



## Research Paper

## Renovascular effects of inorganic nitrate following ischemia-reperfusion of the kidney



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## ABSTRACT

**Background:** Renal ischemia-reperfusion (IR) injury is a common cause of acute kidney injury (AKI), which is associated with oxidative stress and reduced nitric oxide (NO) bioactivity and increased risk of developing chronic kidney disease (CKD) and cardiovascular disease (CVD). New strategies that restore redox balance may have therapeutic implications during AKI and associated complications.

**Aim:** To investigate the therapeutic value of boosting the nitrate-nitrite-NO pathway during development of IR-induced renal and cardiovascular dysfunction.

**Methods:** Male C57BL/6 J mice were given sodium nitrate (10 mg/kg, i. p) or vehicle 2 h prior to warm ischemia of the left kidney (45 min) followed by sodium nitrate supplementation in the drinking water (1 mmol/kg/day) for the following 2 weeks. Blood pressure and glomerular filtration rate were measured and blood and kidneys were collected and used for biochemical and histological analyses as well as renal vessel reactivity studies. Glomerular endothelial cells exposed to hypoxia-reoxygenation, with or without angiotensin II, were used for mechanistic studies.

**Results:** IR was associated with reduced renal function and slightly elevated blood pressure, in combination with renal injuries, inflammation, endothelial dysfunction, increased Ang II levels and Ang II-mediated vasoreactivity, which were all ameliorated by nitrate. Moreover, treatment with nitrate (in vivo) and nitrite (in vitro) restored NO bioactivity and reduced mitochondrial oxidative stress and injuries.

**Conclusions:** Acute treatment with inorganic nitrate prior to renal ischemia may serve as a novel therapeutic approach to prevent AKI and CKD and associated risk of developing cardiovascular dysfunction.

## 1. Introduction

Ischemia-reperfusion (IR) injury of the kidney, associated with transplantation, cardiac bypass surgery and shock, is a major risk for developing acute kidney injury (AKI) and subsequent chronic kidney

disease (CKD) [1]. Underlying mechanisms are complex and involve reperfusion-associated oxidative stress, nitric oxide deficiency and immune cell activation, which together leads to endothelial dysfunction [2, 3]. Ongoing research has been focused on finding new and effective therapies for preventing and treating AKI and its progression to CKD,

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such as remote ischemic preconditioning, temporary local hypothermia as well as new pharmacological interventions [3,4].

The gaseous signaling molecule nitric oxide (NO) importantly contributes to the regulation of cardiovascular and renal homeostasis and has been shown to play a protective role during IR injuries [5,6]. However, during the ischemic event, the lack of oxygen compromises the function of endothelial NO synthase (eNOS), which may contribute to the “no-reflow phenomenon” in the ischemic tissue during the reperfusion phase. This in turn propagates endothelial and tubular epithelial cell injuries [7], which contribute to the development of reduced kidney function. In addition to hypoxia during the ischemic period, excessive production of reactive oxygen species (ROS) generated in excess by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and mitochondria during the reperfusion phase scavenges NO [8]. New approaches that reduce oxidative stress and maintain NO bioactivity may have therapeutic value in the battle against IR-induced kidney failure.

Inorganic nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ) were previously viewed as relatively inert products derived from NO metabolism. However, research conducted for more than two decades has shown that these anions can undergo bioconversion, via serial reductions, and again form NO and other bioactive nitrogen oxide species [9]. The activity of this alternative nitrate-nitrite-NO pathway is enhanced during conditions with hypoxia and low pH when the classical NOS system can become dysfunctional [10].

The diet, in addition to the NOS system, is an important source of circulating nitrate and nitrite, due to high levels of nitrate for example in green leafy vegetables. Supplementation with inorganic nitrate and nitrite has been associated with favorable cardiovascular effects, including blood pressure reduction, in both experimental and clinical studies [11]. Organ protective effects have also been shown in experimental IR injury models of the liver [12], brain [13], lung [14], and heart [12,15]. However, the potential therapeutic value of inorganic nitrate and nitrite in kidney IR injury remains controversial. As recently reviewed [5], variable outcomes in IR models, using nitrite therapy, may be due to differences in the dosage used, route of administration, treatment duration, as well as the experimental model itself [16,17]. Several experimental studies have demonstrated protective effects of dietary nitrate supplementation in models of cardiorenal disease, via mechanisms that involve dampening of oxidative stress and improvement of NO bioactivity as well as modulation of inflammatory responses [5]. To date, there is limited knowledge about the effects of inorganic nitrate therapy in renal IR injury. We have previously demonstrated that chronic dietary pretreatment with nitrate attenuated subsequent IR-induced renal injuries [18]. However, there is less knowledge regarding the potential value of boosting the nitrate-nitrite-NO pathway at the onset of IR to reduce the risk of developing AKI and CKD. If protective, this could have wide clinical applications in patients at risk of developing AKI for example those undergoing major surgery.

In this study we used a mouse model of renal IR injury to investigate the potential benefits of acute nitrate treatment immediately prior to the ischemic event. This was combined with mechanistic *ex vivo* vessel studies and *in vitro* cell culture experiments with nitrite treatment. We hypothesized that boosting the nitrate-nitrite-NO pathway would promote kidney protection against IR injury via modulation of oxidative stress and NO bioactivity as well as mitochondrial function.

## 2. Methods

### 2.1. Reagents

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich, Sweden.

### 2.2. Animals and kidney ischemia-reperfusion model

C57BL/6 J mice (Male, 10 weeks) were obtained from Janvier Laboratories (France) and housed in our animal facilities at the Karolinska Institutet under climate-controlled conditions with a 12-h light/dark cycle and provided with standard rodent chow and tap water. All animal procedures were approved by the Regional Ethical Committee for Animal Experiments (Protocol ID: N139/15) and were carried out according to the National Institutes of Health (NIH) guidelines and with the EU Directive 2010/63/EU for the conduct of experiments in animals.

After 1 week of acclimatization, mice were administered placebo (NaCl) or sodium nitrate ( $\text{NaNO}_3$ ) intraperitoneally (10 mg/kg) 2 h prior to unilateral ischemia of the left kidney. The right kidney was inspected but otherwise left intact. Sham surgeries were performed in the same way, but without clamping the left kidney. Mice were anesthetized with isoflurane and the body temperature was kept at  $37 \pm 0.5$  °C using a heating lamp and a self-monitored heating pad throughout the surgery. After the surgery, mice were treated with either sodium nitrate (1 mmol/kg/day) or placebo for 2 weeks until termination.

### 2.3. Blood pressure measurement

Blood pressure was measured in mice using a non-invasive tail-cuff method before termination. Mice were conditioned on a warm plate at 35 °C for 10 min and then placed in plastic restrainers. A cuff with a pneumatic pulse sensor was attached to the tail, and blood pressure values were recorded with the CODA system (Kent Scientific, Torrington, CT, USA). Animals were trained to the restrainers on the warming plate at least three days before blood pressure recordings. Systolic, diastolic and mean arterial pressure were collected over 3 consecutive days and individual median of all accepted values were compiled for assessment.

