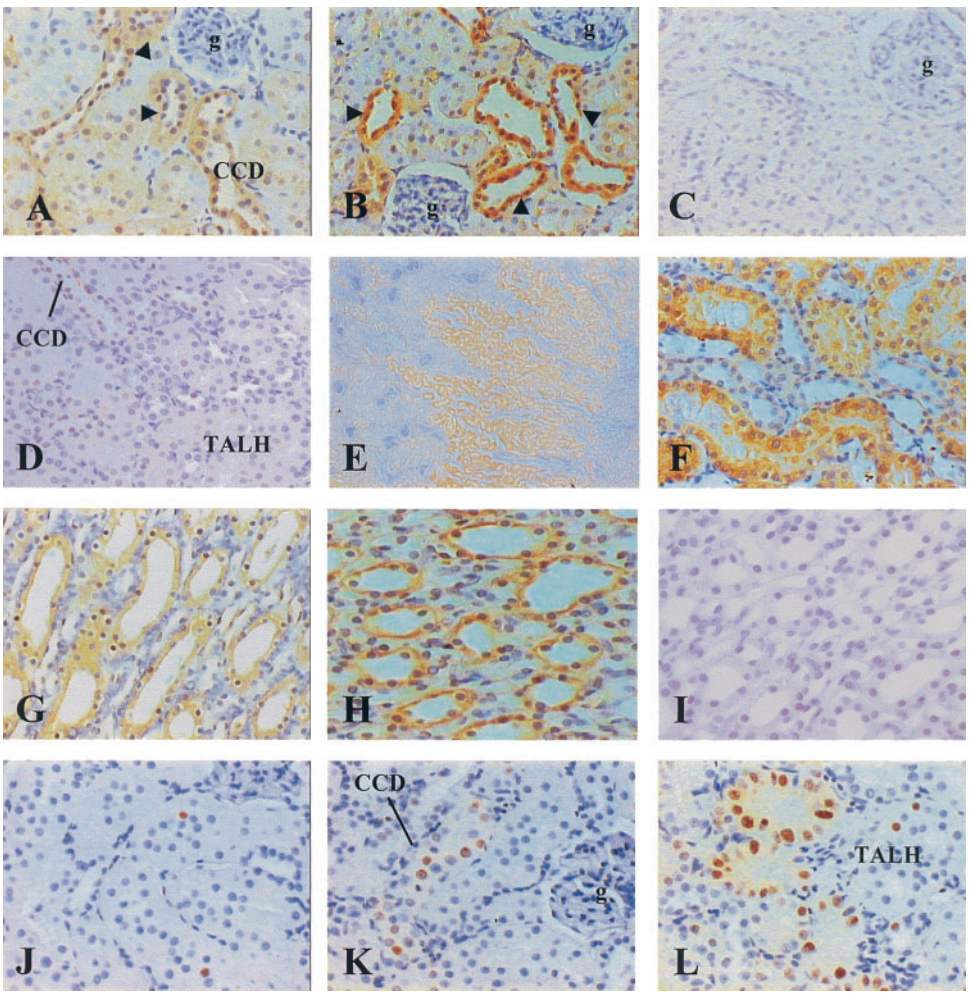

Fig. 2. Polycystin-2 (**A–I**) and PCNA (**J–L**) expression in ischaemic and non-ischaemic kidney. In normal rat kidney cortex (A), polycystin-2 expression is just detectable in the proximal tubules but is more prominent in the distal convoluted tubules (arrows) and cortical collecting ducts (CCD). No expression was seen in glomeruli (g). At the corticomedullary junction, faint expression was also seen in the thick ascending limbs (TALH) (D) and in the medullary collecting ducts (G). In ischaemic kidney at 48 h (B), expression was increased in the distal convoluted tubules (arrows) and cortical collecting ducts with patchy expression in the proximal tubules. Expression was markedly increased in the thick ascending limbs (E,F) and medullary collecting ducts (H). Non-immune rabbit IgG showed no detectable expression in the cortex (C) or medulla (I). In non-ischaemic kidney cortex (J) and medulla, only occasional PCNA-positive cells were found. At 48 h post-ischaemia, there was a 6-fold increase in PCNA positive nuclei in ischaemic cortex (K) and medulla (L). Original magnification ×400 for all except for E (×100).

kidney at 24 h and 48 h in both cortex (24 h NI = 1.1%, 24 h I = 4.9%, 48 h NI = 2.2%, 48 h I = 6.6%) and medulla (24 h I = 2%, 24 h I = 5.5%, 48 h N = 1.5%, 48 h I = 6%) (Figure 1C). As can be seen from Figure 1C, the time course and magnitude of PCNA expression mirrors that of polycystin-2 expression in the ischaemic kidney. However, unlike the widespread increase in polycystin-2 expression by all tubular segments, PCNA-positive cells were mostly found in tubular segments adjacent to the corticomedullary junction: these are most likely to represent S3 segments and thick ascending limbs.

