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ORGAN PHYSIOLOGY

Renal ischemia-reperfusion injury impairs renal calcium, magnesium, and phosphate handling in mice

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Abstract

Fibroblast growth factor 23 (FGF23) levels are elevated in patients with acute kidney injury (AKI). The consequences on renal Ca^{2+}, Mg^{2+}, and P_i regulatory mechanisms are unknown. We hypothesized that renal ischemia-reperfusion (I/R) injury alters the expression of important renal Ca^{2+}, Mg^{2+}, and P_i transport proteins. I/R injury was induced in male C57BL/6 mice by clamping both renal arteries for 27 min. Mice were investigated 18 h later. The mRNA and protein levels of renal Ca^{2+}, Mg^{2+}, and P_i transport proteins were measured by RT-qPCR and western blot analysis. I/R injury-induced hyperphosphatemia and hypermagnesemia were paralleled by a decrease in glomerular filtration rate and an increase in the fractional excretion of Ca^{2+}, Mg^{2+}, and P_i. I/R injury affected the fibroblast growth factor 23 (FGF23)-klotho-vitamin D axis by increasing plasma levels of FGF23 and downregulation of renal klotho expression. Plasma levels of PTH and 1,25-dihydroxyvitamin D_3 were unchanged. Further, downregulation of key genes for paracellular reabsorption of Ca^{2+} and Mg^{2+} (claudin (Cldn)2, Cldn10b, Cldn16, Cldn19) and for active transcellular transport of Ca^{2+}, Mg^{2+}, and P_i (calbindin-D_{28K}, Ncx1, Pmca4, Cnnm2, Trpm7, NaP_i-2a, and NaP_i-2c) was observed. However, renal expression of Trpv5 and Trpv6 was increased. In vitro studies support a direct effect of proinflammatory cytokines on the mRNA expression of Cldn16, Cldn19, and Trpv6. Our findings indicate that renal I/R injury increases FGF23 blood levels independent of PTH and 1,25-dihydroxyvitamin D_3. This increase is associated with hypermagnesemia, hyperphosphatemia, and increased or decreased expression of specific renal Ca^{2+}, Mg^{2+}, and P_i transporters, respectively.

Keywords Acute kidney injury · Klotho · FGF23 · Inflammation · Parathyroid hormone

Introduction

Acute kidney injury (AKI) is a major clinical problem without effective therapy and may lead to chronic kidney disease (CKD) [66]. Ischemia-reperfusion (I/R) injury causes AKI in various clinical settings including hypotension, sepsis, kidney transplantation, and aortic bypass surgery [6], and is associated with high morbidity and mortality [9]. The clinical consequences of renal I/R injury depend on the severity of the injury, and ranges from minor changes in renal function to a requirement for dialysis or transplantation. Renal I/R injury results from a generalized or localized reduction in renal blood flow leading to hypoxia. As a result, tubular epithelial cell injury with functional impairment of water and electrolyte homeostasis, including calcium (Ca^{2+}), magnesium (Mg^{2+}), and phosphate (P_i) deregulations, develops [5, 11, 20, 62]. However, the underlying mechanisms for electrolyte disturbances have not been fully defined.

Fibroblast growth factor (FGF) 23 was originally described as a bone-derived hormone that mainly affects renal function via the FGF receptor (FGFR). FGF23 is involved in renal phosphate reabsorption, the modulation of the production of parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), and in the control of mineral homeostasis. FGF23 and PTH increase renal P_i excretion mainly due to a downregulation of type II sodium-dependent P_i co-transporters (NaPi-2a and NaPi-2c) in proximal tubules [22, 29]. Further, FGF23, PTH, and $1,25(OH)_2D_3$ modulate renal Ca^{2+} reabsorption by increasing the apical membrane expression of transient receptor potential vanilloid-5 (Trpv5) abundance and activity

