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Chronic treatment with tempol does not significantly ameliorate renal tissue hypoxia or disease progression in a rodent model of polycystic kidney disease

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Running title: Oxidative stress and hypoxia in polycystic kidney disease

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Abstract

1. We tested whether polycystic kidney disease (PKD) is associated with renal tissue hypoxia and oxidative stress, which in turn contribute to the progression of cystic disease and hypertension.

2. Lewis polycystic kidney (LPK) rats and Lewis control (Lewis) rats were treated with tempol (1 mM in drinking water) from 3-13 weeks of age, or remained untreated.

3. LPK rats developed polyuria, uremia and proteinuria. At 13 weeks of age LPK rats had greater mean arterial pressure (1.5-fold), kidney weight (6-fold) and plasma creatinine (3.5-fold) than Lewis rats. LPK kidneys were cystic and fibrotic.

4. Renal hypoxia was evidenced by staining for pimonidazole adducts and hypoxia-inducible factor-1α (HIF-1α) in cells lining renal cysts, and up-regulation of HIF-1α and its downstream targets vascular endothelial growth factor (VEGF), glucose transporter-1 (Glut-1), and heme-oxygenase 1 (HO-1). Total HO activity, however, did not differ greatly in LPK compared to Lewis kidney tissue.

5. Renal oxidative/nitrosative stress was evidenced by 9-fold greater immunofluorescence for 3-nitrotyrosine in LPK kidney tissue than Lewis and more than 10-fold upregulation of mRNA for p47phox and gp91phox. Total renal superoxide dismutase (SOD) activity was 7-fold less, while SOD-1 mRNA expression was 70% less, in LPK than Lewis kidney tissue. In LPK rats, tempol treatment reduced immunofluorescence for 3-nitrotyrosine and mRNA for HIF-1α, while up-regulating VEGF and p47phox mRNA expression, but otherwise had little impact on disease progression, renal tissue hypoxia and hypertension.

6. Our findings do not support the hypothesis that oxidative stress drives hypoxia and disease progression in PKD.

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Keywords: heme-oxygenase, hypoxia, hypoxia inducible factors, nitrotyrosine, oxidative and nitrosative stress, polycystic kidney disease, superoxide dismutase.
Introduction

Polycystic kidney diseases (PKD) are common genetic conditions characterized by gradually
developing parenchymal cysts and nephromegaly.\textsuperscript{1, 2}. They account for 7-10% of all patients on
dialysis and present as the fourth most common form of end stage renal disease\textsuperscript{1, 2} Current
therapeutic approaches for cystic kidney disease (e.g. those targeting the renin-angiotensin system)
are chiefly aimed at control of hypertension and its deleterious effects on the progression of kidney
disease, rather than the primary disease process; cyst formation.\textsuperscript{1}

Clinical and experimental studies of various forms of acute kidney injury and chronic kidney
disease have provided evidence to support a role of a vicious cycle of tissue hypoxia and oxidative
stress as a final common pathogenic pathway. Tissue hypoxia has been detected in virtually all
forms of clinical and experimental kidney disease in which it has been assessed.\textsuperscript{3-6} We are aware of
no direct measurements of renal tissue oxygen tension (PO\textsubscript{2}) in PKD, but indirect evidence of
hypoxia comes from observations of intense pimonidazole staining and activation of hypoxia
inducible factors (HIFs) in animal models of PKD.\textsuperscript{7-9} Tissue hypoxia in PKD is likely due partly to
reduced microvascular perfusion, as evidence by observations of reduced total renal blood flow\textsuperscript{10-13}
and structural microvascular abnormalities\textsuperscript{14-16} in human PKD.

In turn, tissue hypoxia stimulates the progression of kidney disease. For example, hypoxia, through
multiple HIF signaling pathways, stimulates epithelial-to-mesenchymal transition and
fibrogenesis.\textsuperscript{17, 18} Hypoxia also reduces the anti-inflammatory influence of peroxisome proliferator-
γ activation\textsuperscript{19} and can induce generation of reactive oxygen species.\textsuperscript{20} It is also plausible that renal
tissue ischaemia and/or hypoxia could contribute to development of hypertension through
stimulation of renal afferents.\textsuperscript{21}
There is strong evidence of oxidative stress in both human PKD and animal models of PKD. Oxidative stress can directly promote progression of renal disease by stimulating apoptosis, inflammatory cell infiltration, extracellular matrix synthesis and cellular proliferation. It can also exacerbate development of hypoxia by promoting fibrosis and through reduced nitric oxide bioavailability. Nitric oxide normally inhibits sodium reabsorption and mitochondrial respiration, so oxidative stress can increase renal oxygen consumption. Thus, as in other forms of chronic kidney disease, there is potential for a vicious cycle of oxidative stress and hypoxia to exacerbate disease progression in PKD. This is supported by the observation that inhibition of synthesis of the antioxidant glutathione exacerbated progression of disease in the Han:SPRD model of PKD. A range of agents which have pleiotrophic actions including antioxidant effects have also been shown to have beneficial effects in animal models of PKD, including the lipophilic phenol probucol. We hypothesized that PKD is associated with an increase in renal tissue oxidative stress and hypoxia. We further considered that if this hypothesis is true, administration of the antioxidant superoxide dismutase (SOD) mimetic tempol should break the vicious cycle of oxidative stress and tissue hypoxia, thereby reducing hypoxia-mediated progression of renal disease and development of hypertension.

We have undertaken these studies in the Lewis PKD (LPK) rat and the corresponding Lewis control (Lewis) rat. The LPK model is a model of nephronophthisis 9 (NPHP9), arising from a single nucleotide polymorphism in the NIMA (never in mitosis gene a)- related kinase 8 (Nek8) gene on chromosome 10. LPK rats develop renal cysts from 3 weeks of age and as with human PKD, hypertension and sympathetic overactivity, reduced arterial compliance and cardiac hypertrophy are key features as the disease progresses. We assessed tissue hypoxia by analysis of the formation of pimonidazole adducts, an acknowledged marker of severe tissue hypoxia, and expression of hypoxia-inducible factor-1α (HIF-1α) and its downstream targets. Oxidative stress
was assessed through expression of critical subunits of NADPH oxidase, the major source of renal superoxide production; SOD-1, which is responsible for reactive oxygen species dismutation; expression of heme-oxidase-1 (HO-1), a cellular protective enzyme, the expression of which is enhanced by oxidative stress; and levels of 3-nitrotyrosine as a marker of oxidative/nitrosative stress. Renal pathology was assessed both histologically and functionally, and arterial pressure was determined to allow assessment of the antihypertensive efficacy of chronic tempol treatment in the LPK model.

Methods

Study design
All experimental procedures were approved by the Animal Ethics Committee of the School of Biomedical Sciences, Monash University (reference 2009/84), and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Three week old male Lewis rats (70 ± 1 g, n = 10) and LPK rats (64 ± 3 g, n = 10) were obtained from the Animals Resources Centre, Perth Australia and housed in the Monash University Large Animal Facility at an ambient temperature of 21-23°C with a 12-hour light, 12-hour dark cycle. Food (Barastoc, product code 8720610, Australia) and water were available ad libitum. Five rats of each genotype were treated with 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol, Aldrich Chemistry, St Louis, MO, USA, 1 mM in drinking water) from 3-13 weeks of age. Body weight was measured weekly throughout the study. At 13 weeks of age, arterial pressure was measured in the conscious rat and renal tissue was collected at post-mortem for subsequent analysis. Unless otherwise stated, reagents were purchased from Sigma Aldrich (Australia).

