Nephrol Dial Transplant (2020) 1–10
doi: 10.1093/ndt/gfaa236

ndt
Nephrology Dialysis Transplantation

ORIGINAL ARTICLE

Downloaded from https://academic.oup.com/ndt/advance-article/doi/10.1093/ndt/gfaa236/5959832 by University of New England user on 09 November 2020

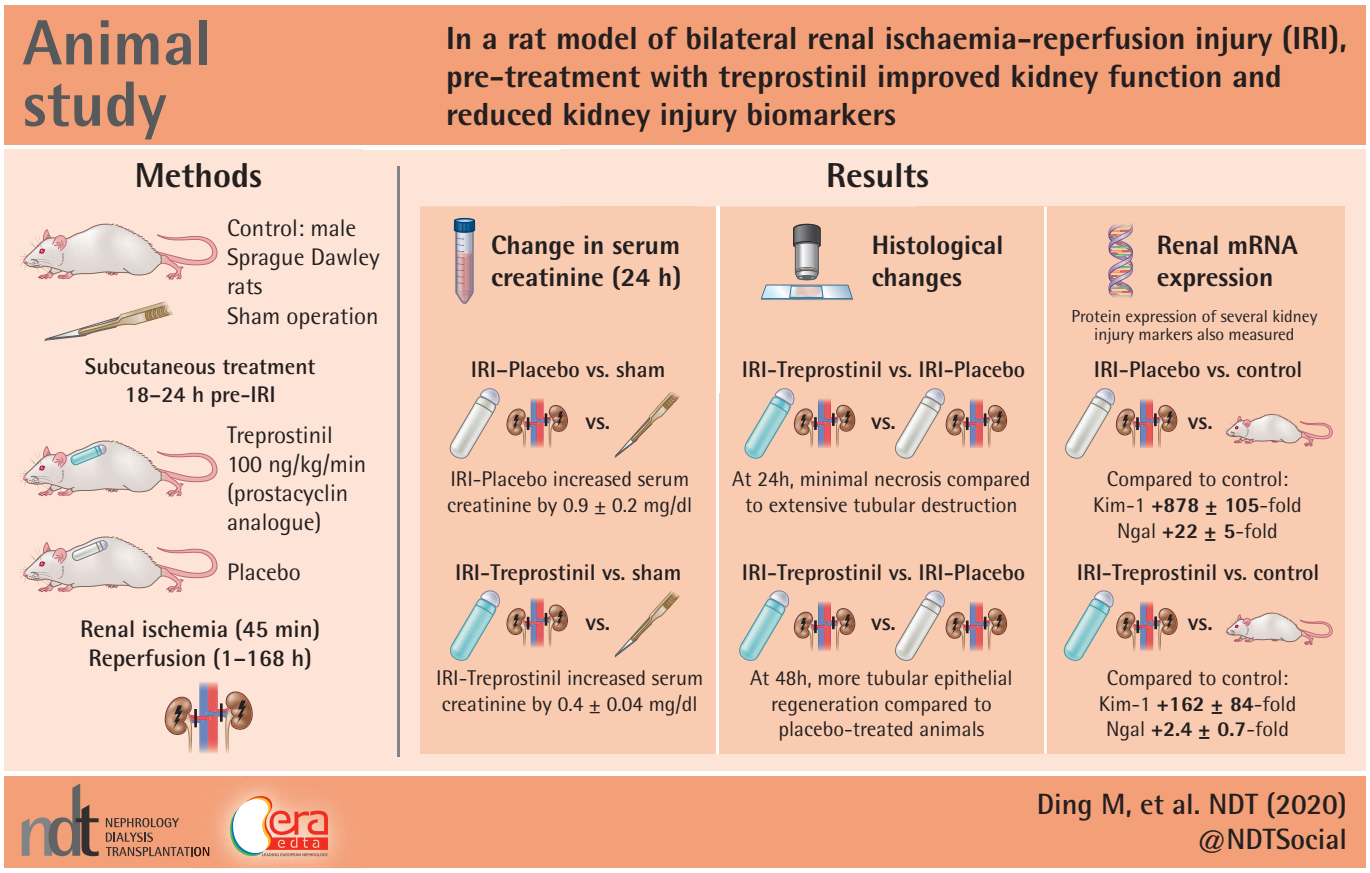
Treprostinil, a prostacyclin analog, ameliorates renal ischemia–reperfusion injury: preclinical studies in a rat model of acute kidney injury

Meiwen Ding[1], Evelyn Tolbert[2], Mark Birkenbach[3], Fatemeh Akhlaghi[1], Reginald Gohh[4] and Nisanne S. Ghonem [1]

[1]Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, RI, USA, [2]Division of Renal Disease, Department of Medicine, Warren Alpert School of Medicine, Brown University, Providence, RI, USA, [3]Department of Pathology, Warren Alpert School of Medicine, Brown University, Providence, RI, USA and [4]Division of Organ Transplantation, Rhode Island Hospital, Warren Alpert School of Medicine, Brown University, Providence, RI, USA

Correspondence to: Nisanne S. Ghonem; E-mail: nghonem@uri.edu

GRAPHICAL ABSTRACT



© The Author(s) 2020. Published by Oxford University Press on behalf of ERA-EDTA. All rights reserved.

KEY LEARNING POINTS

What is already known about this subject?

- Renal ischemia–reperfusion injury (IRI) is a major cause of acute kidney injury that significantly contributes to morbidity and mortality in clinical settings, including kidney transplantation. Currently no pharmacological treatment for renal IRI is available.

What this study adds?

- This study demonstrates the efficacy of treprostinil, a prostacyclin analog, in ameliorating renal IRI in rats and provides evidence of the inhibitory effects of treprostinil on IR-induced renal inflammation, oxidative stress and apoptosis. IR-induced renal inflammation, oxidative stress and apoptosis.

What impact this may have on practice or policy?

- The significance of these findings includes identifying a novel treatment and the potential for treprostinil to ameliorate renal IRI *in vivo*.

ABSTRACT

Background. Renal ischemia–reperfusion injury (IRI) is a major factor causing acute kidney injury (AKI). No pharmacological treatments for prevention or amelioration of I/R-induced renal injury are available. Here we investigate the protective effects of treprostinil, a prostacyclin analog, against renal IRI *in vivo*.

Methods. Male Sprague Dawley rats were subjected to bilateral renal ischemia (45 min) followed by reperfusion for 1–168 h. Treprostinil (100 ng/kg/min) or placebo was administered subcutaneously for 18–24 h before ischemia.

Results. Treatment with treprostinil both significantly reduced peak elevation and accelerated the return to baseline levels for serum creatinine and blood urea nitrogen versus I/R-placebo animals following IRI. I/R-treprostinil animals exhibited reduced histopathological features of tubular epithelial injury versus I/R-placebo animals. IRI resulted in a marked induction of messenger RNA coding for kidney injury biomarkers, kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin and for pro-inflammatory cytokines chemokine (C-C motif) ligand 2, interleukin 1β, interleukin 6 and intracellular adhesion molecular 1 in animals treated with placebo only relative to sham controls. Upregulation of expression of all these genes was significantly suppressed by treprostinil. Treprostinil significantly suppressed the elevation in renal lipid peroxidation found in the I/R-placebo group at 1-h post-reperfusion. In addition, renal protein expression of cleaved poly(ADP-ribose) polymerase 1 and caspase-3, -8 and -9 in I/R-placebo animals was significantly inhibited by treprostinil.

Conclusions. This study demonstrates the efficacy of treprostinil in ameliorating I/R-induced AKI in rats by significantly improving renal function early post-reperfusion and by inhibiting renal inflammation and tubular epithelial apoptosis. Importantly, these data suggest that treprostinil has the potential to serve as a therapeutic agent to protect the kidney against IRI *in vivo*.