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in distal tubules [5]. In addition, FGF23 suppresses and PTH stimulates renal 1,25(OH)$_2$D$_3$ synthesis, which depends on Cyp24a1 and Cyp27b1 gene expression. Cyp24a1 and Cyp27b1 encode the enzymes 24-hydroxylase and 1α-hydroxylase, respectively, which are responsible for renal 1,25(OH)$_2$D$_3$ degradation and synthesis. Both renal Cyp24a1 and Cyp27b1 are highly regulated: PTH stimulates the expression of Cyp27b1 in the kidney, whereas FGF23 downregulates it, and FGF23 strongly induces the expression of Cyp24a1, whereas PTH reduces its expression [3, 4, 43, 50, 58].

Increased FGF23 levels have been observed after dietary phosphate loading, administration of interleukin-1β (IL-1β), IL-6, PTH, or 1,25(OH)$_2$D$_3$, and most notably during CKD [12, 16, 53]. Subsequently, also non-osseous tissues, including the kidney, for example, have been reported to express and secret FGF23 in response to inflammation or AKI, for example [16, 19, 49]. In proximal and distal renal tubules, the FGFR1 is probably the dominant FGF receptor mediating the effects of FGF23 [21]. Renal FGF23 signaling requires the presence of the co-receptor αKlotho (klotho) on target cells [21]. In CKD, the decrease in klotho expression has been suggested to contribute to renal FGF23 resistance and a maladaptive increase in circulating FGF23, leading to an impaired mineral metabolism [48, 51]. Thus, FGF23 has been implicated in the development of secondary hyperparathyroidism in CKD [48]. Increased levels of FGF23 and decreased levels of klotho also are well-described findings in patients with AKI and in animal models of AKI, and are associated with an adverse outcome [10, 16, 26, 34, 45, 57]. More recently, we found that lipopolysaccharide-induced AKI, which is associated with hypocalcemia and hyperphosphatemia and a disturbed FGF23-klotho-vitamin D signaling, was accompanied by an altered expression of specific renal Ca^{2+} and P_i transporters [42]. Therefore, one may assume that AKI due to renal I/R injury could also be associated with an impaired FGF23-klotho-vitamin D signaling [23, 26], leading to an alteration in Ca^{2+} and P_i homeostasis. However, the consequences of such alterations on renal Ca^{2+} and P_i regulatory mechanisms in response to I/R injury are still unclear. We therefore hypothesized that I/R injury alters the expression of important renal Ca^{2+} and P_i transport proteins. In addition, we characterized the impact of I/R injury on FGF23-klotho-vitamin D signaling and on renal Mg^{2+} transport regulation.

Methods

Animals

All animal experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local animal protection committee. Male C57BL/6J mice (7 weeks old) were obtained from Charles River (Sulzfeld, Germany), and were acclimated for at least 1 week in the animal facility with free access to standard chow (V1534-300; Ssniff Spezialdäten GmbH Soest, Germany) containing 1.00% Ca^{2+}, 0.22% Mg^{2+}, and 0.70% phosphate and received water ad libitum. Mice were kept in cages in groups of two to four individuals at 23 °C under a 12-h/12-h dark (6 p.m.–6 a.m.)/light (6 a.m.–6 p.m.) cycle.

Induction of renal I/R injury model

Mice were anesthetized with isofluorane and I/R was undertaken as described previously [54]. In brief, mice received subcutaneous injections of 250 μL of 0.9% saline and buprenorphine (0.05 mg/kg) as analgesic substance. Briefly, after visualizing both renal pedicles through a mid-line abdominal incision, the arteries of both kidneys were clamped individually with a non-traumatic microvascular clamp for 27 min. The time of ischemia was chosen to obtain a reversible model of ischemic acute renal failure and to avoid animal mortality [31]. Renal ischemia was confirmed by blanching of kidneys. In sham controls, renal arteries were only touched with forceps. After ischemia, the clamps were released for the kidneys to start the reperfusion. Thereafter, the abdominal wound was sutured and the mouse was kept on a heating pad until it gained full consciousness. Mice were killed after 18 h of reperfusion, blood was collected in tubes containing lithium-heparin, and the kidneys were quickly removed, frozen in liquid nitrogen, and stored at − 80 °C until extraction of total RNA or protein.