Because the experimental protocol described above required two bouts of anaesthesia and injection of pimonidazole prior to tissue collection (see details below), an additional 6 untreated Lewis and 7
untreated LPK rats were euthanized at 10 weeks of age, by decapitation under brief isofluorane anaesthesia (5% v/v in 100% O₂, Isoflo, Abbot, Australasia, Kurnell, Australia). This enabled us to determine whether these pre-treatments confounded measurement of renal gene expression and antioxidant enzyme activity in the primary arm of the study.

**Metabolic cage studies**
At 4, 7 and 12 weeks of age the rats were housed individually in metabolic cages for 24 h to allow measurement of food and water intake and collection of urine. Two days prior to each metabolic cage study, rats were separated from their group and housed in individual cages to acclimatise them to isolation. Urine samples were centrifuged for 10 min at 3,000 g and the supernatant frozen at -20°C for later analysis.

**Terminal measurements**
At 13 weeks of age, arterial pressure was measured in conscious rats via tail artery catheterization as previously described. In brief, the catheter was inserted under brief anaesthesia (isoflurane, 1-4% v/v in 100% O₂, Isoflo). One hour later, the catheter was connected to a pressure transducer (Cobe, Arvada, USA) and bridge amplifier (QA1, Scientific Concepts, Mount Waverley, Australia) interfaced with a computer running a LabView-based data acquisition program (Universal Acquisition, University of Auckland). Mean arterial pressure and heart rate were recorded for 60 min, after which 2-pimonidazole (60 mg/kg, Hydroxyprobe Inc, U.S.A) was administered i.p. Sixty minutes after pimonidazole injection, rats were anaesthetized with pentobarbitone sodium (60 mg/kg i.p.). Once a surgical level of anaesthesia was established, blood (1 mL) was drawn from the catheter placed in the tail artery, and after centrifugation at 3,000 x g for 10 min, plasma was stored at -20 °C for later analysis using previously described techniques.
Kidney perfusion and tissue collection
Immediately after collection of arterial blood (above), a midline incision was made to expose the rat’s abdominal aorta and inferior vena cava, to allow retrograde perfusion of the abdominal aorta. The kidneys were cleared of blood by perfusion for 30 s with ice-cold 0.1 M phosphate buffered saline (PBS, pH 7.4) at a pressure of 150 mmHg for Lewis rats and 200 mmHg for LPK rats. The left renal artery and vein were then ligated and the kidney removed, decapsulated, weighed, snap-frozen in liquid nitrogen, and stored at -70º C for later analysis. The right kidney was then perfusion fixed with 300-500 mL of ice-cold 4% w/v paraformaldehyde (PFA) and post-fixed in a 4% PFA solution at 4º C overnight.

Renal histopathology
Perfusion fixed kidneys were transferred into 70% v/v ethanol and processed and embedded in paraffin. Paraffin-embedded kidneys were sectioned at 4 µm using a microtome (Leitz Wetzlar, Germany) and left to adhere to slides (Superfrost Plus, Menzel-Glaser, Germany) for 3 h at 70 ºC. The slides were dewaxed in xylene and then rehydrated through a series of graded alcohols prior to staining. Sections were stained with haematoxylin and eosin. Collagen deposition was assessed by staining with 1% w/v picrosirus red, and reticular fibers assessed by staining with Gordon’s silver salts. Slides were then dehydrated through a series of graded alcohols, cleared in xylene and then cover slipped. The stained slides were viewed under a microscope (Olympus Provis ax70, Olympus, Oakleigh, VIC) and photomicrographs were taken at magnifications ranging from 100x to 1000x.

Immunohistochemistry
Paraffin embedded PFA-fixed tissue blocks were sectioned at 5 µm. Kidney sections were deparaffinized with xylene, and hydrated in a series of graded ethanol solutions.

(1) Pimonidazole adduct straining. Antigen retrieval was performed in a boiling solution of 10 mM sodium citrate buffer which was left to cool to room temperature before washing in PBS (3 x 5
Endogenous peroxidase activity was quenched with 0.3% v/v hydrogen peroxide solution. Sections were blocked with either tris PBS solution (TPBS-Tx; 10 mM tris base, 0.1 M phosphate buffer, 0.9% w/v NaCl, pH 7.4, 0.1% v/v Triton X 100 and 0.1% w/v sodium azide) with 10% v/v donkey serum or 1% w/v bovine serum albumin (BSA) in PBS for 1 h at room temperature. Sections were incubated with a mouse primary antibody against pimonidazole (Hypoxyprobe Inc., USA; dilution 1:250), diluted in the blocking solution. As a negative control, primary antibody was omitted from the blocking solution. After incubation, the sections were washed thrice in 0.01 M PBS for 15 min. Sections were incubated in biotinylated donkey anti-mouse secondary antibody (Jackson Immunology, USA; 1:200) in TPBS and 1% v/v donkey serum at 4 °C overnight. Slides were then washed thrice in 0.01 M PBS and incubated at 4 °C overnight in Vectastain ABC reagent (Vectastain, USA). Immunoreactive products were developed using 3, 3-diaminobenzidine (DAB) (Vectastain, USA) as the chromogen. Tissues were washed, cover-slipped and examined under bright field conditions (Axio Imager, Z1; Zeiss, Germany). Images were captured with an Axiocam MR3 Digital camera (Zeiss) and adjusted for brightness and contrast with Axiovision 4.5 software (Zeiss).

(2) Immunofluorescence microscopy for 3-nitrotyrosine and HIF-1α. Detection of 3-nitrotyrosine was performed as previously described, using fresh-frozen kidney sections (10 μm) fixed in acetone for 15 min and washed in 0.01 M PBS (3 x 10 min). Tissue sections were then incubated in a Mouse on Mouse Ig blocking reagent (Vector Laboratories, Switzerland) for 1 h to reduce nonspecific binding. Detection of HIF-1α was performed using 5 μm sections of PFA-fixed tissue.

Tissue mounted sections were incubated with a mouse monoclonal anti-3-nitrotyrosine antibody (final dilution 1:100 v/v, AbCAM, England) or with anti-hypoxia inducible factor (HIF-1α; 1:500 dilution, Novus Biologicals, CO, USA) at 4°C overnight. For nitrotyrosine detection, sections were then washed in PBS (3 x 10 min) and incubated in a biotinylated anti-mouse IgG reagent for 10 min
and a Fluorescein Avidin DCS (Vector Laboratories, Switzerland). Sections labeled with HIF-1α were incubated with a secondary anti-mouse 568 antibody (1:10,000; Molecular Probes, OR, USA) for 30 min then mounted with fluorescent mounting medium (DakoCytomation, Glostrup, Denmark) and incubated with 4′-6-diamidino-2-phenylindole (DAPI, Molecular Probes) to detect cell nuclei. Sections were washed, cover slipped and photographed on a Provis fluorescence microscope (Olympus, Oakleigh, VIC). Image settings were identical for each image acquired and all the appropriate primary and secondary controls were performed. The intensity of 3-nitrotyrosine fluorescence was quantified in 3 randomly selected regions (the total area of each region was 14 mm²) from 3 randomly selected kidney sections, from each animal, using analySIS software (Soft Imaging System, Singapore) with identical measurement settings.