Keywords: acute kidney injury, apoptosis, ischemia–reperfusion injury, oxidative stress, prostacyclin

INTRODUCTION

Renal ischemia–reperfusion (I/R) injury (IRI) is a major cause of patient morbidity and mortality in a variety of clinical settings and is virtually unavoidable during kidney transplantation. Renal IRI is characterized by the restriction of blood flow followed by restoration of perfusion and reoxygenation. This sequence initiates oxidative injury that results in apoptosis, necrosis and inflammation with an associated rapid onset of renal dysfunction. During renal IRI, the initial decline in tissue oxygen reduces oxidative phosphorylation and depletes intracellular adenosine triphosphate (ATP) stores, ultimately leading to mitochondria damage and disruption of renal tubular epithelial cell structure [1, 2]. Injury to either vascular tissues or renal tubular epithelial cells generates pro-inflammatory cytokines and chemokines, including interleukin (IL)-6, tumor necrosis factor, monocyte chemoattractant protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1), all of which are involved in initiating an early pro-inflammatory response in the kidney [3, 4].

Mitochondria play a pivotal role I/R-induced cellular injury [5]. Following an ischemic insult, mitochondria release reactive oxygen species (ROS), resulting in the loss of antioxidants such as superoxide dismutase (SOD) [6], leading to renal lipid peroxidation [7], DNA damage [8] and apoptosis [9]. The signaling pathways of apoptosis, including the intrinsic and extrinsic pathways are involved in renal IRI and are both capable of activating the apoptotic executioner caspase-3 [10, 11]. Caspase-3 is a critical upstream target of renal microvascular and epithelial injury post-IRI [12].

Prostacyclin (PGI_2) is a member of the prostaglandin family, with potent vasodilatory and antiplatelet actions ∼30 times more potent than prostaglandin E1 [13]. A key step in promoting reperfusion-related injury has been attributed to a decreased ratio in vasodilatory mediators such as PGI_2 relative to vasoconstrictor mediators such as thromboxane [14]. The role of PGI_2 in increasing renal blood flow has been demonstrated in animal renal failure [15–17] and clinical studies [18, 19]. Also, Johannes *et al.* [20] reported that iloprost, a PGI_2 analog, preserved intrarenal oxygenation during acute kidney injury (AKI). Furthermore, animal [21] and clinical [22] studies have shown that PGI_2 analogs reduce pro-inflammatory cytokines and adhesion molecules during AKI. Additionally, PGI_2 inhibits AKI-induced proximal tubular cell apoptosis by inhibiting DNA damage and downregulating pro-apoptotic gene and protein expression [23, 24]. Collectively, these findings suggest that PGI_2 may have an important role in reducing renal IRI. However, while PGI_2 analogs have been tested for their renoprotective effects, poor chemical stability side effects of earlier analogs and short half-lives necessitating continuous infusion have limited their clinical application during AKI. Thus there are currently no approved pharmacological agents to reduce renal IRI. Treprostinil (Remodulin), a US Food and Drug Administration (FDA)-approved PGI_2 analog, is stable at room

Downloaded from https://academic.oup.com/ndt/advance-article/doi/10.1093/ndt/gfaa236/5959832 by University of New England user on 09 November 2020

temperature and has an increased potency and longer elimination half-life than other commercially available PGI_2 analogs [25], which allows for lower and more tolerable dosing to achieve therapeutic efficacy and fewer side effects. This study investigates the efficacy of treprostinil in reducing IRI in a rat model of bilateral renal IRI.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats weighing 200–300 g (Charles River Laboratories, Wilmington, MA, USA) were housed in a laminar-flow, specific pathogen-free atmosphere in the Central Research Facilities of Rhode Island Hospital (RIH, Providence, RI, USA).

Bilateral kidney IRI model

Animals were randomly divided into control, sham, I/R-placebo and I/R-treprostinil groups. Under isoflurane anesthesia, the left and right artery and vein as well as right and left kidney were isolated and exposed from surrounding tissues and occluded with clamps for 45 min. The clamps were then removed to allow reperfusion. Control animals were not subjected to any surgical manipulation and serve as a baseline; sham animals had incisions only. All surgical procedures were performed by the same surgeon blinded to the treatment. All procedures involving animals were performed with approval from the RIH Institutional Animal Care and Use Committee in accordance with the National Institues of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Experimental design

Treprostinil and placebo (sodium chloride, metacresol, sodium citrate and water for injection) manufactured by United Therapeutics (Durham, NC, USA) were administrated subcutaneously (100 ng/kg/min) via osmotic minipumps (Alzet, Cupertino, CA, USA). Pumps were filled with treprostinil or placebo and implanted ∼18–24 h before renal IRI to ensure steady-state concentrations at the time of IRI. Post-reperfusion animals were kept under a heating lamp for 2 h and were given standard diet and water *ad libitum*. Blood and kidney tissue were collected at 1–168 h post-reperfusion.

Biochemical analysis

Serum creatinine (SCr) concentrations were measured on a Prominence ultrafast liquid chromatography (UFLC) system (Shimadzu, Kyoto, Japan) coupled to a 4500 Q-Trap ESI (Sciex, Framingham, MA, USA) using a Kinetexhydrophilic interaction liquid chromatography column (100 × 2.1 mm, 1.7 µm; Phenomenex, Torrance, CA, USA). Creatinine-d3 (Cayman Chemical, Ann Arbor, MI, USA) was used as an internal standard. The coefficient of variation was <3.3% for this assay. Serum blood urea nitrogen (BUN) was measured by a QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA, USA). Renal lipid peroxidation was measured by formation of malondialdehyde (MDA) using a thiobarbituric acid reactive substances assay (BioAssays Systems, Hayward, CA, USA). Fluorescence ($\lambda_{ex/em} = 530/580$ nm) was detected using a SpectraMax iD5 microplate reader (Molecular Devices, San Jose, CA, USA).

Histopathology

Kidneys were harvested, bisected through the hilum in the coronal plane and fixed in 10% formalin and paraffin embedded. Whole-mount coronal sections (1–2 µm) were stained with hematoxylin and eosin (H&E) and evaluated by light microscopy by a renal pathologist who was blinded to all animal groups. Renal tubular epithelial cell injury was evaluated semiquantitatively on the basis of the proportion of all tubules in each whole-mount section exhibiting the following: (i) features of mild or early tubular injury consisting of tubular ectasia (flattening of intact epithelium); (ii) features of moderate to severe tubular epithelial cell injury consisting of nuclear pyknosis, cytoplasmic eosinophilia, epithelial cell detachment and appearance of necrotic cells within tubule lumens and (iii) features of severe, advanced injury consisting of loss of tubular epithelium and accumulation of occlusive casts consisting of necrotic debris within tubule lumens. The following semiquantitative grading system was used: 0 (<1% of all tubules), 1 (1–10%), 2 (10–25%), 3 (25–50%) and 4 (>50%). Separate injury scores were generated for cortex and outer stripe of the outer medulla. Neutrophil analysis was performed using Naphthol AS-D Chloroacetate (Specific Esterase) kit (Sigma-Aldrich, St. Louis, MO, USA).

Quantitative real-time polymerase chain reaction (PCR) analysis

RNA was extracted from snap-frozen renal cortex segments using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The purity and concentration of RNA were measured at 260/280 nm by Nanodrop (Thermo Scientific, Waltham, MA, USA). Two micrograms of total RNA from each sample were used to generate cDNA using the SuperScript IV First-Strand Synthesis System kit (Invitrogen). Renal messenger RNA (mRNA) levels of kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), chemokine (C-C motif) ligand 2 (CCL2), interleukin-6 (IL-6), interleukin-1β (IL-1β) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were analyzed using Taqman probes (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR (qPCR) was performed using a ViiA 7 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Relative mRNA expression was calculated using the ΔΔCt method and normalized to Gapdh.

Western blot analysis

Renal cortical tissue was homogenized in tissue protein extraction reagent with protease inhibitor (Thermo Scientific). Protein (100–150 µg) was separated by 4–20% Tris-glycine gel electrophoresis (Thermo Scientific), transferred to polyvinylidene difluoride membrane, incubated overnight with primary antibodies for NGAL (AF3508, R&D Systems, Minneapolis, MN, USA), β-actin (sc-47778), GAPDH (sc-32233), poly(ADP-ribose) polymerase 1 (PARP-1) (sc-74470), SOD1

Downloaded from https://academic.oup.com/ndt/advance-article/doi/10.1093/ndt/gfaa236/5959832 by University of New England user on 09 November 2020

(sc-271014), caspase-9 (sc-133109, Santa Cruz Biotechnology, Dallas, TX, USA), caspase-3 (14220) and caspase-8 (4790, Cell Signaling Technology, Danvers, MA, USA), followed by IRDye goat antimouse IgG(H + L) 680RD, goat antirabbit IgG(H + L) 680RD, IRDye donkey antigoat IgG(H + L) 680RD and goat antimouse (800CW). Blots were visualized using the Odyssey CLx scanner (LI-COR Biosciences, Lincoln, NE, USA) and protein band density was determined using ImageJ.