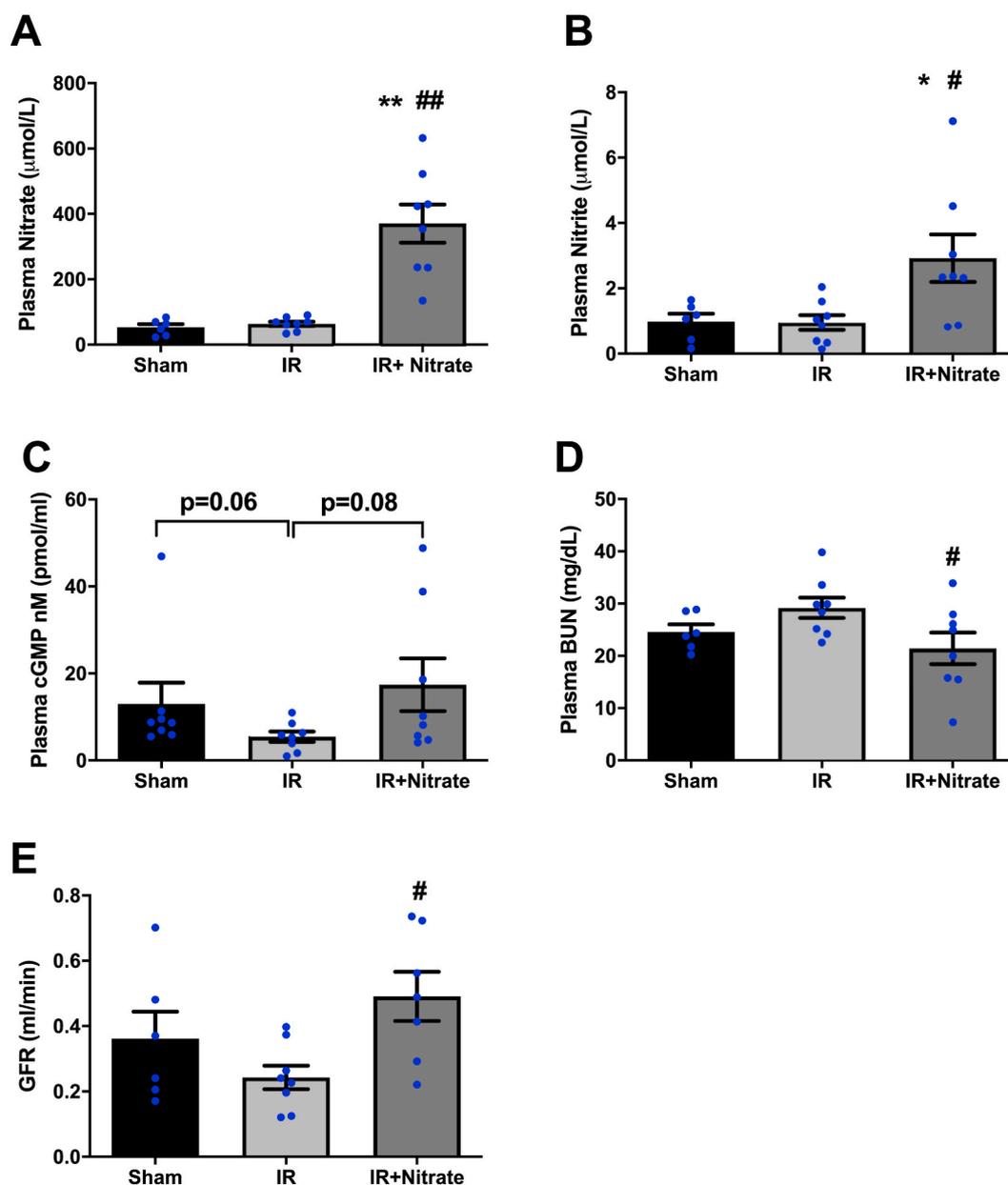
### 2.4. Tissue harvest

Before termination, the mice were put into metabolic cages to collect urine for calculation of glomerular filtration rate (GFR). Animals were then anesthetized with isoflurane and blood samples were collected through the inferior vena cava. All samples were centrifuged immediately at  $6000 \times g$ , 4 °C for 5 min in the presence of EDTA (Sigma-Aldrich, Stockholm, Sweden), final concentration of 2 mM. Plasma samples were transferred into new tubes and immediately snap-frozen in dry ice and finally stored at  $-80$  °C. Kidneys were extracted and cut into several parts for *ex vivo* vascular function experiments (interlobar arteries and afferent arterioles), histology (HE and PAS staining), and quantification of apoptosis and inflammation (Tissue-Tek optimum cutting temperature (OCT) compound embedding and frozen for TUNEL and F4/80 staining).

### 2.5. Measurements of nitrate, nitrite, cGMP, creatinine, blood urea nitrogen (BUN) and angiotensin II

Nitrite and nitrate were analyzed by HPLC (ENO-20) as described previously [19]. Plasma samples (10  $\mu\text{l}$ ) were injected into the HPLC system with a Hamilton syringe. Subsequently, nitrite and nitrate were separated by reverse phase/ion exchange chromatography followed by nitrate reduction to nitrite by cadmium and reduced copper. Nitrite was derivatized using Griess reagent to form diazo compounds and detected at 540 nm. To prevent degradation of cGMP, the plasma was transferred to tubes containing a PDE inhibitor, IBMX (3-Isobutyl-1-methylxanthine; Sigma-Aldrich #15879) to give a final concentration (10  $\mu\text{M}$ ). Samples were thereafter frozen and stored at  $-80$  °C before analyzing cGMP with an ELISA kit (GE Healthcare #RPN226), according to the manufacturers' instructions.

The plasma and urine creatinine levels were detected by using Creatinine Colorimetric Assay Kits (Cayman Chemical, #700460 and



**Fig. 1.** Effects of nitrate on nitric oxide bioactivity and kidney function following ischemia-reperfusion of the kidney. Plasma nitrate, nitrite, and cGMP levels (A–C), and BUN and GFR (D–E) following ischemia-reperfusion (IR) in mice treated with placebo or nitrate. Data are shown as mean  $\pm$  SEM. \*, \*\* $p$  < 0.05 and 0.01 vs. Sham group; #, ## $p$  < 0.05 and 0.01 vs. IR group, respectively.  $n$  = 6–8/group.

#500701). Creatinine clearance formula ( $CL_{Cr} = \text{Urine}_{Cr} \times \text{Urine flow} / \text{Plasma}_{Cr}$ ) was used to estimate GFR. The plasma BUN levels were detected by using BUN Colorimetric Detection Kit (Invitrogen, #EIA-BUN). The levels of Ang II in plasma and kidney tissues were measured by using Ang II ELISA kit (Cloud-Clone Corp., #CEA005Mu) according to the manufacturers' instructions. However, the preparation of the tissue extract differed slightly from the recommendation. 10 vol 50 mM HCl in 50% EtOH were added to the tissue and heated up for 10 min at 100 °C, centrifuged 10 000 $\times$ g 10 min. Thereafter the samples were frozen and stored at  $-80$  °C before analyzed. All absorbance readings were done in SpetraMax iD3 from Molecular Devices.

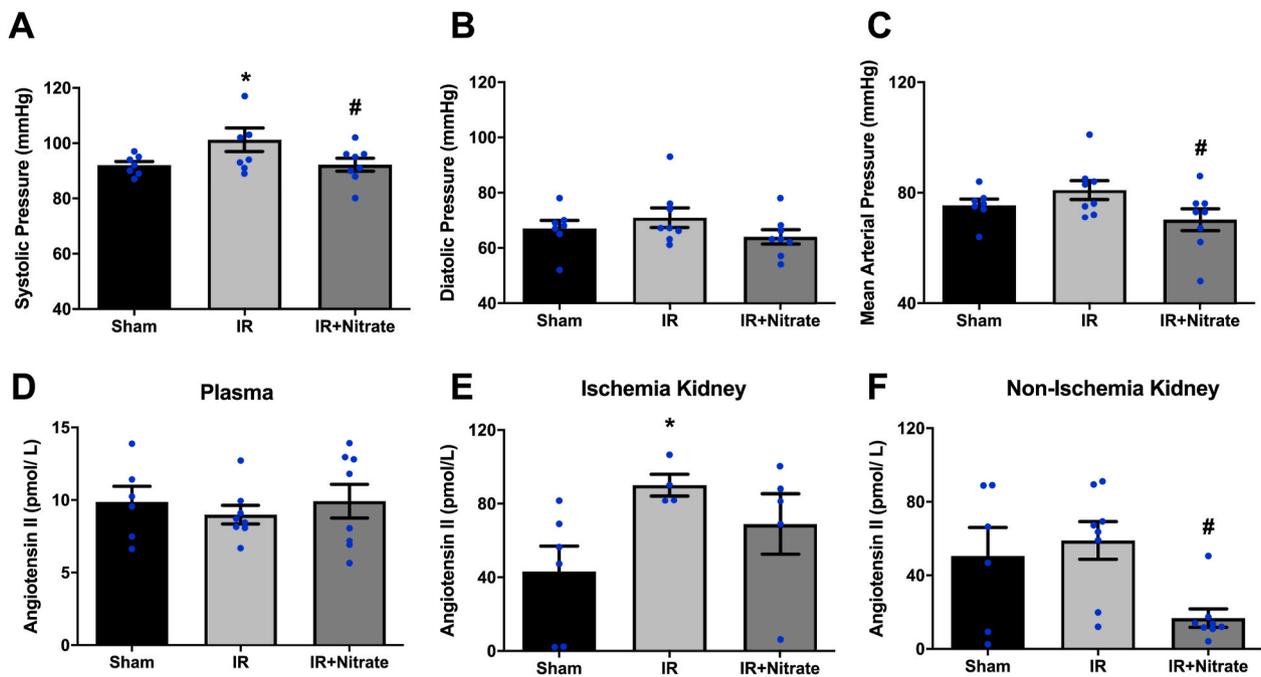
## 2.6. Isolation and perfusion of interlobar arteries