In addition, both sham control and I/R injury mice were placed in metabolic cages for 24 h on the day before the surgery for acclimatization. Two hours after the surgery, these mice were replaced in metabolic cages for 16 h to collect urine. No mouse died before the study was finalized.

Histology

Kidneys from sham-operated and I/R-treated mice were fixed in 4% paraformaldehyde solution by retrograde perfusion through the abdominal aorta [55]. The fixed kidneys were then dehydrated, embedded in paraffin, and cut into 5 μm sections. To evaluate morphological damage, paraffin-embedded sections containing all kidney zones were stained with periodic acid-Schiff (PAS) stain according to the manufacturer's protocol (Sigma-Aldrich, Germany).

Primary proximal tubule and distal tubule cells and mouse kidney thick ascending limb cell line

Primary proximal tubule (PT) cells and distal tubule cells (DTCs) were isolated and cultured as described previously [42, 55]. Mouse kidney thick ascending limb (MKTAL) cells were cultured in DMEM/HAM-F12 (1:1) (Lonza) containing

5% (vol/vol) FCS and 2 mM l-glutamine, at 37 °C in a humidity-controlled incubator with 5% (vol/vol) CO_2 [7]. The cells were grown in culture medium until confluent and then in serum- and hormone-free medium for 24 h before use. Thereafter, cells were treated with or without a mixture of TNFα and IL-1β (100 ng/mL each) for 24 h. Murine recombinant cytokines were used for the experiments from PeproTech.

Biochemical and analytical methods

Urea, P_i, and total Mg^{2+} and Ca^{2+} levels were measured using colorimetric assays according to the manufacturer's protocol (ArborAssays, Ann Arbor, MI; BioAssay Systems, Hayward, CA). Plasma creatinine concentrations were determined using a commercially available enzymatic colorimetric assay according to the manufacturer's protocol (LT-SYS, Labor + Technik, Berlin, Germany). Urine creatinine concentrations were determined using a commercially available colorimetric assay according to the manufacturer's protocol (ArborAssays, Ann Arbor). Plasma PTH was measured with a mouse PTH 1-84 ELISA kit (Immutopics, San Clemente, CA). Plasma 1,25-dihydroxyvitamin D_3 was determined with a mouse 1,25-dihydroxyvitamin D_3 ELISA kit (Cusabio Biotech Co., LTD., Wuhan, P.R.China). Plasma levels of FGF23 were determined with a mouse/rat FGF23 (Intact) ELISA kit (Immutopics, San Clemente, CA). FE of Ca^{2+} was calculated using the formula: ([urine Ca^{2+}] × [serum creatinine] × 100) / ([urine creatinine] × [serum Ca^{2+}]). FE of Mg^{2+} and P_i were calculated in accordance.

Real-time quantitative PCR

Total tissue and cell RNAs were extracted with TRIzol Reagent (Invitrogen) according to the manufacturer's instruction. Total RNA was reverse transcribed into cDNA according to standard protocols as described previously [39]. Real-time PCR was performed in a LightCycler 480 (Roche, Mannheim, Germany). All PCR experiments were conducted using the LightCycler DNA Master SYBR Green I kit provided by Roche Molecular Biochemicals (Mannheim, Germany) as described previously [25]. PCR primers are listed in Table 1 or have been described previously by us [42, 54].