Glycosylation analysis of polycystin-2 in the post-ischaemic kidney

The glycosylation profile of polycystin-2 was analysed in the post-ischaemic kidney using the glycosidase enzyme, EndoH. As shown in Figure 3A, polycystin-2 remained fully EndoH sensitive at 48 h in the post-ischaemic as in the non-ischaemic kidney even though its absolute level was increased. No change in EndoH sensitivity could be demonstrated at either earlier or later time points following renal ischaemia. The band shift observed was specific to EndoH activity rather than non-specific protease digestion since stripping and reprobing of the same membranes for the unglycosylated α1 subunit of Na^+-K^+-ATPase showed no change in molecular weight at each time point.

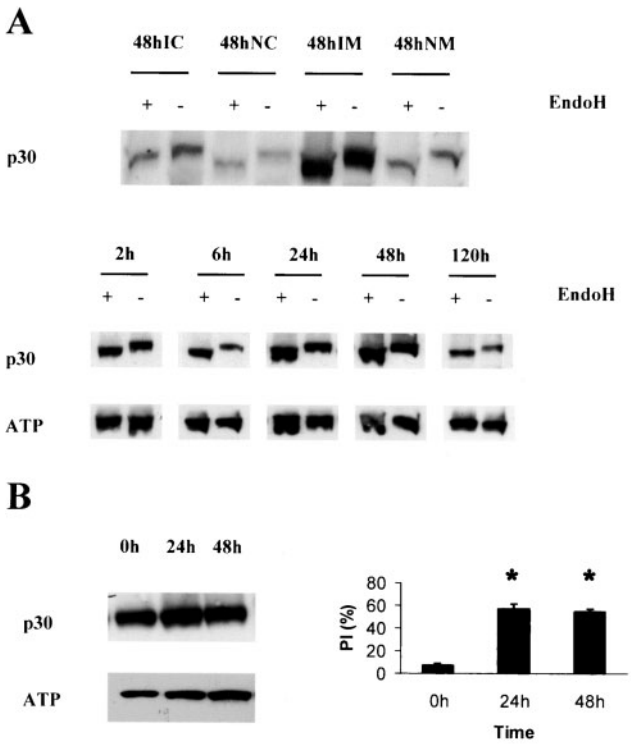

Fig. 3. (**A**) Glycosylation analysis of endogenous polycystin-2 following renal ischaemia. Although polycystin-2 expression was maximal at 48 h following ischaemia, no change in EndoH sensitivity was detected in ischaemic cortex (IC) or medulla (IM) compared to the contralateral non-ischaemic control cortex (NC) or medulla (NM). Similarly, no change in EndoH sensitivity for polycystin-2 could be detected in ischaemic medulla at earlier periods of reperfusion. Kidney membranes were pre-treated (+) with EndoH or enzyme buffer (–), precipitated and loaded at 100 µg per lane (cortex) or 50 µg per lane (medulla). The α1 subunit of Na^+-K^+-ATPase (ATP) was used as control for protein loading and integrity. No change in molecular weight was detected for the latter after EndoH treatment, as it is unglycosylated. (**B**) Polycystin-2 (p30) and PCNA expression (PI) in quiescent (0 h) and proliferating (24 h, 48 h) COS-1 cells. The α1 subunit of Na^+-K^+-ATPase (ATP) was used as control for protein loading and integrity. $^*P < 0.05$ compared to quiescent cells. Bars indicate SEM.

The effect of proliferation on polycystin-2 expression in vitro

To test directly whether polycystin-2 expression was increased in proliferating cells, total cellular polycystin-2 was measured by immunoblotting and compared to changes in PCNA expression by immunostaining. Following stimulation with 10% FCS, PCNA expression (expressed as PI, %) was increased by 8-fold over quiescent COS-1 cells by 24 h (Figure 3B). In contrast, no difference in polycystin-2 levels was observed at 24 h or 48 h between quiescent COS-1 cells and those stimulated to proliferate (Figure 3B). Identical results were found with a human proximal tubular cell line, CL11 (not shown).

Discussion

The identification of the two genes mutated in ADPKD, *PKD1* and *PKD2*, have been major steps in facilitating the study of the cellular and molecular basis of this common kidney disease. Nonetheless, the functions of the two proteins encoded, polycystin-1 and polycystin-2, have not been fully clarified. A native polycystin-1/polycystin-2 complex has been identified [10], and polycystin-2 either alone or co-expressed with polycystin-1 can reconstitute a non-selective Ca^{2+} channel [1,2].