Kidneys were harvested after sacrifice and kept in ice-cold physiological salt solution (PSS) until isolation of the interlobar arteries. Renal interlobar arteries were dissected (2 mm in length) and mounted in a

myograph chamber (Model 620 M, Danish Myo Technology, Denmark) with PSS as described previously [20]. Isometric tension was recorded with a Powerlab system (Powerlab 4/30, AD Instruments, Australia). After being mounted, the vessels were equilibrated for 20 min in PSS bubbling with carbogen (95% O<sub>2</sub>; 5% CO<sub>2</sub>) at 37 °C, pH 7.4. Then resting tension of the arteries was set as described previously [21], which is in accordance with Mulvany's normalization procedure [22]. After a second equilibration, 0.1 mol/L KCl was applied to assess arterial ring viability.

## 2.7. Isolation and micro-perfusion of afferent arterioles

C57BL/6 J mice were anesthetized with inhaled isoflurane and kidneys were removed and sliced quickly as described previously [23–25]. Kidney slices were placed in ice-cold Dulbecco's modified Eagle's medium (DMEM). A single afferent arteriole with attached glomerulus was micro-dissected under a stereomicroscope and transferred to a



**Fig. 2.** Effects of nitrate on blood pressure and Ang II levels following ischemia-reperfusion of the kidney. Systolic, diastolic and mean arterial pressure (A–C), and plasma and intrarenal levels of Ang II (D–F) following ischemia-reperfusion (IR) in mice treated with placebo or nitrate. Data are shown as mean  $\pm$  SEM. \* $p < 0.05$  vs. sham group, # $p < 0.05$  vs IR group,  $n = 4$ –8/group.

temperature-regulated chamber on the stage of an inverted microscope. The glomerulus was fixed with a holding micropipette and the afferent arteriole was cannulated and perfused with a set of micropipettes. The intraluminal pressure of the perfused afferent arteriole was maintained at 60 mmHg during experiments. Time for dissection and the following micro-perfusion was limited to 120 min after the mouse was sacrificed as described previously [26]. After 30 min equilibration period at 37 °C, cumulative dose response curves to Ang II ( $10^{-12}$  to  $10^{-8}$  mol/L) were obtained. Each concentration of Ang II was perfused for 2 min, the constrictive response was recorded and then the diameter of the afferent arteriole was measured.

## 2.8. Histological examination

The kidney samples were harvested and fixed in 4% paraformaldehyde solution. Fixed kidney tissues were embedded in paraffin and thereafter sliced and stained with Hematoxylin-Eosin (H&E) or Periodic Acid Schiff (PAS) for histological assessment. Four randomly selected animals from each experimental group were used for analysis. Ten randomly chosen fields from each section were captured under 400 $\times$  magnification. All morphometric analyses were performed in a blinded manner.

## 2.9. TUNEL staining

OCT-embedded kidney samples were used for TUNEL staining. 6- $\mu$ m thick sections were incubated with 20  $\mu$ g/mL proteinase K for 15 min at room temperature, and TUNEL reaction were performed by using the Click-iT™ Plus TUNEL Assay (Invitrogen C10618, USA) following the manufacturer's instructions. At the end of the protocol the DAPI staining was performed. For quantification, three animals and three field area of image (200  $\times$ ) were selected randomly and photographed using the Zeiss LSM800-Airy confocal microscope, the positive signals were quantified using ImageJ/Fiji software, and were calculated as the ratio of the positive signals to DAPI.

## 2.10. Tissue immunofluorescence staining and confocal microscopy (F4/80)

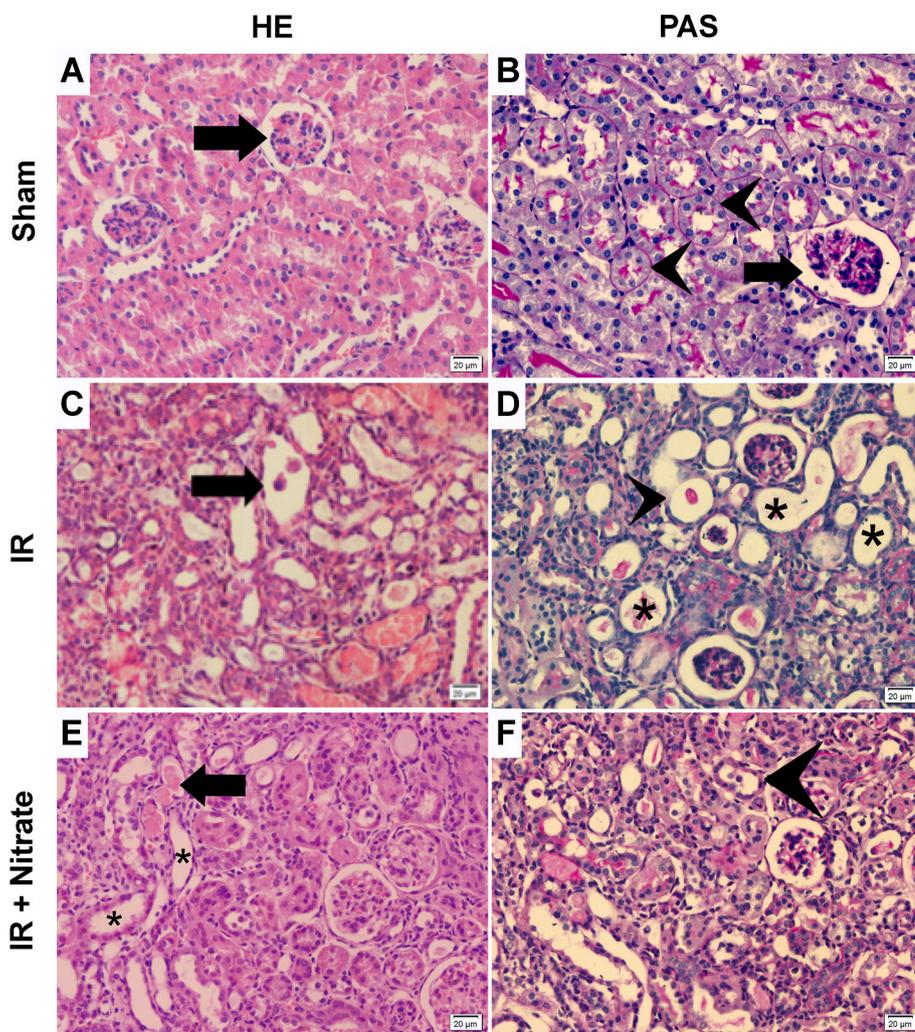
Kidneys were frozen in OCT (Tissue-Tek, Torrance, CA, USA) and sections (6  $\mu$ m) were prepared and further processed. Frozen sections were washed with 1  $\times$  PBS and blocked with 2% BSA in PBS for 30 min and then incubated with anti-F4/80 (1:50, BIO-RAD Cl:A3-1) overnight in a 4 °C cold-room, and subsequently with a secondary antibody (1:200 Cell signaling Tech #4417) at room temperature for 1 h, followed by counterstaining with 300 nmol/L DAPI (4',6'-diamidino-2-phenyl-indole, dihydrochloride, Invitrogen) for 3 min. The immunofluorescence signals were visualized under the Zeiss LSM800-Airy confocal microscope. three animals and three field area of image (200  $\times$ ) were selected randomly. F4/80 positive signals were quantified using ImageJ/Fiji software, and were calculated as the ratio of the positive signals to DAPI.