Immunoblotting

Protein preparation and immunoblotting were performed as described previously [41]. In brief, protein samples were electrophoretically separated on 15 or 10% polyacrylamide gels and transferred to nitrocellulose membranes, which were blocked overnight in 5% nonfat dry milk diluted in Tris-buffered saline with 0.1% Tween-20, and then incubated for 1 h at room temperature with antibodies against calbindin-D_{28K} (CB300, SWANT, Bellinzona, Switzerland; 1:2000; expected size of ~28 kDa), Trpv5 (SC-30187, Santa Cruz Biotechnology, Santa Cruz, CA; 1:1000; expected size of ~75 kDa), Trpv6 (CAT11-A, Alpha Diagnostics International, San Antonio, TX; 1:1000; expected size of ~85 kDa), Trpm6 (Alomone Labs, ACC-046, 1:1000, expected size ~233 kDa), Trpm7 (Alomone Labs, ACC-047, 1:1000, expected size ~212 kDa), NCX1 (π 11-13; Swant, 1:1000; expected size of ~120 kDa for full length protein and of ~70 kDa for active proteolytic fragment), Claudin (Cldn)16 (Zymed Laboratories Inc., 1:500; expected size ~34 kDa), Cldn19 (Sigma-Aldrich, SAB2100440, 1:500, expected size ~ 23 kDa), β-actin (A5316, Sigma-Aldrich; 1:5000; expected size of ~43 kDa), or klotho (ab75023, Abcam plc, Cambridge, UK; 1:1000; expected size of ~116 kDa). After being washed, the membrane was incubated for 2 h with a secondary antibody (Santa Cruz Biotechnology; 1:2000) and subjected to a chemiluminescence detection system. Quantitative assessment of band densities was performed densitometrically using ImageJ Software. The Trpv5 antibody has been used previously by others [37, 47, 52, 65].

Table 1 Sequences of primers for RT-PCR

Gene	Forward primer	Reverse primer
Trpm7	caatccaatgttgttccatctcat	tgtgtatgtgcatctgtaggttcct
Trpm6	aaagccatgcgagttatcagc	cttcacaatgaaaacctgccc
Slc41a1	ctcaccctcatcttcatcatctt	ccagtctgcgatgtacagga
Slc41a3	tgaagggaaacctggaaatg	ggttgctgctgatgattttg
Parvalbumin	gcaagattggggttgaagaa	gtgtccgattggtacagcct
Cnnm2	aaagcccctgagtgctacct	gagggccatcacaccatagta
Cldn2	tgaacacggaccactgaaag	ttagcaggaagctgggtcag
Cldn10b	ggagttcccctccatgct	gcaaaaatggaaccgaaaaa
Cldn14	gtccagctcctaggcttcct	catccacagtcccttcaggt
Cldn16	ggttgctttttggcagga	tagttcctctcaggcccaac
Cldn19	tctccggaggtgctctctt	ggggttgaagaattcctgtg
Casr	aaacacctacggcacctgaa	ttgtagtacccaacttccttgaaca
FGF23	gtatggatctccacggcaac	agtgatgcttctgcgacaagt
FGFR1	tctggcctctacgcttgc	gaggatgggagtgcatctg
CYP24a1	tctgcccattgcgttctgt	tcttgatttgggggtgaaaa
Pmca4	ggtacaggaaatctgcactagac	aaacaagtctacgcttctccaa

Trpm, transient receptor potential channel melastatin; *Slc41*, Na^+-Mg^{2+}-exchanger solute carrier family (Slc) 41; *Cnnm2*, ancient conserved domain protein 2/cyclin M2; *CYP41a1*, cytochrome P450, family 24, subfamily a, polypeptide 1; *Cldn*, claudin; *FGF23*, fibroblast growth factor 23; *FGFR1*, fibroblast growth factor receptor 1; *Casr*, Ca^{2+}-sensing receptor; *Pmca4*, Ca^{2+} transporting plasma membrane 4 (Atp2b4)

Statistical analyses

Results are presented as means ± SEM. Statistical comparisons were made by a two-sided Student's unpaired *t* test. A P value < 0.05 was considered significant.