Statistics

Data are presented as the mean ± standard error of the mean (SEM). Comparisons between the groups were performed using two-way analysis of variance with Turkey's post-test (GraphPad Prism version 7.0, GraphPad Software, San Diego, CA, USA). Significance was defined as $P < 0.05$.

RESULTS

Treprostinil improves renal function early post-renal IRI

SCr and BUN are commonly used as biochemical markers of renal function. Throughout the study period, sham animals that had undergone anesthesia and surgery without vascular ligation showed no change from control (baseline) in SCr (0.16 ± 0.01 versus 0.12 ± 0.01 mg/dL, respectively) and BUN (32.5 ± 1.1 versus 35.5 ± 1.4 mg/dL, respectively) at 24 h post-reperfusion. In contrast, IRI caused by bilateral renal artery ligation resulted in significantly elevated SCr and BUN in I/R-placebo animals, reaching peak levels at 24 h post-reperfusion (0.9 ± 0.2 mg/dL and 151 ± 20.4 mg/dL, respectively) versus sham animals ($P < 0.001$). Pretreatment with treprostinil significantly reduced peak SCr (0.4 ± 0.04 mg/dL) and BUN (74 ± 6.4 mg/dL) versus placebo ($P < 0.001$; Figure 1A and B). Furthermore, treprostinil significantly accelerated recovery of renal function such that peak injury was reached at 6 h compared with 24 h post-reperfusion in the I/R-placebo group. These results indicate that treprostinil improves kidney function in early phases of post-IRI.

Treprostinil reduces renal tubular epithelial necrosis

Histopathological evaluation of whole-mount coronal sections of bisected kidneys stained with H&E revealed preservation of renal tubular epithelium in control- and sham-operated animals at all time points. In contrast, transient renal artery ligation resulted in morphological features of I/R-induced injury that appeared as early as 6 h post-reperfusion in both I/R-placebo and I/R-treprostinil animals. The earliest changes included tubular ectasia and cytological features of cell injury such as nuclear pyknosis, detachment of individual cells from each other and from tubular basement membranes and increased cytoplasmic eosinophilia (Figure 2A). These changes were most prominent in the outer stripe of the outer medulla, with less extensive involvement noted in the renal cortex. Although medullary tubule injury parameters at 6 h were similar between I/R-placebo and I/R-treprostinil animals, significantly more extensive cortical injury was noted in the placebo group at this time point. Histopathological differences between I/R-placebo and I/R-treprostinil animals became most apparent at 24 h post-reperfusion, at which time extensive destruction of tubular epithelium was noted in both the outer medulla and cortex in I/R-placebo animals, while only minimal necrosis was observed in these locations in treprostinil-treated animals ($P < 0.05$). These differences persisted at 48 h post-reperfusion, as treprostinil-treated animals also displayed more prominent features of tubular epithelial regeneration, including increased mitotic activity and nuclear enlargement at earlier time points than placebo animals ($P < 0.01$; Figure 2B–D). Together, the pathology results suggest that treprostinil protects the kidney during IRI by protecting proximal tubular cells from nuclear pyknosis and necrosis.

Treprostinil reduces kidney injury biomarkers

KIM-1 [14] and NGAL [26] are established biomarkers of AKI and have been shown to accumulate in renal tubular epithelium after I/R-induced AKI [27, 28]. In our study, renal mRNA expression of KIM-1 and NGAL was significantly

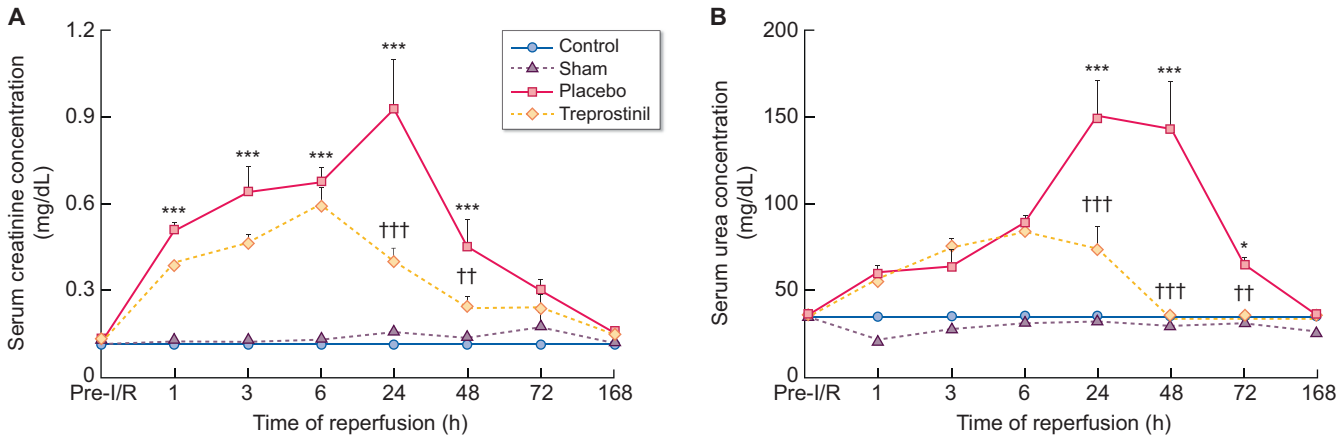


FIGURE 1: Treprostinil attenuates I/R-induced kidney injury. (**A**) SCr and (**B**) BUN, measured at pre-IRI (baseline) and at 1–168 h post-reperfusion in control-, sham-, placebo- and treprostinil-treated animals. Data are presented as mean ± SEM. *$P < 0.05$ and ***$P < 0.001$ versus control; †$P < 0.05$, ††$P < 0.01$ and †††$P < 0.001$ versus I/R-placebo ($n = 5$–12/group).

Downloaded from https://academic.oup.com/ndt/advance-article/doi/10.1093/ndt/gfaa236/5959832 by University of New England user on 09 November 2020