## 2.11. Cell culture

Rat glomerular endothelial cells (GECs) (DSMZ ACC262, Germany) were cultured in RPMI 1640 (Gibco 21870-076) medium with 10% fetal bovine serum and 2 mM L-Glutamine (Gibco 25030-082), penicillin and streptomycin (50 mg/L, Gibco 15140-122). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. In hypoxia experiments, cells were incubated in HBSS (Gibco 14025-092) instead of serum-free medium in a chamber with 1% oxygen at 37 °C for 2 h. Cells were pre-incubated with nitrite (10  $\mu$ M), with/without the xanthine oxidoreductase (XOR) inhibitor febuxostat (100 nM), for 30 min before hypoxia. Cell viability was evaluated by PrestoBlue® assay (Invitrogen, A13261 and A13262), and cell mortality was assessed by Trypan Blue exclusion assay (Sigma, T8154-20 ML).

## 2.12. Detection of mitochondrial ROS

Mitochondrial superoxide levels were detected with a fluorogenic dye (MitoSOXTM, Invitrogen M36008). After treatment, the endothelial cells were incubated with 2  $\mu$ mol/L MitoSox in culture medium at 37 °C for 10 min. Then the cells were washed with 1 $\times$  HBSS twice and the



**Fig. 3.** Effects of nitrate on renal histopathology following ischemia-reperfusion of the kidney. Photomicrographs of the left renal cortex from sham and ischemia-reperfusion (IR) mice treated with placebo or nitrate (A–F). Normal renal parenchyma, without glomerular changes, preserved urinary space (arrow), tubules without atrophy or dilation, absence of necrosis and inflammatory cells, and preserved tubular basement membrane (arrowhead) (A–B). Left renal parenchyma submitted to IR showing loss of organ architecture, tubular ectasia (\*), tubules with cells in necrosis and lumen filled of cellular debris (arrow), rupture of the tubular basement membrane (arrowhead) and interstitial inflammatory cells (C–D). Left renal parenchyma submitted to IR and treatment with nitrate, presenting necrosis and tubular ectasia (\*), presence of granular cylinders in the tubular lumen (arrow), regenerating cells and preserved tubular membranes (arrowhead) (E–F). Magnification for all photomicrographs presented is  $\times 400$ .

**Table 1**  
Quantification of the histopathological findings (%) – Mean  $\pm$  SEM.

	Sham	IR	IR + Nitrate
Necrosis	0 $\pm$ 0	40.31 $\pm$ 3.29*	23.62 $\pm$ 2.43*#
Tubular regeneration	0 $\pm$ 0	7.80 $\pm$ 1.46*	14.39 $\pm$ 2.11*#
Necrosis/regeneration ratio	0 $\pm$ 0	25.76 $\pm$ 7.09*	60.88 $\pm$ 5.33*#
Tubular ectasia	0 $\pm$ 0	33.87 $\pm$ 3.13*	12.71 $\pm$ 1.78*#
Rupture of tubular basement membrane	0 $\pm$ 0	24.91 $\pm$ 2.80*	8.35 $\pm$ 1.07*#

\*p < 0.05 vs Sham group; #p < 0.05 vs IR group.

fluorescent signal was measured (SpectraMax iD3 reader, Molecular Devices, USA) and normalized to cell viability.

### 2.13. Immunoblotting analysis

Frozen kidney samples or endothelial cells were lysed in buffer containing 10 mmol/L Tris-HCl pH 8, 150 mmol/L NaCl, 5 mmol/L EDTA, 60 mmol/L *N*-octyl-glucoside, 1% Triton X-100, protease inhibitor cocktail and protein phosphatase inhibitors. The cell or tissue lysates were centrifuged 5 min at 10,000 $\times$ g at 4  $^{\circ}$ C. The supernatants/proteins were transferred into new tubes and quantified by using the Bio-Rad

protein assay. Protein extracts containing equivalent amount of proteins were analyzed by using 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to an immobilon polyvinylidene difluoride membrane for 1 h at 300 mA. Membranes were blocked in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20 (TBS-T) containing 5% fat-free milk powder for 1 h and immunodetected with antibodies to *anti*-TFAM (1:2000, ab131607), *anti*-OXPHOS (1:1000, ab110413), *anti*-vinculin (1:5000, ab129002) (Abcam Cambridge, UK) and *anti*-Cleaved caspase-3 (1:1000, #9664) (Cell Signaling Technology, Beverly, MA, USA). All secondary antibodies for immunoblot analysis were from Cell Signaling Technology. For Western blots, uncropped, annotated, full-length images with MW markers are shown in the Supplementary File.

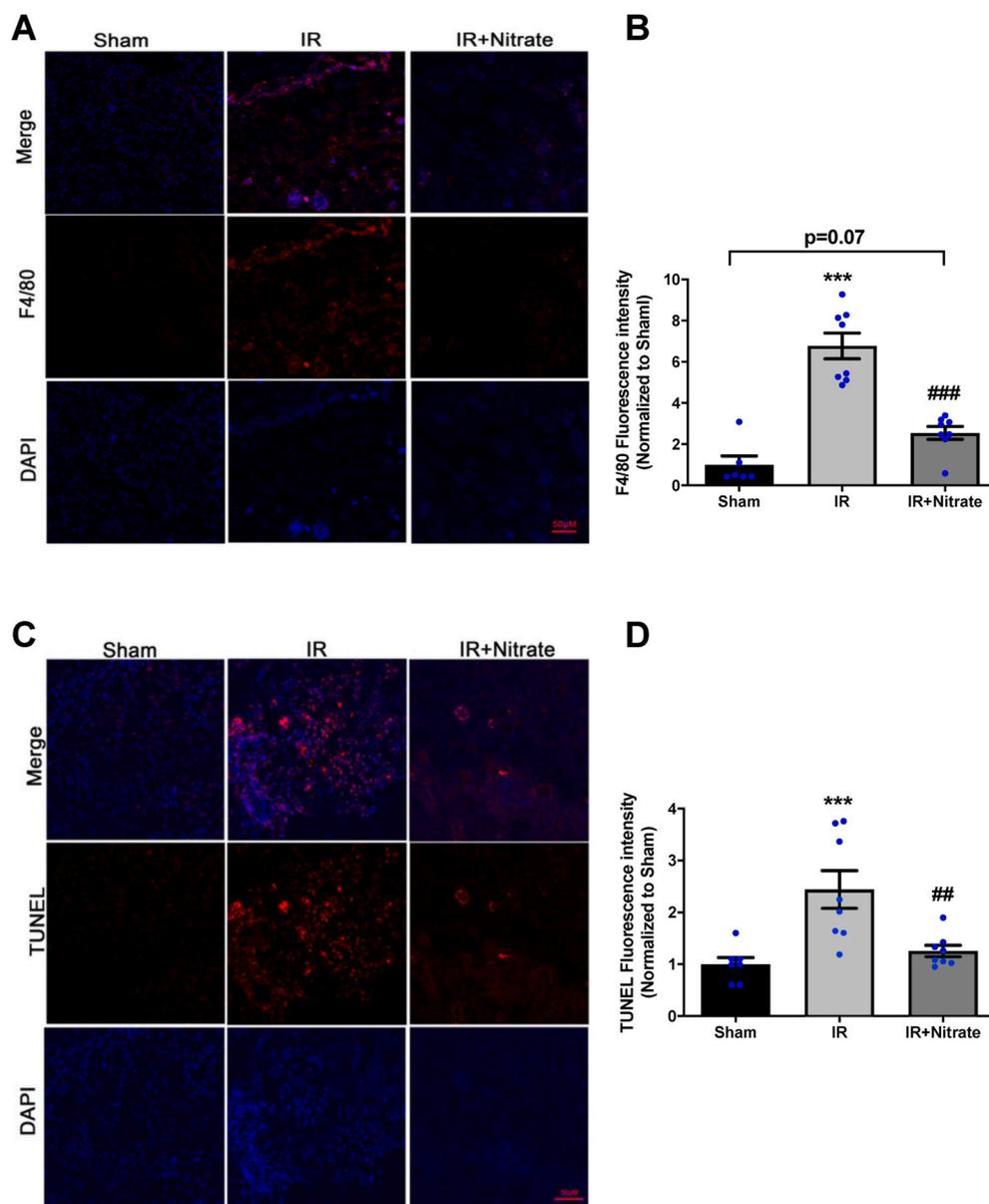
### 2.14. Statistical analysis

Multiple comparisons among groups were analyzed by one- or two-way ANOVA followed by recommended post-hoc test. Data are presented as mean  $\pm$  SEM. All statistical calculations were made using Graphpad Prism 8.0. Statistical significance was defined as p < 0.05.