Results

I/R injury worsens renal function

I/R injury did not alter food and water intake, but clearly induced AKI, which is reflected by a decrease in creatinine clearance (Table 2). As expected, sections from sham-operated mice exhibited no evidence of structural damage (Fig. 1a). I/R injury resulted in extensive changes in renal morphology, including destruction of tubules and cast formation (Fig. 1a), and an elevation of plasma creatinine and urea concentration (Fig. 1b). In addition, renal mRNA expression of the tubular injury markers neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) was increased in the kidneys with I/R injury (Fig. 1c). The increase in tubular injury markers was paralleled by an increase in the renal mRNA expression of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)α (Fig. 1d).

I/R injury induces hypermagnesemia and hyperphosphatemia

To investigate the consequences of I/R-induced AKI on electrolyte homeostasis, we determined blood levels and urinary excretion of Ca^{2+}, Mg^{2+}, and P_i. I/R injury caused an increase in plasma levels of P_i (Fig. 1e) and Mg^{2+} (Fig. 1g). Blood Ca^{2+} levels were not different from control levels 18 h after induction of I/R injury (Fig. 1f). To investigate the effect of I/R injury on the urinary excretion of Ca^{2+}, Mg^{2+}, and P_i, we placed mice in metabolic cages and collected urine over a period of 16 h. We found that I/R injury increased the FE of P_i, Ca^{2+}, and Mg^{2+} (Table 2). However, the total urinary excretion of Ca^{2+} and Mg^{2+} was decreased in response to I/R injury (Table 2). I/R injury increased total urinary excretion of P_i (Table 2).

Table 2 The effect of ischemia-reperfusion (I/R) injury on deregulated Ca^{2+}, Mg^{2+}, and PO_4^{3-} homeostasis

	Sham control	I/R
Water intake (mL)	2.23 ± 0.23	2.44 ± 0.12
Food intake (mg)	2.13 ± 0.28	2.09 ± 0.33
Urine output (mL)	1.06 ± 0.11	1.34 ± 0.17
Plasma Mg^{2+} (mmol L^{-1})	0.71 ± 0.01	0.76 ± 0.01*
Plasma Ca^{2+} (mmol L^{-1})	2.37 ± 0.06	2.37 ± 0.03
Plasma PO_4^{3-} (mmol L^{-1})	2.92 ± 0.07	4.87 ± 0.14*
Urine creatinine (mg dL^{-1})	31.7 ± 2.6	13.4 ± 1.1*
Creatinine clearance (μL min^{-1})	106 ± 7	32 ± 8*
Urine Mg^{2+}/creatinine	0.36 ± 0.04	0.34 ± 0.07
Urinary Mg^{2+} (mg 16 h^{-1})	0.12 ± 0.01	0.06 ± 0.01*
FE Mg^{2+} (%)	6.6 ± 0.9	11.5 ± 0.9*
Urine Ca^{2+}/creatinine	0.19 ± 0.03	0.20 ± 0.04
Urinary Ca^{2+} (mg 16 h^{-1})	0.06 ± 0.01	0.03 ± 0.01*
FE Ca^{2+} (%)	0.63 ± 0.07	1.36 ± 0.18*
Urine PO_4^{3-}/creatinine	7.98 ± 1.41	27.20 ± 4.22*
Urinary PO_4^{3-} (mg 16 h^{-1})	2.56 ± 0.40	4.69 ± 0.72*
FE PO_4^{3-} (%)	8.9 ± 1.0	40.3 ± 5.6*

Parameters are collected after 16 h housing in individual metabolic cages. Data are expressed as mean ± SEM for six animals. *$P < 0.05$ vs. control

I/R injury increases blood levels of fibroblast growth factor 23 and decreases renal klotho expression

In order to determine whether renal I/R results in altered FGF23-klotho signaling, we investigated blood FGF23 levels. FGF23 levels were increased in response to I/R injury (Fig. 2a). Renal mRNA levels of FGF23 and of the primary FGF23 receptor FGFR1 were also increased (Fig. 2b), while klotho expression was decreased (Fig. 2b, c).