**Gene expression**

For RNA extraction, 30 mg of kidney was transferred into RNA lysis buffer and total RNA extracted according to the manufacturer’s instructions (Promega SV Total RNA Isolation System, Promega, WI, USA). Contaminating genomic DNA was destroyed using DNase1 as part of the extraction protocol. RNA was then eluted into MilliQ H₂O and assayed for RNA concentration and purity using a NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific, Scoresby, Vic, Aust). The AffinityScript QPCR cDNA synthesis kit (Stratagene, USA) was then used to generate cDNA using 1 µg of extracted RNA and 15 ng/µL of random primers (Agilent Affinity Script, USA) in a 50 µL reaction volume. Negative controls that did not contain either starting RNA (water controls) or RT enzyme (-RT controls) were also generated at this stage and processed concurrently with kidney RNA samples through the PCR process.

Real time quantitative polymerase chain reaction (qPCR) was performed using 1 µL of cDNA mix in a 25 µL reaction with each forward and reverse primer (300 nM final concentration) using SYBR green mastermix as provided by the manufacturer (Stratagene, Agilent Technologies, Inc., USA).
Primers used are listed in Table 1. Conditions for qPCR (40 cycles) were as follows: 95 °C for 30 s, 60 °C for 1 min, then 72 °C for 30 s. Each reaction was performed as two or three replicates and the average taken for each animal. RNA levels were normalised to cytochrome C1 (CYC1) as an endogenous control, selected from the geNorm reference gene selection kit as per the manufacturer’s instructions (Primer Design Ltd, Southampton United Kingdom UK). For all primer sets, water and -RT controls were clean for all samples, indicating lack of DNA or other sources of PCR contamination.

Two steps were undertaken to validate the specificity of PCR products. For all PCRs, a dissociation melt curve analysis was conducted, whereby a final and additional PCR cycle was conducted over the temperature range of 55°C – 95°C and the melt curve was calculated from the first derivative of the fluorescence response. All gene products yielded a single specific peak for each primer at a temperature greater than 80°C, indicating the presence of a single amplicon. Amplified fragments from selected Lewis and LPK kidney samples for all primer pairs were also run on a 2% agarose (70V, 1 h) gel post-stained with ethidium bromide and viewed under UV light in order to confirm the presence of a single amplicon that corresponded to the predicted product size.

Cycle threshold (Ct) values (representing cycle number at which fluorescence emission data exceeded a threshold limit) were determined for each gene and CYC1 Ct values were subtracted from the Ct value for each gene (to give ΔCt values). These values were then used to carry out statistical comparisons between and within strains. Fold variation was then determined using the $2^{-ΔΔCt}$ method according to published protocols$^{40}$ and as we have described previously.$^{41}$ The $ΔΔCt$ values (difference in ΔCt values between a chosen reference and test sample) were calculated, using vehicle-treated Lewis animals as the reference group (equal to 1) and applying the $2^{-ΔΔCt}$ formula. Range values were then determined using the formula $2^{(\Delta C_t \pm SD \Delta C_t)}$.$^{40}$ For the 10-week old untreated
group, Lewis rats served as the reference group (equal to 1) for the LPK animals. Data are presented as normalised values and fold variations.

**Antioxidant enzyme activity**

*Superoxide dismutase activity:* The total SOD activity assay is based on the ability of SOD to inhibit auto-oxidation of pyrogallol at alkaline pH and has been described in detail previously. Increased SOD activity is therefore inferred by lesser rates of pyrogallol auto-oxidation and vice versa. In brief, kidney homogenate was subjected to three cycles of freeze-thawing in liquid nitrogen and 2 µL of each lysate was loaded in duplicate into a 96-well plate and diluted to a final volume of 20 µL. SOD protein standard was prepared with known concentrations of SOD protein in duplicate. A pyrogallol stock solution (0.2 M) was prepared and degassed with nitrogen for 1 h to remove all dissolved oxygen. Tris-cacodylic acid buffer (50 mM pH 8.2) was prepared in diethylenetriamine penta-acetic acid (DPTA) and equilibrated for 1 h at room temperature before use. To each well, 180 µL of 0.2 mM Tris-cacodylic acid buffer was added and pyrogallol auto-oxidation was determined from rate of absorbance at 405 nm measured 0, 5, 15, 20, 25, 30, and 35 min later. Pyrogallol auto-oxidation was normalized to protein concentration, determined with bicinechonic acid as described by the manufacturer’s notes (Sigma-Aldrich).

*Total heme oxygenase activity:* Determination of total HO activity in kidney homogenate was based on the yield of bilirubin per mg microsomal protein produced per min from freshly isolated rat liver microsomes. Kidney tissue (90-130 mg) was suspended in 1 mL of lysis buffer (50 mM PBS, pH 7.4 containing 1 mM EDTA, 10 mM BHT and a Protease Inhibitor Cocktail Tablet (Roche Diagnostics, Switzerland)) and homogenized for 5 min in a glass tube with a rotating piston at 500 rpm (Wheaton, USA). The kidney homogenate was subjected to three cycles of freeze-thawing in liquid nitrogen and then centrifuged at 5000 x g at 4 °C for 5 min. To remove any solid material, the supernatant was taken and centrifuged twice more at 5000 g at 4 °C for 5 min. To isolate the
microsomal fraction, the clarified supernatant was mixed with buffer A (250 mM sucrose; 20 mM Tris pH 7.4) and centrifuged at 100,000 x g at 4 °C for 1 h using an Optima™ TLX Ultracentrifuge (Beckman-Coulter, Australia). The pellet containing the microsomal fraction was resuspended in Buffer A and 5 µL was taken to determine protein concentration using a bicinchoninic acid (BCA) protein assay. For total HO activity, the reaction was assembled on ice as follows: 100 µg of microsomal protein, 600 µg of freshly isolated rat liver microsomes and 1 µL of 2.5 mM hemin, in 25% dimethyl sulfoxide (DMSO) in 99 µL of ice-cold Buffer B (250 mM sucrose, 20 mM Tris pH 7.4 1 mM NADPH), 2 mM D-glucose-6-phosphate, and 1 U glucose-6-phosphate dehydrogenase. Reaction mixtures were incubated in the dark for 1.5 h at 37 °C. An equal volume of ethanol:DMSO (95:5; v/v) was added to stop the reaction and the mixture was centrifuged at 13,000 x g at 4°C for 5 min. Bilirubin, the primary product of hemin catabolism, was determined with reversed-phase HPLC as previously described. The total HO-1 activity of kidney homogenate was reported as the yield of bilirubin/mg microsomal protein/min. Protein concentration was determined by bicinchoninic acid as described above.