## 3. Results

### 3.1. Nitrate supplementation increases markers of NO bioactivity and attenuates renal dysfunction following IR

Levels of nitrate and nitrite were increased and cGMP trended to be



**Fig. 4. Effects of nitrate on renal inflammation and apoptosis following ischemia-reperfusion of the kidney.** Immunostaining images of macrophage infiltration (F4/80, red) in kidneys following ischemia-reperfusion (IR), counterstained with nuclear marker DAPI (scale bar, 50  $\mu$ m) (A). Quantification of normalized F4/80 intensity (B). Immunostaining images of TUNEL (red) in kidneys following IR injury (counterstained with nuclear marker DAPI, scale bar, 50  $\mu$ m) (C). Quantification of normalized TUNEL intensity (D). Data in B and D are shown as mean  $\pm$  SEM. \*\*\* $p$  < 0.001 vs. sham group, ### $p$  < 0.0001 vs IR group,  $n$  = 6–8/group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

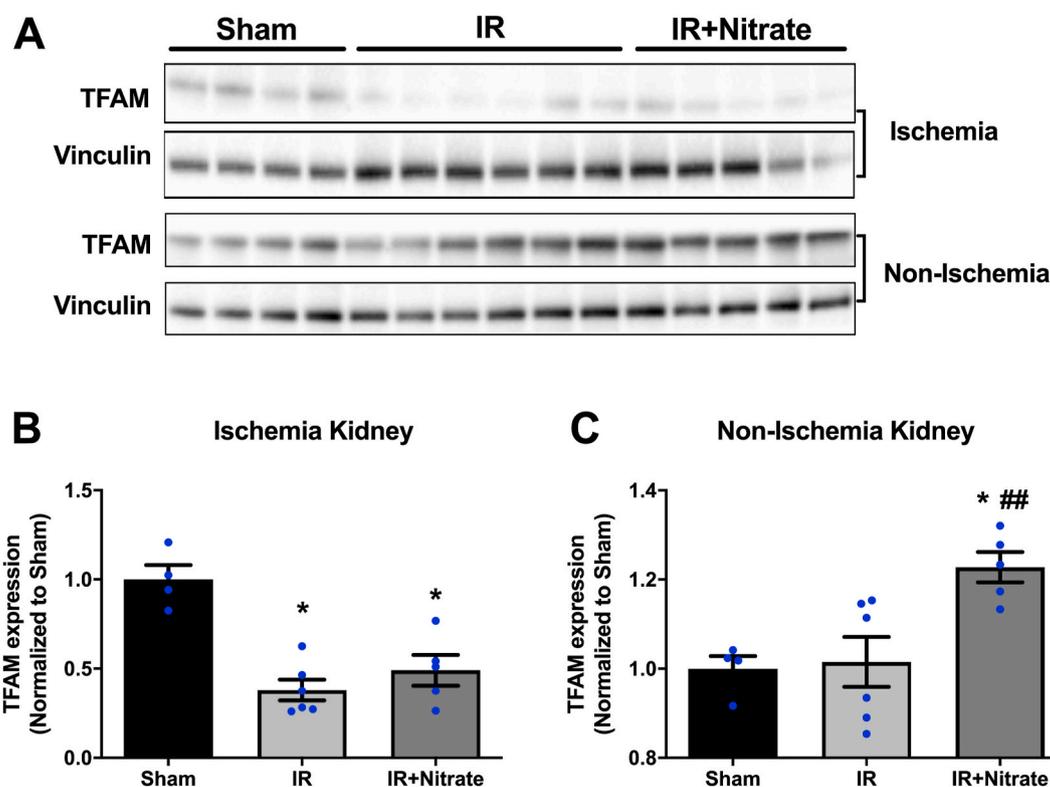
increased in mice following nitrate treatment (Fig. 1A–C). Renal dysfunction, as reflected by changes in BUN and GFR, were also improved by nitrate (Fig. 1D and E).

### 3.2. Nitrate attenuates the blood pressure response and renal Ang II levels following IR

Following two weeks of reperfusion, the blood pressure was slightly elevated in the IR group compared with sham-operated controls. Treatment with nitrate prevented this elevation of blood pressure (Fig. 2A–C). To investigate the link to changes in renin-angiotensin system we measured Ang II levels in plasma and kidney tissues. Our data shows that Ang II in the plasma was similar among the groups, increased in the ischemic kidney, but not significantly changed in the non-ischemic kidney (Fig. 2D–F). Nitrate prevented the increase of Ang II in the renal tissues following IR, which may contribute to its effects on blood pressure.

### 3.3. Nitrate ameliorates renal histopathological changes, inflammation and apoptosis following IR

Next, we analyzed the renal histopathological changes after IR by using H&E and PAS staining. The IR group displayed profound glomerular damage combined with tubular necrosis compared with sham-operated controls (Fig. 3A–F and Table 1). IR mice treated with nitrate had significantly less histopathological changes, as evident from less cast formation, necrosis, ectasia rupture of tubular basement membrane and more tubular regeneration and necrotic tubules in regeneration (Fig. 3A–F and Table 1). Moreover, F4/80 immunostaining showed substantial infiltration of macrophages in the ischemic kidney following IR, which was prevented by nitrate treatment (Fig. 4A and B). TUNEL staining, to detect apoptosis, was significantly elevated after IR injury, which again was significantly reduced by nitrate (Fig. 4C and D) [6].



**Fig. 5.** Effects of nitrate on TFAM expression following ischemia-reperfusion of the kidney. Immunoblotting images of TFAM, indicating mitochondrial biogenesis, following ischemia-reperfusion (IR) (A). Quantification of normalized TFAM in the ischemia (B) and non-ischemia (C) kidneys following IR (B–C). Data in B and C are shown as mean  $\pm$  SEM. \* $p < 0.05$  vs. Sham, ## $p < 0.01$  IR + Nitrate vs. IR group.  $n = 4$ – $6$ /group.

### 3.4. Nitrate improves mitochondrial injury following renal IR via mechanisms that involve restoration of TFAM protein levels

Renal mitochondrial abnormalities and dysfunction, coupled with excessive ROS production, are involved in the pathogenesis of renal disease, including AKI [27,28]. Our data shows that the mitochondrial transcription factor A (TFAM), which is a key regulator of mitochondrial gene expression [29], was significantly decreased in the IR kidney (Fig. 5A–C). Nitrate treatment was not associated with any significant changes of TFAM in the IR kidney, but increased its expression in the non-ischemic kidney following IR (Fig. 5A–C).

### 3.5. Nitrate treatment attenuates Ang II-induced contractility of interlobar arteries and afferent arterioles following renal IR

Next, we used the myography technique to assess the effects of IR on vascular function in large intrarenal vessels of the left kidney following reperfusion. Contractility to phenylephrine was not significantly different between the experimental groups (Fig. 6A). However, Ang II-induced contractility was significantly increased in the IR kidney (Fig. 6B), which was prevented by nitrate treatment. In isolated and perfused afferent arterioles (Fig. 6C), the sensitivity and maximal contractile responses to Ang II were also increased in the IR kidney, which was largely prevented by nitrate treatment (Fig. 6D).

### 3.6. Hypoxia-reoxygenation-induced cell apoptosis and mitochondrial abnormalities in glomerular endothelial cells are ameliorated by nitrite

In further vascular mechanistic studies, aimed at mimicking the in vivo IR model, we used GECs. In these cell studies we used nitrite instead of nitrate to bypass the first step in the nitrate-nitrite-NO pathway which requires commensal bacteria in vivo. Hypoxia followed by reoxygenation in GECs reduced cell viability/increased mortality (Fig. 7A and B).

This was associated with increased levels of cleaved caspase-3, reduced mitochondrial complex II and TFAM expression (Fig. 7C–G), as well as mitochondrial ROS production, as indicated by MitoSox (Fig. 7H). All of these abnormalities associated with hypoxia and reoxygenation were ameliorated by simultaneous treatment with nitrite.