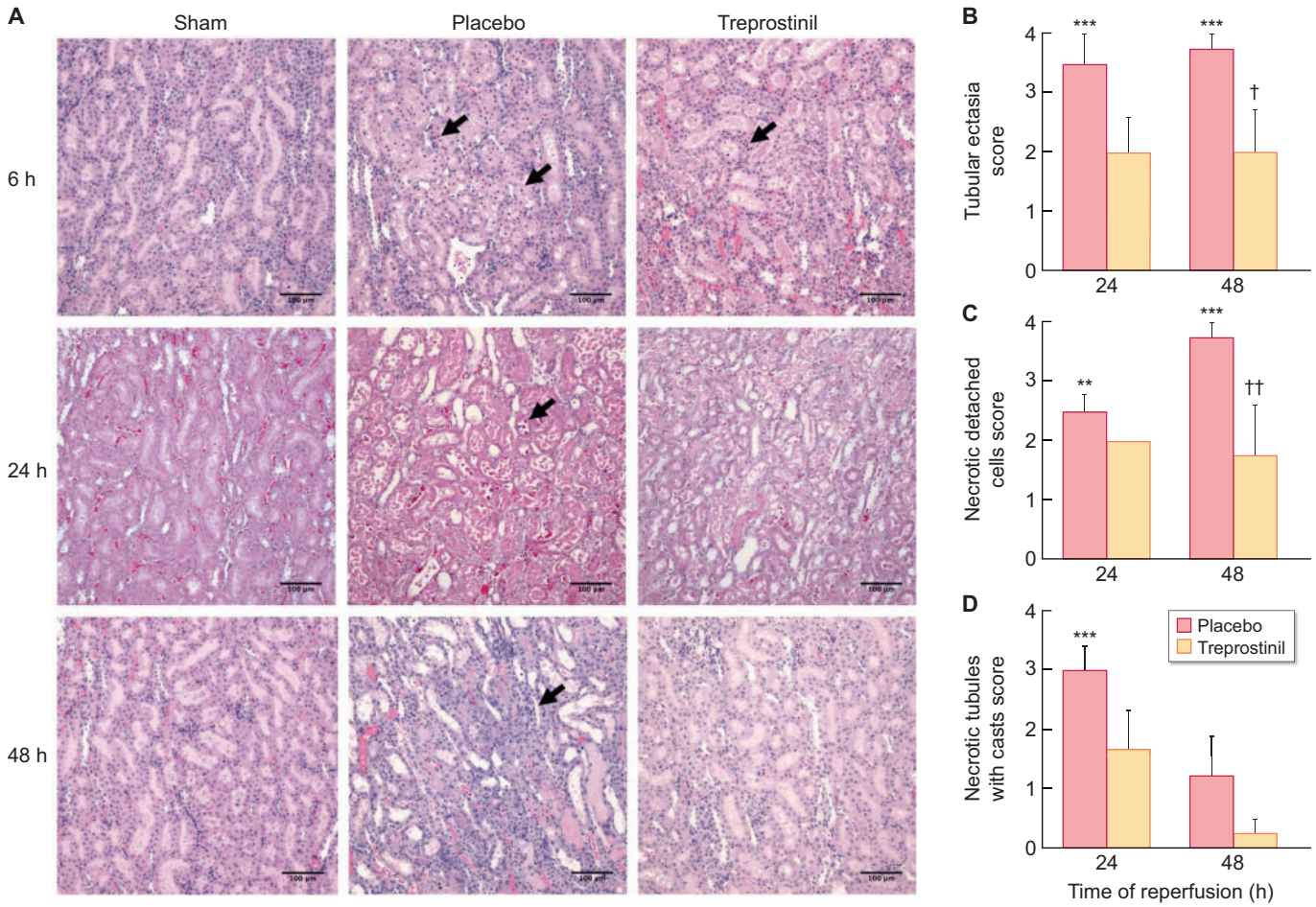


FIGURE 2: Treprostinil reduces renal tubular epithelial necrosis. (**A**) Representative histopathological H&E staining of outer medullary region in rat kidney sections at 6, 24 and 48 h post-reperfusion (×200, scale bar = 100 μm). Yellow arrows indicate nuclear pyknosis and basement membrane detachment in placebo-treated animals, which was reduced in treprostinil-treated animals. Semiquantitative analysis of histological changes of the outer stripe of outer medulla between the I/R-placebo and I/R-treprostinil group at 24 and 48 h post-reperfusion: (**B**) tubular ectasia, (**C**) necrotic detached cells and (**D**) necrotic tubules with casts, evaluated in a blinded manner using the grading system: 0 (<1), 1 (1–10), 2 (10–25), 3 (25–50%), 4 (>50%). Data are presented as mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus sham; $^{\dagger}p < 0.05$ versus I/R-placebo ($n = 3$–5/group).

increased in the I/R-placebo group relative to controls, reaching peak levels at 48 h post-reperfusion (KIM-1: 878 ± 105-fold, NGAL: 22 ± 5-fold; $P < 0.001$). In contrast, treprostinil significantly reduced the magnitude of renal KIM-1 and NGAL mRNA induction to 162 ± 84-fold and 2.4 ± 0.7-fold, respectively, relative to controls ($P < 0.001$ versus placebo; Figure 3A and B). In addition, Ngal protein expression significantly increased by 2.9-fold in I/R-placebo versus sham animals at 24 h post-reperfusion, while treprostinil restored Ngal protein to sham animals ($P < 0.05$ versus placebo; Figure 3C). These results support the renoprotective role of treprostinil during renal IRI.

Treprostinil reduces renal lipid peroxidation and oxidative stress early post-IRI

Healthy kidneys usually have enough endogenous free radical scavengers to mitigate ROS. However, during renal IRI, the depletion of antioxidants such as SOD1 results in the accumulation of ROS and promotes lipid peroxidation to form toxic aldehydes, notably MDA. Our study showed significant renal MDA concentrations at 1 h post-reperfusion in the I/R-placebo compared with sham mice (7.3 ± 1.4 versus 2.5 ± 1.1 μM/mg protein, respectively; $P < 0.05$). In contrast, treprostinil significantly reduced MDA elevations to nearly that of sham animals (3.8 ± 0.5 μM/mg protein; $P < 0.05$ versus placebo; Figure 4A). Consistent with oxidative injury, SOD1 protein levels were reduced by 52% in the I/R-placebo group at 6 h post-reperfusion, relative to the sham group, whereas treprostinil maintained baseline SOD1 levels (Figure 4B). In addition, neutrophil infiltration increased at 6- and 24-h in the I/R-placebo group, while treprostinil exhibited less neutrophil infiltration (Figure 4C). These findings indicate that treprostinil protects the kidney from IRI by reducing renal lipid peroxidation and oxidative stress.

Treprostinil reduced renal pro-inflammatory cytokine levels

Pro-inflammatory cytokines and chemokines play an important role in the pathophysiology of renal IRI. IRI upregulates mRNA levels of cytokines and chemokines in the early stage of

Downloaded from https://academic.oup.com/ndt/advance-article/doi/10.1093/ndt/gfaa236/5959832 by University of New England user on 09 November 2020