**Statistical analyses**

Data are expressed as between animal mean ± standard error of the mean (SEM). Hypothesis testing was performed using the software package SYSTAT (Version 13, Systat Software Inc., Chicago, IL, USA). Data were subjected to analysis of variance (ANOVA), the factors including time, genotype (Lewis or LPK) and treatment (tempol or vehicle). P values for within-subjects factors were conservatively adjusted by the Greenhouse-Geisser method. When multiple comparisons were made within a single ANOVA, P values were conservatively adjusted using a Bonferroni post-hoc test. Student’s unpaired t-test was used for dichotomous data. Two-tailed P ≤ 0.05 was considered statistically significant.
Results

Morphometric, haemodynamic and plasma variables
At 4 weeks of age, body weight of the Lewis (69 ± 1 g) and LPK (66 ± 2 g) rats did not differ significantly, but by 12 weeks of age, the LPK rats weighed 23% less than the Lewis rats (Table 2). Chronic treatment with tempol had little or no effect on body weight. Food intake was similar in all four groups at 7 weeks of age. However, at 12 weeks of age, LPK rats consumed on average 25% less food than Lewis rats and tempol treatment did not significantly affect food intake.

Water intake expressed as a proportion of body weight decreased progressively with age in Lewis rats, but not in LPK rats. Urine flow expressed as a proportion of body weight remained relatively constant in Lewis rats but increased progressively with age in LPK rats. Thus, at 12-weeks of age water intake was 3-fold greater and urine flow was 4.2-fold greater in LPK rats compared to Lewis rats. Daily urea excretion, expressed as a proportion of body weight, increased progressively with age in Lewis rats, but not in LPK rats, so that it was 36% less in LPK than Lewis rats at 12 weeks of age. Daily protein excretion, expressed as a proportion of body weight, increased progressively in LPK rats, but not Lewis rats, so that it was 190% greater in LPK rats than Lewis rats at 12 weeks of age. Tempol treatment did not significantly affect water intake, urine flow or daily excretion of urea or protein (Fig. 1).

At 13 weeks of age, mean arterial pressure was significantly greater in LPK than Lewis rats (46%), while heart rate was slightly (5%) but significantly greater in LPK than Lewis rats. Tempol treatment did not significantly affect either variable (Fig. 2).

At 13 weeks of age, the ratio of kidney weight to body weight was more than 8-fold greater in LPK rats than Lewis rats and tempol treatment did not significantly influence these morphometric indices (Table 2). Plasma urea concentration was 6-fold greater while plasma creatinine concentration was 3.5-fold greater in LPK than Lewis rats, and neither were significantly altered by tempol treatment.
Neither total plasma protein nor plasma albumin concentration differed significantly according to genotype, but plasma globulin concentration was on average 13% less in LPK than Lewis rats. Tempol treatment did not significantly alter plasma total protein or globulin concentration, but was associated with 49% greater plasma albumin in Lewis rats and 8% lesser plasma albumin in LPK rats (Table 2). Plasma sodium concentration was similar in LPK and Lewis rats, and not significantly altered by tempol treatment. In contrast, plasma potassium concentration was 24% greater in LPK than Lewis rats. Furthermore, plasma potassium concentration was slightly (~8%) but significantly greater in tempol-treated rats than untreated rats, an effect which was apparent in both LPK and Lewis rats (Table 1).

**Histopathology**
As previously reported, the most characteristic feature of the kidneys of LPK rats is the presence of large cysts (Fig 3). At 13 weeks of age, the epithelial lining of the cystic tubules appeared damaged, with numerous vacuolized cells. The brush border lining the tubule epithelia was often absent. Glomeruli appeared normal. There was considerable interstitial expansion and fibrosis within LPK kidney tissue, including interstitial collagen deposition as indicated by staining with picrosirius red (Fig. 3). Reticular fibers, as identified by Gordon’s silver salt staining, were widely dispersed within the interstitium of the LPK kidneys. This pattern contrasted with that observed in the kidneys of Lewis rats, in which reticular fibers were restricted to the basement membranes of glomeruli and tubules (Fig. 3). Tempol treatment did not appear to alter the observed histopathological features of the kidneys of LPK or Lewis rats (Online Supplementary Fig. 1).

**Hypoxia**
In Lewis rats, immunohistochemistry for pimonidazole adducts was greatest in the papillary region of the inner medulla and at the immediate border of the outer and inner medullary regions. Very little staining was seen in the outer medulla or cortical regions. This gradient of staining was less
evident in the kidneys of LPK rats, where there was instead a high level of staining for pimonidazole in the epithelial cells lining numerous cysts, particularly in the cortical and outer medullary regions (Fig. 4). Immunoreactive HIF-1α protein was shown to localize to the epithelial cells lining cysts in LPK rats, but not in the epithelial lining of tubules in the kidneys of Lewis rats (Fig. 5). Tempol treatment did not appear to affect the pattern or intensity of pimonidazole (Online Supplementary Fig. 2) or HIF-1α staining, in either the Lewis or LPK rats (Online Supplementary Fig. 3).

Expression of mRNA for HIF-1α was 4.1-fold greater in kidney tissue from vehicle-treated LPK rats than vehicle treated Lewis rats (Table 3). A similar pattern was seen in 10-week old rats. In LPK kidney tissue, HIF-1α mRNA expression was 40% less after tempol treatment than after vehicle treatment, yet tempol treatment did not significantly alter HIF-1α mRNA expression in Lewis rats. Expression of mRNA for VEGF, a downstream target of HIF-1α, was 64% greater in kidney tissue from vehicle-treated LPK rats than Lewis rats but this was not observed in 10-week old rats. Tempol treatment appeared to increase VEGF mRNA expression in both Lewis (56%) and LPK (49%) 13-week old rats.

Expression of the glucose transporter GLUT-1 (another protein under transcriptional regulation of HIF-1α) was greater in kidney tissue from LPK rats than corresponding Lewis rats at both 10 (38%) and 13 weeks of age (84%), but was not significantly affected by tempol treatment (Table 3).

**Oxidative stress**

Immunofluorescence for 3-nitrotyrosine was 10-fold greater in the kidneys of 13-week old vehicle-treated LPK rats than in the kidneys of vehicle-treated Lewis rats. When compared to the corresponding vehicle-treated rats, tempol appeared to cause a substantial reduction in 3-nitrotyrosine staining in both the LPK (74% reduction) and Lewis rats (89% reduction; Fig. 6).
The expression of mRNA for p47phox and gp91phox subunits of the pro-oxidant enzyme NADPH oxidase was significantly greater in the kidneys of 13-week old vehicle-treated LPK compared with 13-week old vehicle-treated Lewis rats (14.8-fold and 10.5-fold, respectively; Table 3). Expression of p47phox and gp91phox was also significantly greater in the 10-week old LPK relative to Lewis rats, but not to the same degree (5.5 and 6.3 fold greater, respectively). Tempol treatment was associated with significantly less p47phox mRNA expression in the Lewis animals (50%), but if anything a tendency for greater p47phox mRNA expression (40%) in LPK rats. Expression of SOD-1 mRNA was 70% less in the kidneys of vehicle-treated 13-week old LPK rats when compared to vehicle-treated Lewis rats, and a comparable difference was present in the 10-week old animals (48% less in PK than Lewis). HO-1 gene expression was 5.4-fold greater in kidney tissue from 13-week old vehicle-treated LPK rats than Lewis, and 11.7 fold greater in 10 week old LPK compared to Lewis. Tempol did not significantly affect either SOD-1 or HO-1 mRNA expression levels in either the LPK or Lewis animals (Table 3).