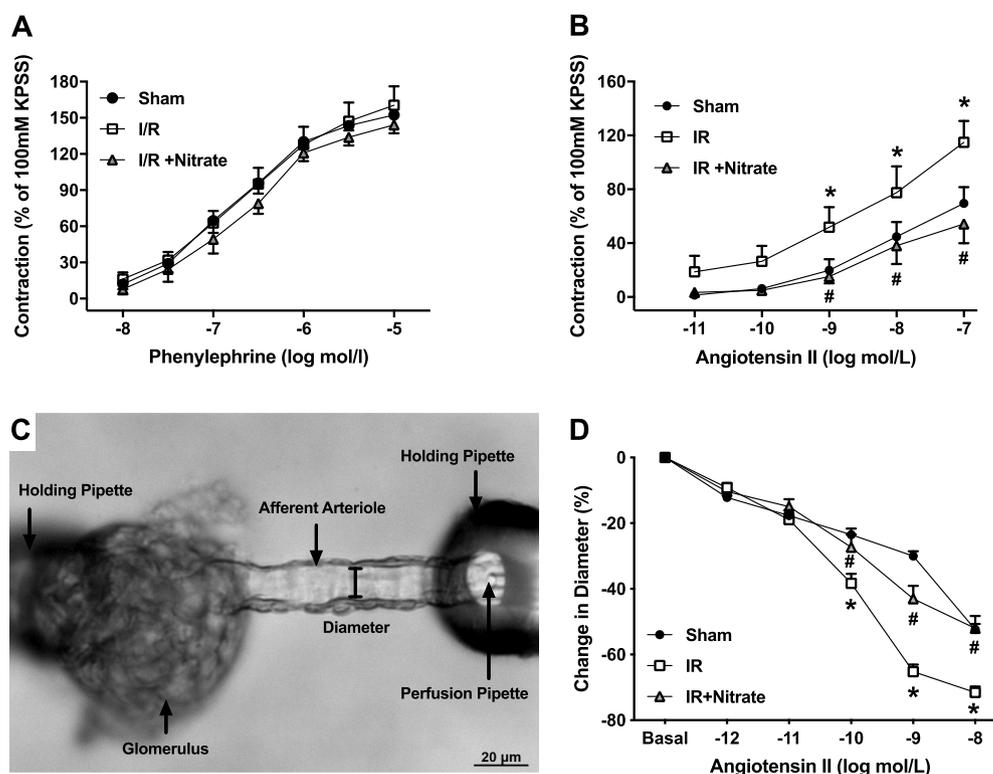
Our in vivo data indicated that IR-induced vascular injuries and dysfunction were coupled with increased renal Ang II levels and sensitivity (Figs. 2E and 6B and D). In the presence of Ang II, hypoxia-reoxygenation reduced cell viability and further increased mitochondrial ROS production (Fig S1 and Fig. 8), which again was reduced by nitrite treatment.

### 3.7. The protective effects of nitrite following hypoxia-reoxygenation in glomerular endothelial cells are abolished by inhibition of xanthine oxidoreductase

XOR is considered as one of the key enzymes involved in the reduction of nitrite to NO and other bioactive nitrogen oxide species. Here we show that, in the presence of the selective XOR inhibitor febuxostat, the beneficial effects of nitrite on cell viability, TFAM expression and mitochondrial ROS (Fig. 9A–D) following hypoxia-reoxygenation combined with Ang II in GECs were abolished. Data on cell viability in the absence of Ang II in the vehicle group is shown in Fig S2.

## 4. Discussion

In this study we demonstrate that inorganic nitrate treatment immediately prior to renal ischemia significantly reduces glomerular and tubular damages as well as the loss of renal function following 2 weeks of reperfusion. These favorable effects of nitrate are associated with dampened inflammatory and angiotensin II responses, coupled with modulation of mitochondrial oxidative stress and cell viability.



**Fig. 6.** Effects of nitrate on vascular function following ischemia-reperfusion of the kidney. Phenylephrine (PE) (A) and Angiotensin II (B) induced contractions of renal interlobar arteries following ischemic-reperfusion (IR). Photomicrograph demonstrating the isolated and micro-perfused afferent arteriolar system (C). Angiotensin II-induced contractions of afferent arterioles from the ischemic kidney following IR (D). Changes in luminal diameters are presented as percent of the control diameter. Data are shown as mean  $\pm$  SEM. \* $p < 0.05$  IR vs. Sham; # $p < 0.05$  IR + Nitrate vs. IR.  $n = 9$ –16 vessels/group in interlobar arteries.  $n = 5$ –6 vessels/group in afferent arterioles.

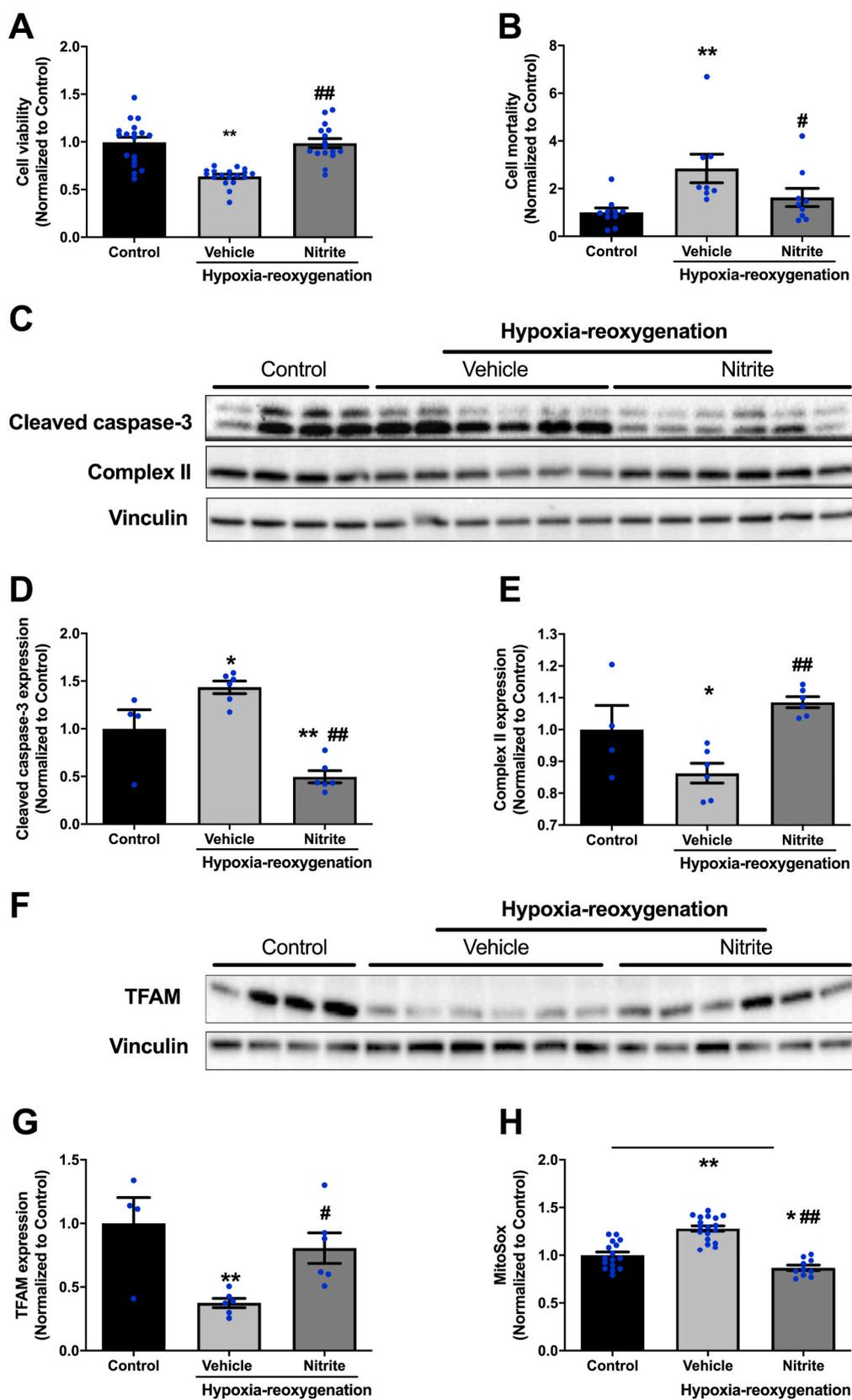
This is the first description of how boosting of the nitrate-nitrite-NO pathway prior to an ischemic event can protect the kidney against subsequent reperfusion-associated renal and endothelial dysfunction and injuries.