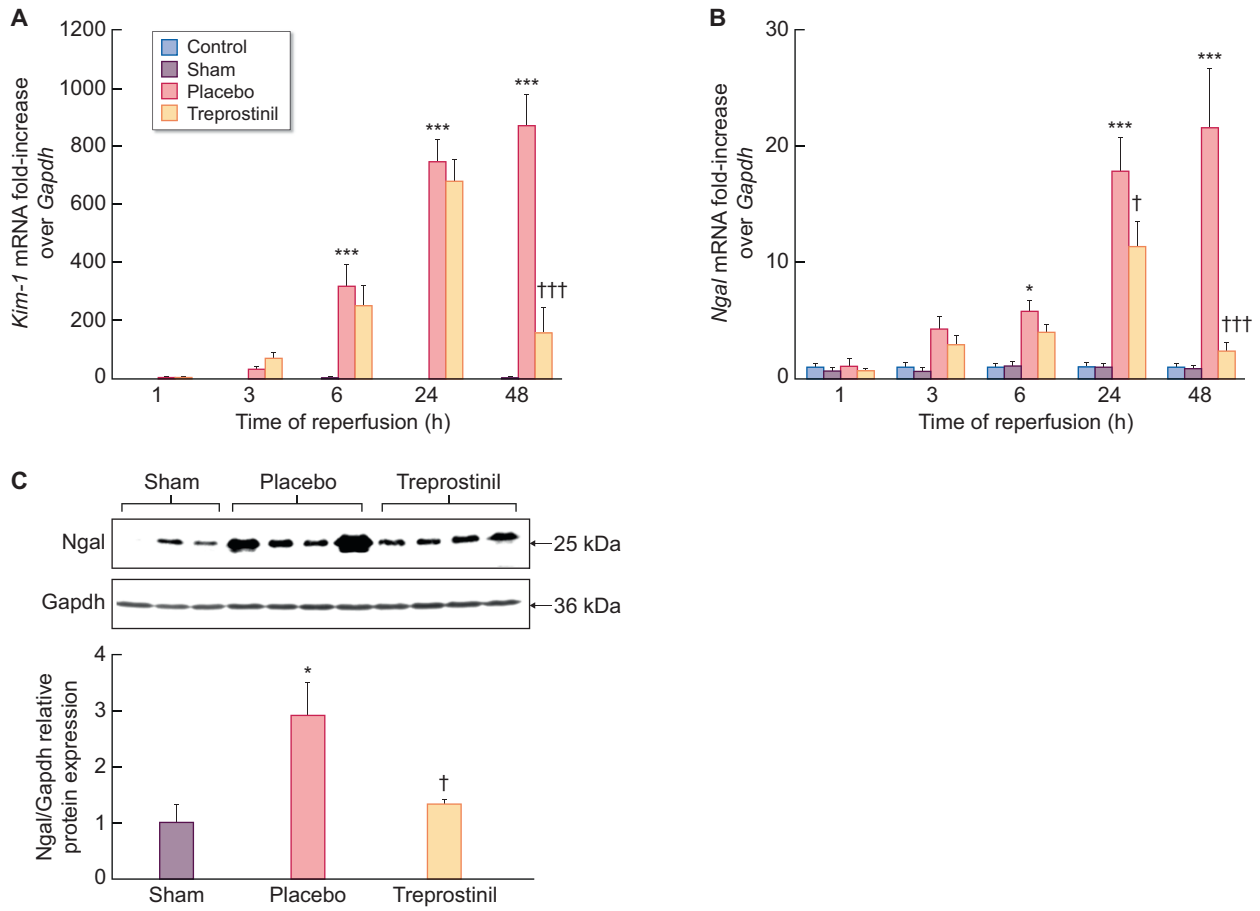


FIGURE 3: Kidney injury marker upregulation post-reperfusion is attenuated by treprostinil. The renal mRNA expression of kidney (**A**) KIM-1 and (**B**) NGAL at 1, 3, 6, 24 and 48 h after reperfusion measured by real-time qPCR, normalized to GAPDH and expressed as a fold increase over control kidney. (**C**) The protein level of NGAL was evaluated at 24 h post-reperfusion by Western blot; quantification was performed using ImageJ software. Data are presented as mean ± SEM. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus control; $^{\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.001$ versus I/R-placebo ($n = 5$–12/group).

injury [29]. We found renal mRNA levels coding for pro-inflammatory cytokines CCL2, IL-1β and IL-6 were significantly increased in I/R-placebo animals at 6 h post-reperfusion relative to sham animals by 3.5-, 2.6- and 90-fold, respectively ($P < 0.001$). In contrast, treprostinil significantly suppressed the elevation in inflammation-associated transcripts, returning mRNA levels of CCL2 and IL-1β nearly to 1.7- and 0.8-fold relative to controls ($P < 0.01$ and $P < 0.001$ versus placebo, respectively) and substantially reducing IL-6 induction (42-fold relative to control; $P < 0.01$ versus placebo; Figure 5A–C). Reduced ICAM-1 mRNA levels by treprostinil were also observed (Figure 5D). Together, these results support the renoprotection of treprostinil against IRI by reducing renal inflammation.

Treprostinil inhibits renal tubular apoptosis

Activation of PARP contributes to and facilitates DNA repair in biological processes. However, I/R-induced oxidative stress cleaves PARP-1 [30], thereby inhibiting PARP-1 activation and accelerating DNA damage, leading to apoptosis [8]. Also, I/R-induced oxidative stress and DNA damage promote mitochondrial damage, leading to the cleavage of caspase-9 [31] and subsequent activation of caspase-3, the apoptosis executioner [32]. Renal IRI activated cleavage of PARP-1 and caspase-3, -8 and -9 by 6.7-, 3.3-, 4.3- and 7.0-fold, respectively, in I/R-placebo animals, whereas treatment with treprostinil suppressed the cleavage of PARP-1 and caspase-3, -8 and -9 relative to placebo (Figure 6A–D). These results indicate that treprostinil strongly inhibits apoptosis induced by renal IRI.

DISCUSSION

PGI_2 analogs have been shown to improve renal function and to inhibit proximal tubule cell death in animal models of AKI [33] and in patients with chronic kidney disease on dialysis therapy [34]. Therefore we hypothesized that treprostinil, an FDA-approved potent PGI_2 analog, would protect the kidney against renal IRI. To test this hypothesis, we used a rat model of bilateral renal IRI to evaluate the effects of treprostinil on kidney function, renal inflammation and apoptosis. Results from the current study demonstrate that treprostinil improved kidney function, reduced the appearance of kidney

Downloaded from https://academic.oup.com/ndt/advance-article/doi/10.1093/ndt/gfaa236/5959832 by University of New England user on 09 November 2020

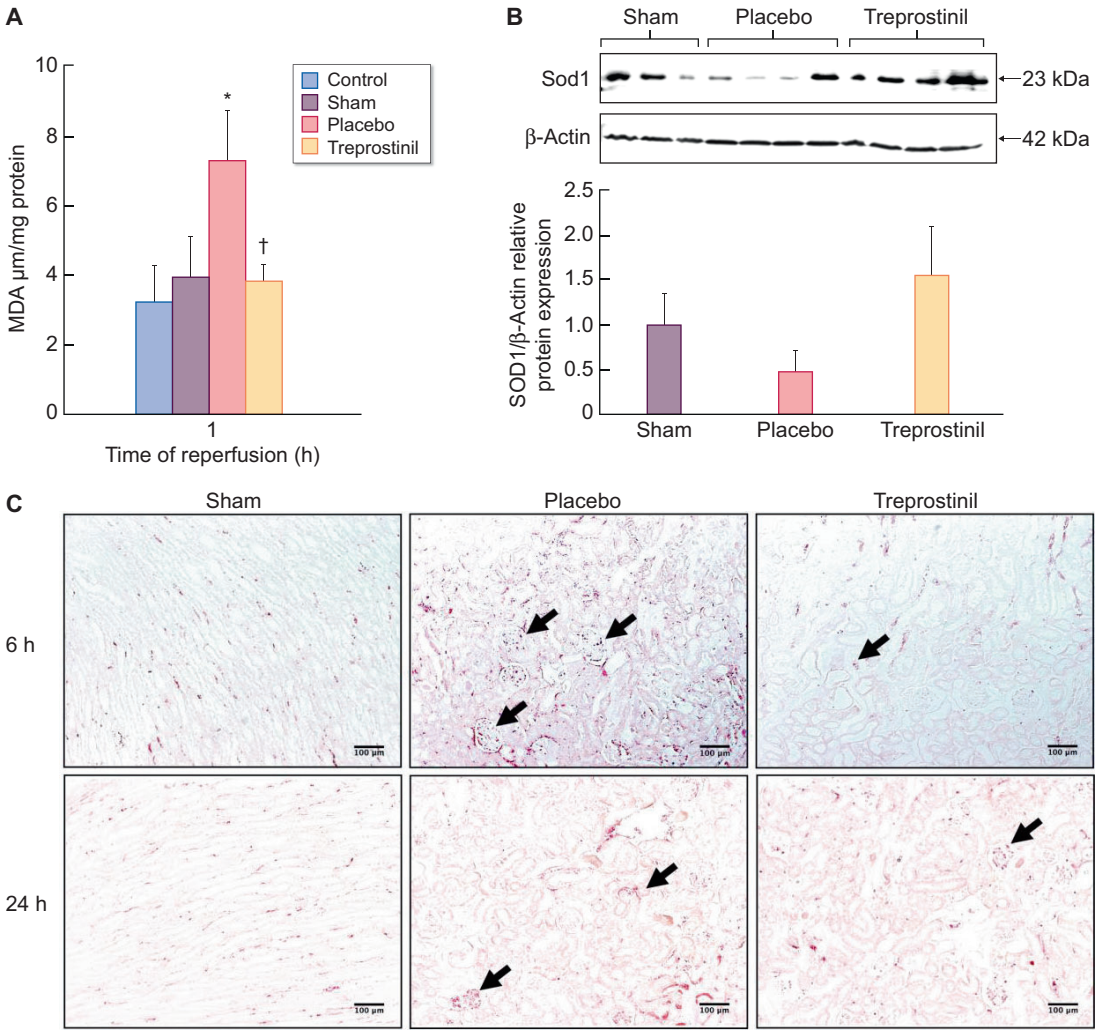


FIGURE 4: Treprostinil significantly attenuates renal lipid peroxidation and oxidative stress post-IRI. (**A**) Production of MDA in kidney tissue at 1 h post-reperfusion was measured using a thiobarbituric acid reactive substance assay; (**B**) protein expression of SOD1 at 6 h post-I/R injury by Western blot, normalized to β-actin; (**C**) neutrophil infiltration in kidney tissue at 6 and 24 h post-reperfusion using Naphthol AS-D Chloroacetate (Specific Esterase) staining (×100, scale bar = 100 µm). Data are presented as mean ± SEM. $^{*}P < 0.05$ versus sham, $^{\dagger}P < 0.05$ versus I/R-placebo ($n = 4$–8/group).

injury biomarkers and induction of pro-inflammatory cytokines induced by IRI and inhibited caspase-3-mediated apoptosis.