Proinflammatory cytokines decrease klotho mRNA expression in vitro

To examine a possible direct effect of proinflammatory cytokines, we incubated primary PT cells and DTCs with a combination of TNFα and IL-1β. Addition of the cytokine mixture decreased klotho mRNA expression in PT cells (Fig. 2d) and DTCs (Fig. 2e). Incubation of primary PT cells with cytokines increased the expression of FGFR1 (Fig. 2d). Further, the combination of TNFα with IL-1β did not alter the expression of FGFR1 mRNA in DTCs (Fig. 2e).

I/R injury does not alter blood levels of parathyroid hormone and renal vitamin D synthesis

Next, we investigated the effect of I/R injury on blood levels of major hormones involved in Ca^{2+} and P_i homeostasis. I/R injury did not alter plasma levels of PTH (Fig. 3a). Similarly, plasma concentration of $1,25(OH)_2D_3$ was not different from controls (Fig. 3b). In accordance with this observation, renal 1,25-dihydroxyvitamin D_3 24-hydroxylase (Cyp24a1) mRNA expression was increased and the mRNA expression of the primary vitamin D regulatory enzyme 25-hydroxyvitamin D_3 1α-hydroxylase (Cyp27b1) was decreased in response to I/R injury (Fig. 3c).

I/R injury alters the expression of crucial renal phosphate, calcium, and magnesium transporters

To characterize the impact of renal I/R injury on tubular electrolyte transport regulation, the renal expression of