Total SOD activity was less in kidney tissue from 13-week old LPK rats compared with 13-week old Lewis rats, as indicated by the observation that pyrogallol auto-oxidation was 7-fold greater in LPK- compared with Lewis-kidney tissue. This observation is consistent with our finding of less mRNA for SOD-1 in kidney tissue from LPK compared with Lewis rats. HO activity was slightly (3%) but significantly greater in kidney tissue from 13-week old LPK compared with Lewis rats. Similar patterns of SOD and HO activity were seen in 10-week old rats. Tempol treatment did not significantly alter renal SOD activity in 13-week old rats, and while HO activity was slightly less in tempol treated rats than vehicle controls (-3% across both Lewis and LPK groups), this small difference is unlikely to be biologically significant (Fig. 7).


Discussion

In the current study, we observed renal tissue hypoxia in the LPK model of PKD, as evidenced by upregulation of mRNA for HIF-1α and its downstream targets VEGF and GLUT-1, along with cortical tissue staining for the hypoxia marker pimonidazole, and punctuate immunohistochemical staining for HIF-1α. Both of these hypoxic markers were significantly expressed in the walls of cysts, indicating that the cystic wall is a critical locus of hypoxia in the LPK kidney. We also observed oxidative stress in the LPK kidney, as evidenced by intense immunoreactivity for 3-nitrotyrosine, upregulation of mRNA expression for the p47phox and gp91phox subunits of NADPH oxidase, the major source of superoxide in the kidney, and decreased SOD-1 mRNA and activity in LPK kidneys compared to Lewis rats. Our data also demonstrate that chronic treatment of LPK rats with tempol from age 3 to 13 weeks significantly reduced renal tissue levels of HIF-1α mRNA and immunofluorescence for 3-nitrotyrosine, suggesting that tempol reduces hypoxic signaling and nitrosative stress in this rat model of PKD. Nevertheless, chronic tempol treatment failed to significantly blunt indices of disease progression, including the extent of hypertension, uraemia, proteinuria, elevated plasma creatinine, or polyuria. Tempol treatment also failed to reduce the degree of fibrosis or the severity of tissue hypoxia as assessed by pimonidazole staining. Thus, while our current findings demonstrate renal tissue oxidative stress and hypoxia in PKD, they do not support the hypothesis that reducing oxidative stress can alleviate hypoxia, or that oxidative stress is a major contributor to the progression of PKD.

Our demonstration of oxidative stress is consistent with the findings of other studies that examined the pathophysiology of PKD. For example, in kidney tissue from the c57BL/6J-cpk mouse and Han:SPRD-cy rat models of PKD, Maser and colleagues observed lipid peroxidation and enhanced expression of HO-1. They also observed reduced expression of mRNA for the antioxidant enzymes extracellular glutathione peroxidase (EGPx), cytosolic α-class glutathione-S-transferase,
manganese SOD and catalase, and reduced plasma and renal levels of EGPx protein and plasma EGPx. Evidence of oxidative stress has also been obtained in human ADPKD, as shown by elevated plasma 8-epi-prostaglandin levels. However, we are not aware of any previous intervention studies that have investigated the effects of inhibiting oxidative stress on PKD progression.

Tempol treatment greatly reduced immunostaining for 3-nitrotyrosine. Tyrosine nitration can be the result of the reaction between tyrosine residues in a protein (or as an isolated amino acid) with peroxynitrite or through an enzymatic process involving reactions between heme proteins and nitrite to yield a nitrating agent that acts as an electrophile that targets tyrosine residues (e.g. myeloperoxidase). Thus, 3-nitrotyrosine immunofluorescence provides an integrated measure of the long-term impact of multiple pro-oxidant factors on cellular protein integrity. Tempol is a stable free radical which has multiple antioxidant actions, including superoxide dismutation, catalase-like activity, scavenging of nitrogen dioxide, thyl and carbon-centered radicals, inhibition of peroxynitrite-mediated tyrosine nitration and inhibition of myeloperoxidase. Thus, tempol could act to reduce 3-nitrotyrosine generation by multiple mechanisms. Nevertheless, our finding that administration of tempol in drinking water over a 10-week period, at a concentration of 1 mM, greatly reduced the build-up of nitrotyrosine, provides strong evidence that this dose chronically reduces oxidative and/or nitrosative stress, consistent with the study design.

Administration of tempol in drinking water at a concentration of 1 mM has previously been shown to decrease arterial pressure and renal excretion of 8-iso prostaglandin F2 when administered over a 2-week period in spontaneously hypertensive rats. Indeed, antihypertensive effects of chronic treatment with 1 mM tempol in drinking water for up to 10 weeks have been observed in many previous studies in rat models of hypertension (see for a systematic review of previous studies). Our current findings, the first to our knowledge in a model of PKD, contrast strongly with these
previous observations, in that we could not detect an antihypertensive effect of tempol treatment. Furthermore, we could not detect marked effects of tempol on histopathological markers of disease progression (cyst formation, extracellular matrix expansion, fibrosis), HO or SOD activity, or expression of mRNA for HO-1, SOD-1, or gp91phox, although tempol treatment appeared to enhance expression of mRNA for p47phox. Our observation that renal 3-nitrotyrosine staining was significantly less in tempol-treated than vehicle-treated LPK rats is consistent however with the chief locus of action of tempol, the dismutation of superoxide radical anion. Note that this conclusion is not necessarily at odds with the lack of effect of tempol treatment on pyrogallol auto-oxidation in kidney homogenates, since the homogenization process would likely have diluted the tempol and led to reduction of tempol to its hydroxylamine form as a result of liberation of intracellular reductants such as ascorbate and glutathione. Thus, chronic treatment of LPK rats with tempol appears to have blunted oxidative/nitrosative stress, but not disease progression, indicating that the dismutation of the superoxide anion radical is dissociated from the pathogenesis of PKD and unlikely to be a useful target to develop therapeutically. A potential explanation for the lack a beneficial effect of tempol in PKD is increased bioavailability of hydrogen peroxide, as has been proposed as an explanation for exacerbation of renal damage by tempol in a porcine model of renovascular disease.

The presence of tissue hypoxia in the kidneys of LPK rats was demonstrated by intense pimonidazole staining in the cystic epithelium of kidneys from LPK rats, increased expression of HIF-1α mRNA and parallel increases in HIF-1α protein immunostaining in the kidneys of LPK rats relative to Lewis rats. Expression of mRNA for two downstream targets of HIF, VEGF and GLUT-1, were also up-regulated in LPK renal tissue. VEGF upregulation was evident in the 13 week old animals, while GLUT-1 expression was increased in both the 10 and 13 week study groups. Both pimonidazole immunostaining and HIF-1α immunofluorescence in the LPK kidney were localized
to the walls of cysts, as has been shown previously in the Han:SPRD rat and in human ADPKD.\textsuperscript{7, 8}

Renal tissue hypoxia in PKD likely results from multiple factors. These include reduced renal oxygen delivery due to ischaemia,\textsuperscript{10-13} in part likely the result of structural microvascular abnormalities,\textsuperscript{14-16} and anaemia.\textsuperscript{30} Increased barriers to oxygen diffusion due to tissue fibrosis and the presence of cysts themselves likely also contribute, as may inefficient oxygen utilization due to the presence of oxidative stress and reduced nitric oxide bioavailability.\textsuperscript{25} An important limitation of our current study is the absence of direct measurements of renal tissue $PO_2$. Such studies are warranted to place our understanding of renal hypoxia in PKD on a more quantitative footing.