Renal IRI is well-known to be associated with an increase of SCr and BUN and corresponding kidney dysfunction. Early and encouraging indications of the treprostinil's efficacy were found by significantly reduced peak SCr and BUN levels as early as 6 h post-reperfusion and returned to baseline by 48 h post-reperfusion and remained at baseline throughout the 7-day study period. Iloprost, another FDA-approved PGI_2 analog, has been shown to reduce SCr and BUN levels and prolong survival from 60% to 100% in a 7-day study using a rat model of unilateral renal IRI [22, 35]. However, administration of iloprost is associated with complications [36] often limiting its clinical application. Although the various PGI_2 analogs display similar efficacies, treprostinil has a higher potency and longer half-life than other commercially available PGI_2 analogs [25], which allows for a lower and more tolerable dose and effective therapy. In the current study, 7-day survival was 100%, indicating that a longer period is needed to determine the efficacy of treprostinil on survival during AKI, for example, 28 days, as others have studied [37]. Nevertheless, and consistent with previous histological findings of significant morphological changes in early (4 h) post-renal IRI [38], we demonstrate by histological evaluation that renal IRI resulted in renal proximal tubular cell injury, nuclear pyknosis and apoptosis in I/R-placebo animals and that treprostinil reduced cellular apoptosis and accelerated cellular recovery in the early stage of renal IRI. Collectively these findings suggest that treprostinil plays an important role in preserving renal cellular structure and function while protecting the kidney against renal IRI.

The depletion of antioxidants during renal ischemic injury prevents ROS from being normally scavenged, leading to a massive generation of ROS. A rapid increase of ROS released by damaged mitochondria leads to renal injury by lipid peroxidation, which can be quantified by reactive aldehydes such as

Downloaded from https://academic.oup.com/ndt/advance-article/doi/10.1093/ndt/gfaa236/5959832 by University of New England user on 09 November 2020

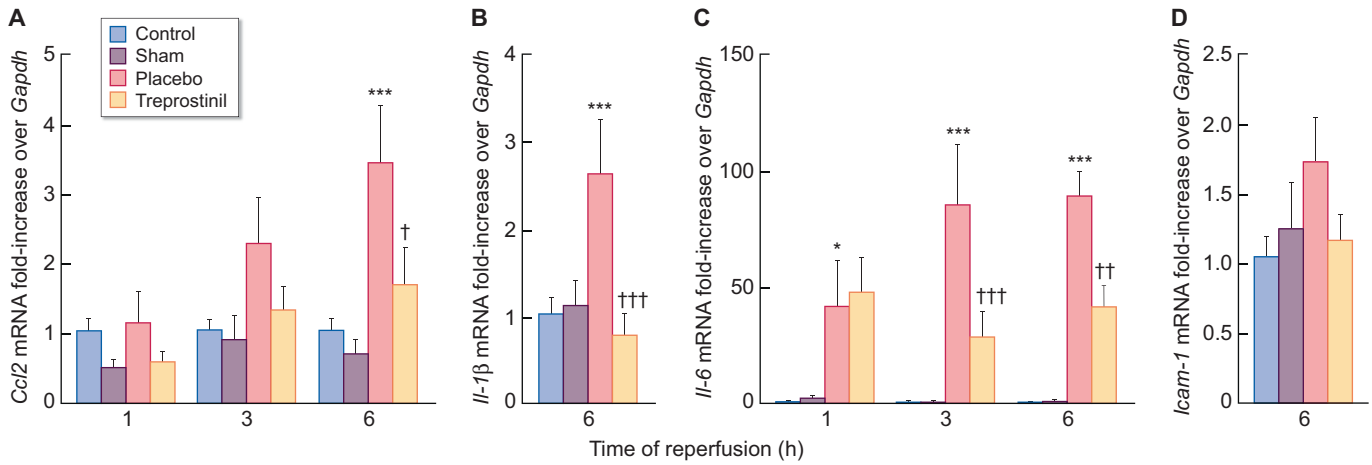


FIGURE 5: Renal IRI-induced pro-inflammatory cytokine upregulation is attenuated by treprostinil. The renal mRNA expression of pro-inflammatory cytokines at 1, 3 and 6 h post-reperfusion: (**A**) CCL2, (**B**) IL-1β, (**C**) IL-6 and (**D**) ICAM-1, measured by real-time qPCR, normalized to GAPDH and expressed as the fold increase over control kidney. Data are presented as mean ± SEM. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus control; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$ versus placebo ($n = 3$–8/group).

MDA. Consistent with previous increases in renal MDA levels early post-reperfusion [39, 40], treprostinil significantly reduced elevated renal MDA levels at 1 h post-IRI compared with I/R-placebo. These results suggest that treprostinil protected renal function via prevention of lipid peroxidation during the early stages of IRI. While Döslüoğlu *et al.* [35] found no difference in renal MDA levels between Iloprost- and placebo-treated animals exposed to renal IRI, MDA was measured only at 5 min and 7 days post-reperfusion, likely omitting peak MDA elevation, for example, 1 h post-reperfusion [40]. Also, a unilateral model of renal IRI was used and the likelihood of renal compensation by the uninjured kidney cannot be excluded. In addition, Yakut *et al.* [41] and Bozok *et al.* [42] found that renal MDA levels were significantly increased after 1 or 2 h of renal ischemia followed by 4 h of reperfusion in rabbit, which were significantly reduced by infusion of iloprost. The protective effects of PGI_2 against lipid peroxidation have also been reported in the setting of hepatic [43] and ovarian [44] IRI. Furthermore, in this study the protein expression of SOD1, a superoxide scavenger known to decrease during renal IRI [6], was significantly decreased at 6 h post-reperfusion in the I/R-placebo group, and treprostinil restored SOD1 protein to that of baseline, thereby protecting the kidney from oxidative stress.

An early pro-inflammatory response plays an essential role in contributing to renal IRI [45]. Vascular and epithelial proximal tubular cells are susceptible to oxidative stress, resulting in the release of pro-inflammatory cytokines and chemokines including IL-1β [46], MCP-1 (CCL2) [47], IL-6 [48] and adhesion molecules [3] during renal IRI. Similarly, in this study, renal mRNA levels of IL-1β, IL-6, CCL2 and ICAM-1 were elevated at 6 h post-reperfusion and treprostinil significantly suppressed upregulation of these cytokines early post-reperfusion. Consistent with the anti-inflammatory effects of treprostinil, Raychaudhuri *et al.* [49] demonstrated that treprostinil reduces human cytokine secretion by inhibiting human nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) nuclear translocation. Lastly, the well-known biomarkers of kidney injury KIM-1 and NGAL accumulate in renal tubular cells post-reperfusion. Our results show that renal IRI induced renal KIM-1 and NGAL mRNA expression at 24 h post-reperfusion, in parallel with upregulated NGAL protein expression. Treprostinil significantly reduced KIM-1 and NGAL accumulation in kidney tissue during renal IRI, thus confirming its renoprotection.

Characteristic features of apoptotic cell death during renal IRI include loss of proximal tubular cells and corresponding kidney dysfunction [10, 11]. Inhibition of apoptotic pathways has important clinical therapeutic prospects in treating renal IRI [50]. Furthermore, increased cleaved PARP-1 and caspase-3, -8 and -9 protein levels were found at 24 h post-reperfusion in I/R-placebo animals, suggesting that both the intrinsic and the extrinsic pathways are involved during renal IRI. Importantly, treprostinil inhibited the cleavage of PARP-1 and caspase-3, -8 and -9 proteins, suggesting that treprostinil has a critical role in reducing apoptosis during renal IRI. In agreement with our results, Hsu *et al.* [24] reported that PGI_2 overexpression prevents renal tubule cell apoptosis by inhibiting the cleavage of caspase-3, -8 and -9 via peroxisome proliferator-activated receptor alpha activation. Overall, our data indicate that treprostinil attenuated the loss of proximal tubular cells and apoptosis after renal IRI.