NOS-derived NO generation is vital for many cardiovascular and renal functions, including modulation of blood pressure and renal autoregulation as well as tubular electrolyte handling [30–32]. However, in conditions with low oxygen tension and low pH this enzymatic system is compromised leading to NO deficiency or even uncoupling of NOS, which increases production of reactive oxygen species [6,30]. The alternative nitrate-nitrite-NO pathway, is independent of the NOS system, and can be boosted via our everyday diet. Importantly, the efficiency of the nitrate-nitrite-NO pathway is greatly enhanced in conditions with ischemia or hypoxia when the NOS system is compromised [9,33]. Experimental and clinical studies have demonstrated favorable cardiovascular effects of nitrate supplementation, including improved endothelial function and blood pressure reduction in normotensive and hypertensive individuals [11]. We have previously shown that the renal microvasculature is exquisitely responsive to nitrite-mediated vasodilatation [23], especially during acidic and low oxygen conditions mimicking the *in vivo* ischemic environment [20]. Taken together, these findings suggest that nitrate treatment may be associated with organ protection following IR.

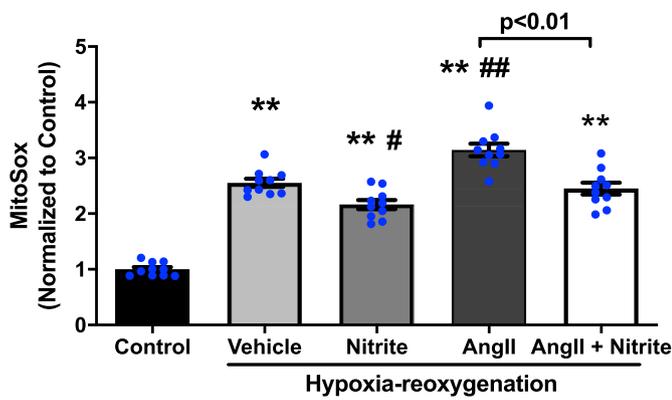
Previous studies using nitrite interventions have generated somewhat conflicting results regarding its therapeutic value in rodent models of IR injury [16,17,34,35]. Triptatara et al. showed that acute renal topical administration of nitrite (30 nmol) protected the rat kidneys against 60 min bilateral ischemia and 6 h reperfusion-induced injuries and dysfunction *in vivo*, at least in part, via XOR-dependent NO production [16]. Using a mouse transgenic approach, findings by Milsom et al. suggested that eNOS is a crucial player, by contributing to nitrite

reduction during IR, thereby mediating protection [35]. In agreement, Cantow and colleagues showed that acute systemic administration of nitrite (0.172 followed by 0.057 mg/h/kg) significantly improved renal reoxygenation and reperfusion following unilateral ischemia (45 min) and 60 min reperfusion in rats [34]. In contrast, Basireddy et al. did not observe any protective effects of acute systemic administration of nitrite (0.12–12 nmol/g/kg) in rats subjected to unilateral nephrectomy followed by 45 min of ischemia of the remaining kidney and 24–48 h reperfusion [17]. Possible explanations for these discrepancies may be different experimental approaches (i.e. IR model, duration of the IR periods), dose regime, administration route and species differences. Currently, there is less knowledge regarding the therapeutic value of using inorganic nitrate instead of nitrite. Nitrate can safely be administered in higher doses than nitrite and has a much longer half-life (6 h vs. 30 min), which allows continuous formation of nitrite, NO and other bioactive nitrogen oxides over a much longer period. In this context nitrate might be considered a prodrug for more continuous generation of nitrite.

In a previous mouse study we showed that dietary nitrate pretreatment for 2 weeks protected the kidney against IR injury via mechanisms that involve modulation of immune cells phenotype and anti-oxidative properties [18]. Instead of mimicking a scenario of chronic high nitrate intake, the current study tested the effects of acute administration prior to an ischemic insult. This approach is more attractive from a clinical and therapeutic perspective, as this could resemble acute intervention prior to ischemia in a patient at risk of developing AKI. Our current study indicated that acute nitrate treatment followed by sustained dietary nitrate supplementation for 2 weeks significantly increased the nitrate, nitrite and cGMP levels in the circulation. The morphological analysis showed that nitrate treatment ameliorated IR-induced injuries compared with non-treated IR controls. Along with



**Fig. 7.** Effects of nitrite on cell viability and mitochondrial function in GECs following hypoxia-reoxygenation. Cell viabilities and mortalities after hypoxia-reoxygenation (A–B). Immunoblotting images (C) and quantification of normalized cleaved caspase-3 and OXPHOS complex II proteins expression (D–E) following hypoxia-reoxygenation. Immunoblotting images (F) and quantification of normalized of TFAM protein expression (G) following hypoxia-reoxygenation injury. Mitochondrial production of reactive oxygen species, indicated by MitoSox, following hypoxia-reoxygenation (H). Data are shown as mean  $\pm$  SEM. \*, \*\* $p < 0.05$ , 0.01 vs Control group respectively; #, ## $p < 0.05$ , 0.01 vs. Vehicle group respectively. A:  $n = 16$ – $18$ /group; B:  $n = 8$ – $10$ /group; C–G:  $n = 4$ – $6$ /group; H  $n = 10$ – $18$ /group.



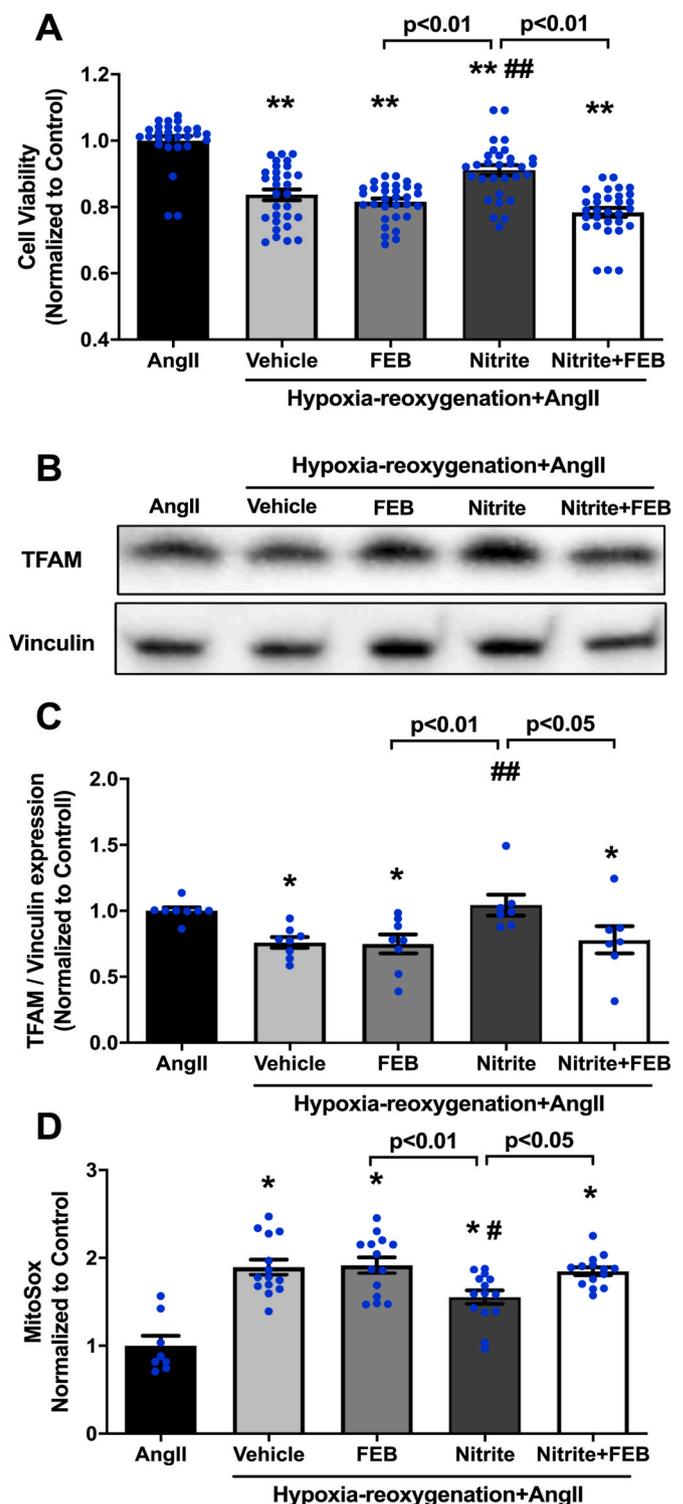
**Fig. 8.** Effects of nitrite in GECs following hypoxia-reoxygenation combined with Ang II. MitoSox levels in glomerular endothelial cells (GECs) following hypoxia-reoxygenation in combination with Ang II. Data are shown as mean  $\pm$  SEM. \*\* $p < 0.05$ , 0.01 vs. Control group respectively; #, ## $p < 0.05$ , 0.01 vs. Vehicle group respectively.  $n = 10$ /group.