A potential role for hypoxia and HIF-signaling in cystogenesis has been proposed, based on the findings of renal cyst development in humans with von-Hippal Lindau (VHL) disease\textsuperscript{53} and in mice with conditional inactivation of the VHL tumor suppressor gene.\textsuperscript{54} VHL tumor suppressor gene inactivation results in activation of HIF and its downstream target genes,\textsuperscript{54} so one might expect HIF activation by hypoxia in PKD to accelerate disease progression. We were unable to adequately test this hypothesis in the current study, since chronic tempol treatment failed to ameliorate the majority of markers of tissue hypoxia. Contrary to a role for HIF-1$\alpha$ in progression of PKD, Belibi and colleagues were unable to demonstrate a beneficial effect of HIF-1$\alpha$ inhibition in multiple rodent models of PKD.\textsuperscript{7} Moreover, while whole kidney levels of HIF-1$\alpha$ increased in severe and advanced PKD, as in our study, they could not demonstrate this in animals with only mild renomegally. However, their findings do not exclude a role for hypoxia signaling pathways in cystogenesis, since cyst development in mice with conditional inactivation of the VHL tumor suppressor gene was blunted by inactivation of the HIF-1$\alpha$ binding partner arylhydrocarbon receptor nuclear translocator (HIF-\(\beta\)) but not HIF-1$\alpha$ itself.\textsuperscript{54} Thus, cyst development may be dependent on hypoxia signaling even if it is independent of HIF-1$\alpha$. This proposition merits further investigation.
One of the hallmarks of PKD is angiogenesis in the cyst wall.\textsuperscript{14} Consistent with this idea VEGF and VEGF receptor 1 are strongly expressed in the epithelial cells of cyst walls in human ADPKD, while VEGF receptor 2 is strongly expressed in the endothelial cells of small vessels surrounding cysts.\textsuperscript{14} Kidney and plasma levels of VEGF, and renal tubular expression of the two VEGF receptors, were found to be enhanced in the Han:SPRD model of ADPKD.\textsuperscript{55} Furthermore, inhibition of VEGF receptor expression was found to blunt disease progression.\textsuperscript{55} Thus, VEGF may facilitate cyst enlargement by promoting both neovascularization in the cyst wall and fluid secretion into the cyst lumen. Our study demonstrated up-regulation of VEGF mRNA in 13-week old LPK rats, although at 10 weeks of age VEGF mRNA expression was not significantly different between LPK and Lewis rats. Levels of VEGF mRNA may have been influenced by the prolonged experimental protocol and potential for anaesthesia to decrease tissue oxygenation/perfusion in the 13-week old animals, as compared to the rapid euthanasia and tissue collection in the 10 week old animals. On the other hand, in the work of Bernhardt et al., VEGF mRNA expression was found to be less in kidney tissue from young Han:SPRD rats than in tissue from wild-type controls.\textsuperscript{8} Thus, VEGF mRNA expression appears to be dependent upon the state of disease progression in PKD.

Interestingly, we found that chronic tempol treatment enhanced renal mRNA expression for VEGF, both in LPK rats and Lewis rats. Given the proposed role of VEGF in progression of PKD,\textsuperscript{55} this effect of tempol would be expected to, if anything, exacerbate disease progression.

The ability of tempol-treatment to increase VEGF expression could be related to its ability to enhance nitric oxide bioavailability by scavenging superoxide. This proposition merits investigation.

Our mRNA data showed markedly greater levels of expression of the NADPH oxidase subunits p47phox and gp91phox in the LPK kidneys. NADPH oxidase is the major source of superoxide in the kidney\textsuperscript{36} and its expression is upregulated in other models of hypertension associated with
oxidative stress, such as the spontaneously hypertensive rat (SHR). Additionally, previous observations of increased HO-1 mRNA expression and reduced manganese SOD mRNA expression in animal models of PKD have been interpreted as evidence of an altered redox state within the kidney of PKD. Our current findings are therefore consistent with the presence of oxidative stress in PKD. However, whereas renal HO-1 mRNA was found to be markedly upregulated in LPK rats, HO activity was at most only slightly upregulated. Mismatches between HO mRNA levels and activity are likely to arise from altered post-translational processing or other factors that alter enzyme activity. For example, peroxynitrite (ONOO−) can inhibit total HO activity in a concentration-dependent manner. Furthermore, in the SOD-3 knock out mouse, chronic oxidative stress resulted in HO-1 induction, yet a reduction in HO activity. Our observation may also be explained in part by the fact that in many instances HO-1 gene expression leads to the expression of the apo-protein that is inactive unless heme biosynthesis is also activated, to allow for incorporation of heme into the holo- or active form of the oxygenase.

In healthy and diseased tissues, protection by SOD is critical for tissue function. SOD exists in two main forms, contingent on the metal ion bound to the active site; copper-zinc SOD (SOD-1) and manganese SOD (SOD-2). In our study, LPK kidneys exhibited decreased SOD-1 mRNA and activity levels compared to control. In association with evidence for increased NADPH oxidase levels, such an imbalance is a likely driver of superoxide accumulation within the kidneys. It has been recently postulated from studies in the diabetic C57BL/6-Akita mice that NADPH oxidase negatively regulates renal SOD. Interestingly, downregulation of SOD-2 mRNA expression has been observed in kidney tissue of the cpk mouse model of PKD so additional studies to examine SOD-2 expression in the LPK model are warranted.

In conclusion, our current findings indicate that the LPK rat model of PKD manifests both oxidative stress and hypoxia within kidney tissue. However, the inability of chronic tempol treatment to
ameliorate the disease process in this model argues against a major role for oxidative stress in disease progression. The role of hypoxia and hypoxia signaling pathways in disease progression remains to be fully elucidated, but our current findings are at least consistent with the proposition that it plays some role, since hypoxia signaling pathways, as assessed by expression of mRNA for HIF-1α, VEGF, and GLUT 1, are up-regulated in 13-week old LPK rats.

**Sources of Funding**

This work was supported by grants from the National Health and Medical Research Council of Australia (384101 & 606601).

**Conflicts of Interest**

None
References

62. Bateman RM, Tokunaga C, Kareco T, Dorscheid DR, Walley KR. Myocardial hypoxia-inducible HIF-1alpha, VEGF, and GLUT1 gene expression is associated with microvascular and


Table 1 Primers for real-time reverse quantitative polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
<th>Size (bp)</th>
<th>Accession No</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>CGAAGAACTCTCACGCCACAG</td>
<td>CGGGCTTTTTTCTAAAGCTTG</td>
<td>204</td>
<td>AF057308</td>
<td>60</td>
</tr>
<tr>
<td>HO-1</td>
<td>ACCCCCGAGTGCAAGCACAG</td>
<td>TTTCTCTCGGCGGTCTCTG</td>
<td>197</td>
<td>NM_012580</td>
<td>61</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>GGTGTCGCACGAGCTGTGTA</td>
<td>GACGAACACGCAGACACCACAG</td>
<td>78</td>
<td>NM_138827</td>
<td>62</td>
</tr>
<tr>
<td>VEGF</td>
<td>GCAATGATGAAGCCCTGAG</td>
<td>GTGAGTTGATCCGCATG</td>
<td>78</td>
<td>AY033506</td>
<td>62</td>
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<tr>
<td>p47phox</td>
<td>CCAGCTCCCAGGTGTATG</td>
<td>TCTTCACCTGCGTCATTG</td>
<td>178</td>
<td>AY029167</td>
<td>63</td>
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<tr>
<td>gp91phox</td>
<td>CGCATGCTTTTGGAGTGGTT</td>
<td>GTGCACAGCAGAAGTGATA</td>
<td>140</td>
<td>AF298656</td>
<td>64</td>
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<tr>
<td>SOD-1</td>
<td>CCACTGACGACCTCATTTT</td>
<td>CACCTTTGCACATCCTTCT</td>
<td>218</td>
<td>NM_017050</td>
<td>65</td>
</tr>
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</table>