In conclusion, our study demonstrates the renoprotective effects of treprostinil during renal IRI, evidenced by improved renal function and reduced pro-inflammatory cytokines, chemokines, adhesion molecules, lipid peroxidation, oxidative stress and apoptosis, thereby accelerating recovery from renal IRI *in vivo*. The significance of this study is in providing data to support treprostinil, an FDA-approved PGI_2 analog, as a

Downloaded from https://academic.oup.com/ndt/advance-article/doi/10.1093/ndt/gfaa236/5959832 by University of New England user on 09 November 2020

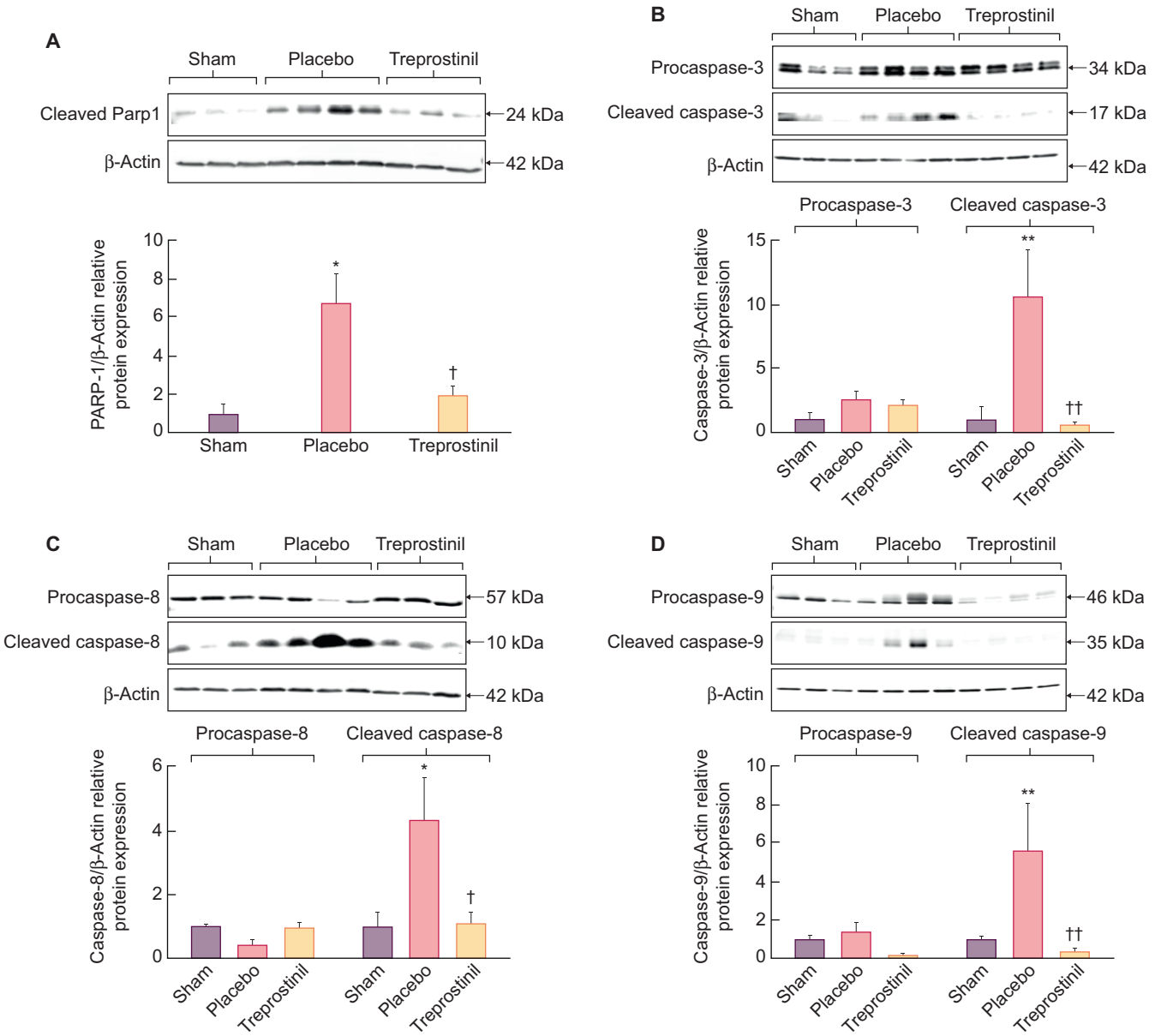


FIGURE 6: Elevated pro-apoptotic proteins during IRI are reduced by treprostinil. Renal protein expression of (**A**) PARP-1, (**B**) caspase-3, (**C**) caspase-8 and (**D**) caspase-9 at 24 h post-reperfusion, measured by Western blot, normalized to β-actin and expressed as a fold increase over sham kidney. Quantification of protein expression was performed using ImageJ. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ versus placebo ($n = 3$–4/group).

potential therapy for reducing renal IRI, which would be beneficial to improve long-term kidney function, for which no treatment is available.

FUNDING

The material presented herein is supported in part by an Institutional Development Award (U54GM115677) from the National Institute of General Medical Sciences of the National Institutes of Health, which funds Advance-CTR (Brown University) and the University of Rhode Island Core Lab (P20GM103430). The content is solely the responsibility of the authors and does not represent the official views of the National Institutes of Health.

AUTHORS' CONTRIBUTIONS

M.D. obtained data and provided data analysis and wrote the manuscript writing. E.T. performed animal surgeries. M.B. obtained data and provided data analysis and wrote the manuscript. F.A. and R.G. provided the study design and reviewed the manuscript. N.G. provided study conception and supervision, data collection and data analysis and wrote the manuscript. All authors approved the final version of the manuscript.

CONFLICT OF INTEREST STATEMENT

None declared. The results presented in this article have not been published previously in whole or part, except partly in abstract format.

Downloaded from https://academic.oup.com/ndt/advance-article/doi/10.1093/ndt/gfaa236/5959832 by University of New England user on 09 November 2020