Our results demonstrate that polycystin-2 expression is increased during the recovery phase after experimental renal injury. We observed an increase in both cortex and medulla detectable by 24 h, peaking at 48 h and which returned to baseline by 120 h. The increase in medullary polycystin-2 at 24 h just failed to reach statistical significance ($P = 0.07$). We do not think this reflects a true pathophysiological difference between cortex and medulla; rather it is likely that a significant increase could have been detected by using a greater number of animals. Unfortunately we were unable to measure changes in polycystin-1 in the same experiment, as our polycystin-1 antibodies did not cross-react with rat polycystin-1 (not shown). The expression pattern of polycystin-1 in rat kidney following ischaemia has been reported to be increased, although polycystin-2 was reported to be unchanged following ischaemia in the same study [12]. Neo-expression of immunoreactive polycystin-1 by regenerating proximal tubules in human kidney has been observed [13].

Since polycystin-2 expression is less segment restricted in fetal kidney than in the adult, it had been postulated that some of the discrepancies in nephron segment localization of polycystin-2 might relate to the state of cellular quiescence, proliferation or differentiation [5,14,15]. Although the magnitude and time course of increased polycystin-2 expression mirrored that seen for cellular proliferation as measured by PCNA-positive cells, we did not detect a change in segment localization of immunoreactive polycystin-2 following renal ischaemia. Moreover, we did not find a direct correlation between polycystin-2 expression and PCNA expression in cultured kidney cells, even though the magnitude of increase in PCNA expression (8-fold, Figure 3B) was comparable to that seen in the post-ischaemic kidney (6-fold, Figure 1C). This suggests that the increase in polycystin-2 observed *in vivo* may instead relate to a different cellular process such as repair or differentiation. The increases we observed in the post-ischaemic kidney were also highest in the same segments that expressed the highest levels in normal kidney. No clear evidence of surface-membrane staining was observed although this does not rule out the existence of a minor population of surface-bound polycystin-2 [10].

At present, the major site of polycystin-2 location and action remains controversial. There is strong biochemical evidence favouring an exclusive ER location for polycystin-2 in both cultured cells and native kidney [16]. However, polycystin-2 immunoreactivity has also been noted at the basolateral cell surface of normal tubules in mouse, rat and human kidney, suggesting that it may be expressed at the cell surface under certain conditions [4,5,17]. In support of this, polycystin-2 has been identified in Golgi and plasma-membrane fractions isolated both from native kidney and cultured kidney cells in more recent studies [10,18]. Thus there may be increased recruitment of polycystin-2 to the plasma membrane under conditions of increased cell adhesion or membrane turnover, possibly in association with polycystin-1. To assess if polycystin-2 might have translocated to the plasma membrane at any point following ischaemia, we utilized sensitivity to the endoglycosidase enzyme EndoH as a biochemical assay of the subcellular membrane location of polycystin-2. Resistance to EndoH is typically acquired as a protein undergoes post-translational modification of carbohydrate residues in the Golgi, and an Endo H-resistant protein has potentially undergone maturation through the Golgi and could potentially be targeted to the plasma membrane. Conversely, proteins that are retained in the ER are typically Endo H sensitive (although there are some exceptions) [10]. Our results indicate that polycystin-2 remains completely EndoH sensitive following renal ischaemia and this is consistent with its predominant intracellular location as visualized by immunohistochemistry. We also confirmed previous findings that both endogenous and recombinant polycystin-2 are EndoH sensitive and mainly localized to the ER [10,16].

In conclusion, our results demonstrate for the first time that polycystin-2 expression is significantly upregulated in adult rat kidney following ischaemia–reperfusion injury. However, a direct correlation between polycystin-2 expression and cellular proliferation was not found. One possibility is that polycystin-2 may play an important role in other processes that are prominent after injury, such as cellular repair or differentiation. An alternative, but not exclusive possibility, is that it may act as a suppressor of cellular proliferation. Cystic epithelial cells, both from ADPKD patients and rodent models, are hyperproliferative and display abnormal upregulation of several proto-oncogenes, including c-erB-2, c-Fos, c-Ki-ras, and c-myc. These findings suggest that polycystin-1 and polycystin-2 function in part as tumour suppressor genes, controlling proto-oncogenes and cellular programmes that direct cell cycle progression and cellular differentiation [19]. If this is the case, then the up-regulation of polycystin-2 may act as a brake on unwanted cell proliferation to allow the proliferative index of the ischaemic kidney to return to baseline. It has been reported that polycystin-2 stimulates the phosphorylation of c-Jun and the induction of AP-1 activity through signalling molecules partially distinct from those involved in polycystin-1-mediated activation [20]. It is therefore possible that polycystin-2 could act both dependently and independently of polycystin-1 in regulating cell proliferation and differentiation [19,20]. Future studies should address how polycystin-2 expression and activity are regulated following cellular injury.

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