the effects of reducing renal structural damage the nitrate treatment preserved kidney function after IR injury. Because plasma creatinine usually returns to normal levels one week after reperfusion [36,37], we measured BUN and GFR to assess renal function following 2 weeks of reperfusion. GFR and BUN were not significantly changed following IR compared to sham-operated controls, which is likely explained by the contralateral compensating kidney. However, our data show that both of these parameters were significantly improved by nitrate compared with the non-treated IR group.

Inflammatory responses and cell apoptosis are pivotal biological processes during IR injury, which contributes to cellular debris clearance and adequate tissue repair [18,24]. However, prolonged and unresolved inflammation may further exacerbate tissue injury [7,38,39]. Renal inflammation, as detected by F4/80 staining of macrophage infiltration, was significantly reduced by nitrate following IR. This is in agreement with recently published studies by Yang et al. [18] and Khambata et al. [40] Moreover, TUNEL assay for apoptosis elucidated that IR injury increased the cell apoptosis in mouse ischemic kidney, which was partially prevented by nitrate treatment. Taken together, these results clearly suggest that nitrate treatment can inhibit renal inflammation and cell apoptosis that occur following IR.

Accumulating evidence show that mitochondrial dysfunction importantly contributes to the development of AKI following IR, and new approaches that modulates mitochondrial function may therefore have therapeutic value [41–45]. For example, in an ischemic kidney, mitochondria in endothelial cells are significantly altered both functionally and morphologically, and becomes extremely reactive and damaging themselves and also surrounding mitochondria [44]. We have earlier shown that dietary nitrate improves mitochondrial efficiency in humans [46]. Stimulation of the nitrate-nitrite-NO pathway has in several recent experimental studies been associated with favorable effects on mitochondrial function during metabolic disorders, including type 2 diabetes, via mechanisms that involve dampening of oxidative stress [47]. Moreover, Shiva and colleagues have demonstrated that both pre- and acute treatment with inorganic nitrite has cytoprotective effects and potentially limits cardiac and hepatic reperfusion injury via modulation of mitochondria electron transport chain and reduction of ROS [48]. In our *in vivo* IR model, TFAM was increased in the non-ischemic kidney, but not significantly changed in the ischemic kidney. Considering that nitrite restored TFAM expression *in vitro* following hypoxia-reoxygenation we speculate that this could be related to time-dependent differences in expression. In the *in vivo* model, analysis of TFAM was only made after 2 weeks and it is possible that this would have been different at an earlier time point following reperfusion.

Increased Ang II signaling is widely known to stimulate NADPH



**Fig. 9.** The favorable effects of nitrite on cell viability and mitochondrial function in GECs following hypoxia-reoxygenation combined with Ang II are blocked by febuxostat. Cell viability (A), immunoblotting images of TFAM and quantification of normalized TFAM (B–C) as well as MitoSox levels (D) following hypoxia-reoxygenation in combination with Ang II, with and without inhibition of xanthine oxidoreductase using febuxostat (FEB). Data are shown as mean  $\pm$  SEM. \*, \*\* $p < 0.05$ , 0.01 vs. Ang II group respectively; #, ## $p < 0.05$ , 0.01 vs. Vehicle group. A:  $n = 28$ –30/group; B–C:  $n = 7$ –8/group; D:  $n = 8$ –14/group.

oxidase and mitochondria-derived ROS production, leading to oxidative stress [49–51]. Cao and colleagues showed that a reno-cerebral reflex activated the renin-angiotensin system following IR, which promoted oxidative stress and progression of renal injuries [52]. We have previously demonstrated that nitrate treatment dampens Ang II-mediated contractility in renal afferent arterioles and hypertension via mechanisms that involve reduction of sympathetic hyperactivity and dampening of oxidative stress, as well as reduced expression of the Ang II type 1 receptor [53–55]. Consistent with previous findings, our data show that nitrate treatment reduces blood pressure which was associated with lower Ang II levels in the kidneys following IR and dampened Ang II-mediated contractility in both interlobar arteries and afferent arterioles.

Since endothelial cell abnormalities following IR is closely associated with renal injuries and compromised kidney function, we next used glomerular endothelial cells (GECs) for mechanistic studies. An in vitro hypoxia-reoxygenation model, with and without simultaneous nitrite treatment, was used to mimic the in vivo IR scenario. Our in vitro results show that hypoxia-reoxygenation increased cell mortality, mitochondrial ROS production and cleaved caspase-3, decreased cell viability and protein expression of OXPHOS complex II and TFAM. All these abnormal changes were significantly ameliorated by nitrite.

As our in vivo data showed that Ang II levels were increased in the kidneys following IR, we also studied the effects of nitrite treatment in GECs following hypoxia-reoxygenation in the presence of Ang II. Our data show that hypoxia-reoxygenation in combination with Ang II further decreased cell viability and increased mitochondrial ROS production, which again was prevented by simultaneous nitrite treatment.

During normoxia and normal pH NOS-derived NO is oxidized to nitrite and nitrate, but during hypoxia and acidic conditions, nitrite can be reduced back to NO by the nitrite reductase action of XOR. Co-incubation with the selective XOR inhibitor febusostat abolished the protective effects of nitrite following hypoxia-reoxygenation in GECs. These data suggest that XOR catalyzes nitrite reduction to NO, which in turn mediates the protection of cells from hypoxia-reoxygenation-induced injury.

Some limitations of the current study include that a group with

nitrate treatment alone in sham operated animals was not included. The main reason for this was that we in previous studies in normal rats and mice have not observed any profound effects, using similar doses of nitrate, on blood pressure, renal function or vascular reactivity. Another limitation is that all in vivo characterizations were made only following two weeks of reperfusion. Additional timepoints could potentially have generated more mechanistic insight.

In conclusion, acute treatment with inorganic nitrate prior to a renal ischemia event, followed by sustained dietary nitrate treatment during two weeks of reperfusion, protected against renal dysfunction and injuries via mechanisms that involve reduction of infiltrating inflammatory cells and angiotensin II levels in the ischemic kidney as well as modulation of mitochondrial function. Future clinical trials may elucidate if interventions with inorganic nitrate can provide beneficial effects in patients with high risk of acquiring renal IR injury.

### Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Jon O Lundberg and Eddie Weitzberg are co-inventors on patent applications related to the therapeutic use of inorganic nitrate. The other authors have no conflicts of interest to declare.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2020.101836>.

	Sham	IR	IR + Nitrate
Necrosis	0 ± 0	40.31 ± 3.29*	23.62 ± 2.43*#
Tubular regeneration	0 ± 0	7.80 ± 1.46*	14.39 ± 2.11*#
Necrosis/regeneration ratio	0 ± 0	25.76 ± 7.09*	60.88 ± 5.33*#
Tubular ectasia	0 ± 0	33.87 ± 3.13*	12.71 ± 1.78*#
Rupture of tubular basement membrane	0 ± 0	24.91 ± 2.80*	8.35 ± 1.07*#

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