REFERENCES

1. Lameire N, Van Biesen W, Vanholder R. Acute renal failure. *Lancet* 2005; 365: 417–430
2. Thadhani R, Pascual M, Bonventre JV. Acute renal failure. *N Engl J Med* 1996; 334: 1448–1460
3. Kelly KJ, Williams WW Jr, Colvin RB *et al.* Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury. *J Clin Invest* 1996; 97: 1056–1063.
4. Bonventre JV, Zuk A. Ischemic acute renal failure: an inflammatory disease? *Kidney Int* 2004; 66: 480–485
5. Szeto HH. Pharmacologic approaches to improve mitochondrial function in AKI and CKD. *J Am Soc Nephrol* 2017; 28: 2856–2865
6. Singh I, Gulati S, Orak JK *et al.* Expression of antioxidant enzymes in rat kidney during ischemia-reperfusion injury. *Mol Cell Biochem* 1993; 125: 97–104
7. Bulkley GB. Free radical-mediated reperfusion injury: a selective review. *Br J Cancer Suppl* 1987; 8: 66–73
8. Martin DR, Lewington AJ, Hammerman MR *et al.* Inhibition of poly(ADP-ribose) polymerase attenuates ischemic renal injury in rats. *Am J Physiol Regul Integr Comp Physiol* 2000; 279: R1834–1840
9. Jassem W, Heaton ND. The role of mitochondria in ischemia/reperfusion injury in organ transplantation. *Kidney Int* 2004; 66: 514–517
10. Havasi A, Borkan SC. Apoptosis and acute kidney injury. *Kidney Int* 2011; 80: 29–40
11. Sanz AB, Santamaria B, Ruiz-Ortega M *et al.* Mechanisms of renal apoptosis in health and disease. *J Am Soc Nephrol* 2008; 19: 1634–1642
12. Yang B, Lan S, Dieude M *et al.* Caspase-3 is a pivotal regulator of microvascular rarefaction and renal fibrosis after ischemia-reperfusion injury. *J Am Soc Nephrol* 2018; 29: 1900–1916
13. Bolger PM, Eisner GM, Ramwell PW *et al.* Renal actions of prostacyclin. *Nature* 1978; 271: 467–469
14. Perico N, Cattaneo D, Sayegh MH *et al.* Delayed graft function in kidney transplantation. *Lancet* 2004; 364: 1814–1827
15. Gerber JG, Branch RA, Nies AS *et al.* Prostaglandins and renin release: II. Assessment of renin secretion following infusion of PGI2, E2 and D2 into the renal artery of anesthetized dogs. *Prostaglandins* 1978; 15: 81–88
16. Finn WF, Hak LJ, Grossman SH. Protective effect of prostacyclin on postischemic acute renal failure in the rat. *Kidney Int* 1987; 32: 479–487
17. Tobimatsu M, Ueda Y, Toyoda K *et al.* Effect of a stable prostacyclin analogue on canine renal allograft rejection. *Ann Surg* 1987; 205: 199–202
18. Fujita T, Fuke Y, Satomura A *et al.* PGI_2 analogue mitigates the progression rate of renal dysfunction improving renal blood flow without glomerular hyperfiltration in patients with chronic renal insufficiency. *Prostaglandins Leukot Essent Fatty Acids* 2001; 65: 223–227
19. Nielsen CB, Bech JN, Pedersen EB. Effects of prostacyclin on renal haemodynamics, renal tubular function and vasoactive hormones in healthy humans. A placebo-controlled dose-response study. *Br J Clin Pharmacol* 2003; 44: 471–476
20. Johannes T, Ince C, Klingel K *et al.* Iloprost preserves renal oxygenation and restores kidney function in endotoxemia-related acute renal failure in the rat. *Crit Care Med* 2009; 37: 1423–1432
21. Yamaguchi S, Inada C, Tamura M *et al.* Beraprost sodium improves survival rates in anti-glomerular basement membrane glomerulonephritis and 5/6 nephrectomized chronic kidney disease rats. *Eur J Pharmacol* 2013; 714: 325–331
22. Sahsivar MO, Narin C, Kiyici A *et al.* The effect of iloprost on renal dysfunction after renal I/R using cystatin C and beta2-microglobulin monitoring. *Shock* 2009; 32: 498–502
23. Goto Y, Yamaguchi S, Tamura M *et al.* A prostacyclin analog prevents the regression of renal microvascular network by inhibiting mitochondria-dependent apoptosis in the kidney of rat progressive glomerulonephritis. *Prostaglandins Other Lipid Mediat* 2014; 112: 16–26
24. Hsu YH, Chen CH, Hou CC *et al.* Prostacyclin protects renal tubular cells from gentamicin-induced apoptosis via a PPARα-dependent pathway. *Kidney Int* 2008; 73: 578–587
25. Kumar P, Thudium E, Laliberte K *et al.* A comprehensive review of treprostinil pharmacokinetics via four routes of administration. *Clin Pharmacokinet* 2016; 55: 1495–1505
26. Mori K, Lee HT, Rapoport D *et al.* Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. *J Clin Invest* 2005; 115: 610–621
27. Han WK, Bailly V, Abichandani R *et al.* Kidney injury molecule-1 (KIM-1): a novel biomarker for human renal proximal tubule injury. *Kidney Int* 2002; 62: 237–244
28. Mishra J, Ma Q, Prada A *et al.* Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol* 2003; 14: 2534–2543
29. Takada M, Nadeau KC, Shaw GD *et al.* The cytokine-adhesion molecule cascade in ischemia/reperfusion injury of the rat kidney. Inhibition by a soluble P-selectin ligand. *J Clin Invest* 1997; 99: 2682–2690
30. Zheng J, Devalaraja-Narashimha K, Singaravelu K *et al.* Poly(ADP-ribose) polymerase-1 gene ablation protects mice from ischemic renal injury. *Am J Physiol Renal Physiol* 2005; 288: F387–398
31. Ow YP, Green DR, Hao Z *et al.* Cytochrome c: functions beyond respiration. *Nat Rev Mol Cell Biol* 2008; 9: 532–542
32. Linkermann A, Chen G, Dong G *et al.* Regulated cell death in AKI. *J Am Soc Nephrol* 2014; 25: 2689–2701
33. Tobimatsu M, Ueda Y, Saito S *et al.* Effects of a stable prostacyclin analog on experimental ischemic acute renal failure. *Ann Surg* 1988; 208: 65–70
34. Turney JH, Williams LC, Fewell MR *et al.* Platelet protection and heparin sparing with prostacyclin during regular dialysis therapy. *Lancet* 1980; 316: 219–222
35. Döslüoğlu HH, Aktan Ö, Yeğen C *et al.* The cytoprotective effects of verapamil and iloprost (ZK 36374) on ischemia/reperfusion injury of kidneys. *Transpl Int* 1993; 6: 138–142
36. Polignano R, Baggiore C, Falciani F *et al.* Efficacy, safety and feasibility of intravenous iloprost in the domiciliary treatment of patients with ischemic disease of the lower limbs. *Eur Rev Med Pharmacol Sci* 2016; 20: 3720–3726
37. Jongbloed F, de Bruin RW, Pennings JL *et al.* Preoperative fasting protects against renal ischemia-reperfusion injury in aged and overweight mice. *PLoS One* 2014; 9: e100853
38. Gunthner R, Kumar VR, Lorenz G *et al.* Pattern-recognition receptor signaling regulator mRNA expression in humans and mice, and in transient inflammation or progressive fibrosis. *Int J Mol Sci* 2013; 14: 18124–18147
39. Ergin B, Zuurbier CJ, Bezemer R *et al.* Ascorbic acid improves renal microcirculatory oxygenation in a rat model of renal I/R injury. *J Transl Intern Med* 2015; 3: 116–125
40. Szeto HH, Liu S, Soong Y *et al.* Mitochondria-targeted peptide accelerates ATP recovery and reduces ischemic kidney injury. *J Am Soc Nephrol* 2011; 22: 1041–1052
41. Yakut N, Yasa H, Bahriye Lafci B *et al.* The influence of levosimendan and iloprost on renal ischemia-reperfusion: an experimental study. *Interact Cardiovasc Thorac Surg* 2007; 7: 235–239
42. Bozok Ş, Ilhan G, Yilmaz Y *et al.* Effects of Iloprost and pentoxifylline on renal ischemia-reperfusion in rabbit model. *Eur J Med Res* 2012; 17: 295–299
43. Chen HM, Chen MF, Shyr MH. Prostacyclin analogue (OP-2507) attenuates hepatic microcirculatory derangement, energy depletion, and lipid peroxidation in a rat model of reperfusion injury. *J Surg Res* 1998; 80: 333–338
44. Ozat M, Gungor T, Barun S *et al.* The effects of iloprost, a prostacyclin analogue, in experimental ischaemia/reperfusion injury in rat ovaries. *Exp Toxicol Pathol* 2009; 61: 519–527
45. Rabb H, Griffin MD, McKay DB *et al.* Inflammation in AKI: current understanding, key questions, and knowledge gaps. *J Am Soc Nephrol* 2016; 27: 371–379
46. Haq M, Norman J, Saba SR *et al.* Role of IL-1 in renal ischemic reperfusion injury. *J Am Soc Nephrol* 1998; 9: 614–619
47. Kielar ML, John R, Bennett M *et al.* Maladaptive role of IL-6 in ischemic acute renal failure. *J Am Soc Nephrol* 2005; 16: 3315–3325
48. Rice JC, Spence JS, Yetman DL *et al.* Monocyte chemoattractant protein-1 expression correlates with monocyte infiltration in the post-ischemic kidney. *Ren Fail* 2002; 24: 703–723
49. Raychaudhuri B, Malur A, Bonfield TL *et al.* The prostacyclin analogue treprostinil blocks NFκB nuclear translocation in human alveolar macrophages. *J Biol Chem* 2002; 277: 33344–33348
50. Nicholson DW. From bench to clinic with apoptosis-based therapeutic agents. *Nature* 2000; 407: 810–816

Received: 6.1.2020; Editorial decision: 22.6.2020

Downloaded from https://academic.oup.com/ndt/advance-article/doi/10.1093/ndt/gfaa236/5959832 by University of New England user on 09 November 2020