Primers were designed and/or verified using the NCBI/ Primer-BLAST database (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). Testing included assessment of secondary annealing, mismatching or partial annealing to sequences other than the intended target. Unsuitable primers were excluded. All primers were confirmed to span an intron except for VEGF, P47phox and Gp91phox where intron/exon data were unavailable. Correct product size for each primer pair was confirmed by visualization of amplicons on ethidium bromide.
stained agarose gels. HIF = hypoxia inducible factor, HO = heme oxygenase, GLUT-1 = glucose transporter 1, VEGF = vascular endothelial growth factor, p47phox, gp91phox = subunits of nicotinamide adenine dinucleotide phosphate oxidase, SOD = superoxide dismutase.
Table 2 Morphometry and plasma variables at 13 weeks.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lewis Control</th>
<th>LPK</th>
<th>Outcome of Analysis of Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Tempol</td>
<td>Vehicle</td>
</tr>
<tr>
<td>Body Weight (BW) (g)</td>
<td>355 ± 9</td>
<td>366 ± 7</td>
<td>279 ± 8</td>
</tr>
<tr>
<td>Kidney Weight (KW) (g)</td>
<td>3.36 ± 0.15</td>
<td>3.49 ± 0.21</td>
<td>21.66 ± 0.86</td>
</tr>
<tr>
<td>KW % of BW</td>
<td>0.95 ± 0.05</td>
<td>0.95 ± 0.06</td>
<td>7.76 ± 0.25</td>
</tr>
<tr>
<td>Plasma Urea (mM)</td>
<td>6.77 ± 0.75</td>
<td>5.90 ± 0.31</td>
<td>24.83 ± 1.77</td>
</tr>
<tr>
<td>Plasma Creatinine (µM)</td>
<td>28.0 ± 2.1</td>
<td>28.0 ± 1.1</td>
<td>81.7 ± 10.4</td>
</tr>
<tr>
<td>Plasma Albumin (g/L)</td>
<td>21.0 ± 6.0</td>
<td>31.3 ± 1.0</td>
<td>26.0 ± 1.2</td>
</tr>
<tr>
<td>Plasma Globulin (g/L)</td>
<td>32.7 ± 3.9</td>
<td>27.0 ± 0.6</td>
<td>25.7 ± 0.3</td>
</tr>
<tr>
<td>Plasma Total Protein (g/L)</td>
<td>53.3 ± 2.4</td>
<td>57.8 ± 1.1</td>
<td>52.3 ± 1.5</td>
</tr>
<tr>
<td>Plasma Sodium (mM)</td>
<td>135 ± 1</td>
<td>136 ± 1</td>
<td>138 ± 2</td>
</tr>
<tr>
<td>Plasma Potassium (mM)</td>
<td>3.2 ± 0.1</td>
<td>3.4 ± 0.1</td>
<td>3.9 ± 0.1</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of n = 3-5 (due to missing data in some groups). P values are the outcomes of two-way analysis of variance (degrees of freedom = 1,11-14).
### Table 3 Gene expression in kidneys from Lewis control and LPK rats.

<table>
<thead>
<tr>
<th>Gene</th>
<th>13-Week Old Rats</th>
<th>10-Week Old Rats</th>
<th>P Values from ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lewis</td>
<td>LPK</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>Tempol</td>
<td></td>
</tr>
<tr>
<td>HIF1α</td>
<td>1.00 (0.92-1.09)</td>
<td>1.01 (0.84-1.22)</td>
<td>4.12 (3.62-4.69)</td>
</tr>
<tr>
<td>VEGF</td>
<td>1.00 (0.97-1.03)</td>
<td>1.56 (1.37-1.77)</td>
<td>1.64 (1.46-1.84)</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>1.00 (0.90-1.11)</td>
<td>1.06 (1.04-1.10)</td>
<td>1.84 (1.64-2.26)</td>
</tr>
<tr>
<td>p47phox</td>
<td>1.00 (0.91-1.10)</td>
<td>0.50 (0.39-0.62)*</td>
<td>14.8 (12.4-17.6)</td>
</tr>
<tr>
<td>gp91phox</td>
<td>1.00 (0.88-1.14)</td>
<td>1.18 (1.07-1.31)</td>
<td>10.5 (8.8-12.6)</td>
</tr>
<tr>
<td>SOD-1</td>
<td>1.00 (0.83-1.20)</td>
<td>0.62 (0.44-0.87)</td>
<td>0.30 (0.27-0.33)</td>
</tr>
<tr>
<td>HO-1</td>
<td>1.00 (0.79-1.27)</td>
<td>0.90 (0.79-1.04)</td>
<td>5.43 (4.83-6.12)</td>
</tr>
</tbody>
</table>

Data, expressed as normalized ΔΔCt values and fold variation relative to reference Lewis animals are shown for tissue collected from 13-week old rats (n = 5 animals per group). To control for the potentially confounding effects of multiple anesthesia and perfusion of the kidneys with phosphate buffered saline, renal tissue was collected from an additional 6 Lewis and 7 LPK rats at 10 weeks of age. HIF = hypoxia inducible factor, HO = heme oxygenase, GLUT = glucose transporter, VEGF = vascular endothelial growth factor, p47phox, gp91phox = subunits of nicotinamide adenine dinucleotide phosphate oxidase, SOD = superoxide dismutase. Significant P values (from ANOVA or t-test) are shown in bold. *P ≤ 0.05 for specific contrasts between vehicle-treated and tempol-treated rats, adjusted by the Bonferroni method to account for the fact that two comparisons were made within each analysis of variance (ANOVA).
Figure Legends

Fig. 1. Daily water intake, urine flow and urea and protein excretion. Columns and error bars represent mean ± SEM of n = 5 rats per group for 24 h (h) water intake, urine flow, and urea and protein excretion in Lewis and LPK rats. Data are normalized per gram bodyweight (g/BW). Repeated measures analysis of variance demonstrated highly significant time*genotype interactions for all variables (P always <0.001) but no significant time*treatment or time*genotype*treatment interactions (P always ≥ 0.06).

Fig. 2 Mean arterial pressure and heart rate in conscious rats. Columns and error bars represent mean ± sem of n = 5 rats per group. Measurements were made when rats were 13 weeks of age, after 9-weeks of treatment with either tempol or its vehicle. Analysis of variance demonstrated significant main effects of genotype on both mean arterial pressure (P_{genotype} < 0.001) and heart rate (P_{genotype} = 0.05), but no significant main effects of treatment or genotype*treatment interactions (P always ≥ 0.08).

Fig. 3. Cystic phenotype and fibrosis in the kidney of LPK rats. Representative photomicrographs of kidney sections of Lewis (a, c, e) and LPK (b, d, f) rats stained with hematoxylin and eosin (a, b), picrosirius red (c, d) and Gordon’s silver salts (e, f). Scale bars in each panel are specific for that image. PKD kidney tissue was characterized by the presence of cysts and expanded interstitium (b), collagen deposition (red staining in (d)), and widely dispersed reticular fibres (stained dark brown in (f)). Representative high-magnification photomicrographs of hematoxylin and eosin stained kidney sections from Lewis and LPK rats treated with vehicle or tempol are shown in the online data supplement (Supplementary Figure 1).
Fig. 4. Pimonidazole adduct immunohistochemistry.

Pimonidazole adducts in the kidneys of Lewis and LPK rats are stained brown. Representative images are shown from the kidney of 13-week old vehicle-treated Lewis [(a) (c) and (e)] and LPK rats [(b) (d) and (f)]. Panels (a) and (b) illustrate cortical regions, while (c) and (d) are from the outer medulla region. Note the relative lack of pimonidazole staining in the cortex and outer medulla of Lewis compared to the LPK rats. Panels (e) and (f) are from the inner medulla and show staining in both animals. Scale bar = 200 µm for each panel. Representative photomicrographs of kidney sections from Lewis and LPK rats treated with tempol are shown in the online data supplement (Supplementary Figure 2).

Fig. 5. Hypoxia inducible factor (HIF) immunofluorescent staining.

Panels show representative images of sections from a vehicle treated Lewis rat (a) and a vehicle treated LPK rat (b). Sections were counter-stained for DAPI (blue). Note punctuate HIF-immunostaining in the tissue from an LPK rat, but much less prominent staining in the tissue in the tissue from a Lewis rat. Representative photomicrographs of kidney sections from Lewis and LPK rats treated with tempol are shown in the online data supplement (Supplementary Figure 2).

Fig. 6. 3-nitrotyrosine immunofluorescence.

Panels (a) to (d) show typical images for Lewis rats (a, b) and LPK rats (c, d) treated chronically with tempol (b, d) or its vehicle (a, c). Scale bar (200 µm) is the same for all panels. Panel (e) shows mean values of immunofluorescence expressed as the logarithm to the base 10, determined from the mean of 3 separate images for each of the 5 kidneys in each group. Analysis of variance demonstrated significant main effects of genotype ($P_{\text{genotype}} = 0.002$) and treatment ($P_{\text{treatment}} = 0.005$) but no significant genotype*treatment interaction ($P_{\text{genotype*treatment}} = 0.38$).
Fig. 7. Antioxidant enzyme activity in renal tissue.

Columns and error bars represent mean ± SEM. Total superoxide dismutase (SOD) activity was determined by the rate of pyrogallol auto-oxidation by SOD, normalized to protein concentration. Increased pyrogallol auto-oxidation is reflective of decreased SOD activity. Data in left panels are for tissue collected from 13-week old rats at the completion of the main experimental protocol (n = 5 per group). Analysis of variance demonstrated a highly significant effect on the rate of pyrogallol auto-oxidation of genotype (P_{genotype} < 0.001) but no significant effect of treatment (P_{treatment} = 0.25) or genotype*treatment interaction (P_{genotype*treatment} = 0.13). For total HO activity, both genotype (P_{genotype} = 0.01) and treatment (P_{treatment} = 0.03) were significant, but the interaction term was not (P_{genotype*treatment} = 0.18). Data in right panels are from renal tissue collected from an additional 6 Lewis control and 7 LPK rats at 10 weeks of age, to control for the potentially confounding effects of multiple anesthesia and perfusion of the kidneys with phosphate buffered saline. *P_{genotype} ≤ 0.05 for rats at 10 weeks of age (unpaired t-test).
Fig. 1

![Graph showing water intake, urine flow, urea excretion, and protein excretion over 4, 7, and 12 weeks for Lewis Vehicle, Lewis Tempol, LPK Vehicle, and LPK Tempol groups.](image-url)
Fig. 2

Mean Arterial Pressure (mmHg)

Heart Rate (beats/min)

Vehicle  Tempol  Vehicle  Tempol
Lewis  LPK

Mean Arterial Pressure (mmHg)

Heart Rate (beats/min)
Figure 3
Fig. 5

(a) 50 µm

(b) 50 µm
Fig 6

(a) and (b) show the immunofluorescence images of 3-nitrotyrosine in Vehicle and Tempol groups. The images display a green fluorescent signal, indicating the presence of the protein of interest. The scale bar in both images represents 200 µm.

(c) and (d) are high-magnification images that provide a closer view of the cellular structures, highlighting the distribution of the protein.

(e) is a bar graph illustrating the 3-nitrotyrosine immunofluorescence intensity (in logarithmic scale) for different conditions: Control (Vehicle and Tempol) and LPK (Vehicle and Tempol). The graph shows a significant increase in fluorescence intensity in the LPK group compared to the Control group, with Tempol treatment showing a reduced response in both groups.
Fig. 7

Pyrogallol Auto-Oxidation (Arbitrary Units X 1000)

13-week old rats

10-week old rats

HO Activity (pmol/mg/min)

Vehicle Tempol Vehicle Tempol

Lewis LPK

Lewis LPK

*
Chronic treatment with tempol does not significantly ameliorate renal tissue hypoxia or disease progression in a rodent model of polycystic kidney disease

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Departments of ²Physiology, ⁵Pharmacology and ³Monash Immunology & Stem Cell Laboratories, Monash University, Melbourne, Australia.

⁴Discipline of Pathology, The University of Sydney, Sydney, Australia.

Running title: Oxidative stress and hypoxia in polycystic kidney disease

*† These authors made an equivalent contribution to the work
Supplementary Fig. 1. Cystic phenotype and fibrosis in the kidney of Tempol treated rats.

Representative high power photomicrographs of kidney sections of from vehicle-treated Lewis (a), tempol treated Lewis (b), vehicle-treated Lewis Polycystic Kidney (LPK) (c) and tempol treated LPK (d) rats stained with hematoxylin and eosin. Scale bar = 100 µm for each image. The Figure serves to illustrate the limited impact of tempol on key cystic features of the LPK and provide additional high power images of LPK and Lewis groups.
Supplementary Fig. 2. Pimonidazole adduct immunohistochemistry in kidneys from Tempol treated rats.

Pimonidazole adducts in the kidneys of Lewis and LPK rats treated with Tempol (1mM in drinking water). Representative images are shown from the kidney of 13-week old Tempol-treated Lewis [(a) (c) and (e)] and LPK rats [(b) (d) and (f)]. Panels (a) and (b) illustrate cortical regions, while (c) and (d) are from the boundary of the cortico-medullary region. Images illustrate the relative lack of staining in the Lewis compared to cystic epithelial cell pimonidazole reactivity in the LPK in the cortex and outer medullary region. Panels (e) and (f) are from the inner medulla, both of which show staining, more evident in the Lewis. No difference was noted between vehicle-treated (Fig 4) and Tempol-treated rats. Scale bar = 200 µm for each panel.
Supplementary Fig. 3 Hypoxia inducible factor (HIF) immunofluorescence staining of renal cortical tissue from tempol treated rats.

Panels show representative images of sections from a tempol-treated Lewis rat (a) and tempol-treated LPK rat (b). Sections were counter-stained for DAPI (blue). Scale bar = 50 µm for each image.