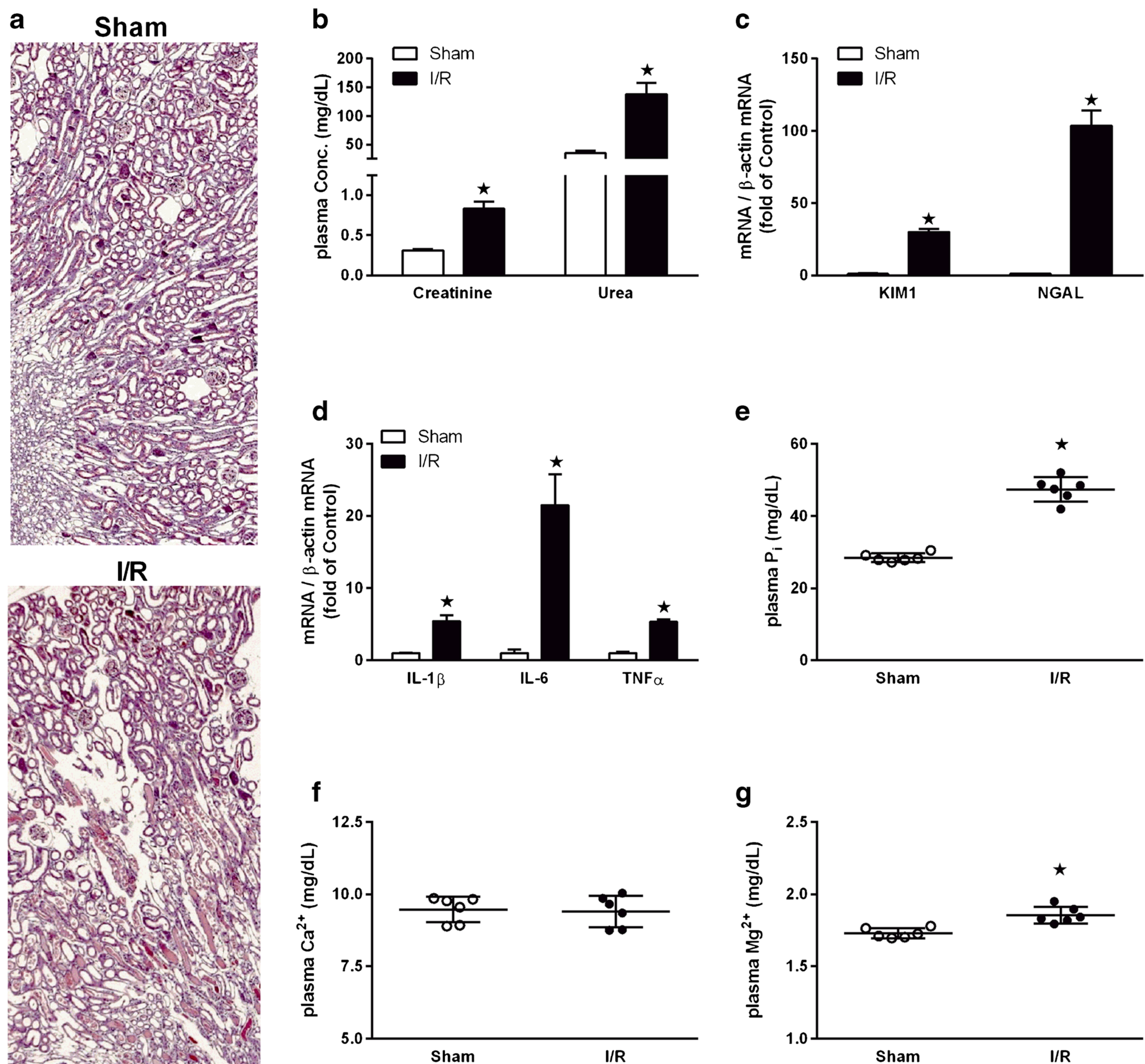

Fig. 1 Ischemia-reperfusion (I/R) injury worsened renal function and induced hyperphosphatemia and hypermagnesemia. Effect of I/R injury (18 h) on **a** renal histological changes (PAS staining of kidneys), plasma levels of **b** creatinine and urea, **c** renal NGAL and KIM-1 mRNA, **d** renal IL-1β, IL-6, and TNFα mRNA expression related to β-actin mRNA expression and on plasma levels of **e** P_i, **f** total Ca^{2+}, and **g** total Mg^{2+}. Values are mean ± SEM for six animals. *$P < 0.05$ vs. control

Ca^{2+}, Mg^{2+}, and P_i transporters was determined. I/R injury decreased the renal mRNA expression of NaP_i-2a and NaP_i-2c (Fig. 4). In contrast, the renal mRNA expression of NaP_i-2b and PiT1 was increased, whereas the mRNA abundance of PiT2 was unaltered (Fig. 4). Further, I/R injury increased renal Trpv5 and Trpv6 mRNA abundance (Fig. 5a), whereas renal calbindin-D_{28K}, plasma membrane Ca^{2+}-ATPase (Pmca) 4, and Na^+/Ca^{2+}-exchanger (Ncx1) mRNA abundance were decreased (Fig. 5a). The renal mRNA expression level of Pmca1b was unaltered (Fig. 5a). In addition, the increase in renal Trpv5 and Trpv6 expression as well as the decrease in renal Ncx1 and calbindin-D_{28K} expression were confirmed on the protein level (Fig. 5b, c). I/R injury did not alter the renal expression of transient receptor potential channel melastatin (Trpm) 6, but decreased abundance of Trpm7 (Fig. 6a, b). Further, I/R injury decreased the mRNA expression of Na^+-Mg^{2+}-exchanger solute carrier family (Slc) 41a3 and of parvalbumin (Fig. 6a). In addition, I/R injury did not alter the renal mRNA expression of Slc41a1, but decreased the mRNA abundance of ancient conserved domain protein 2/cyclin M2 (Cnnm) 2 (Fig. 6a).

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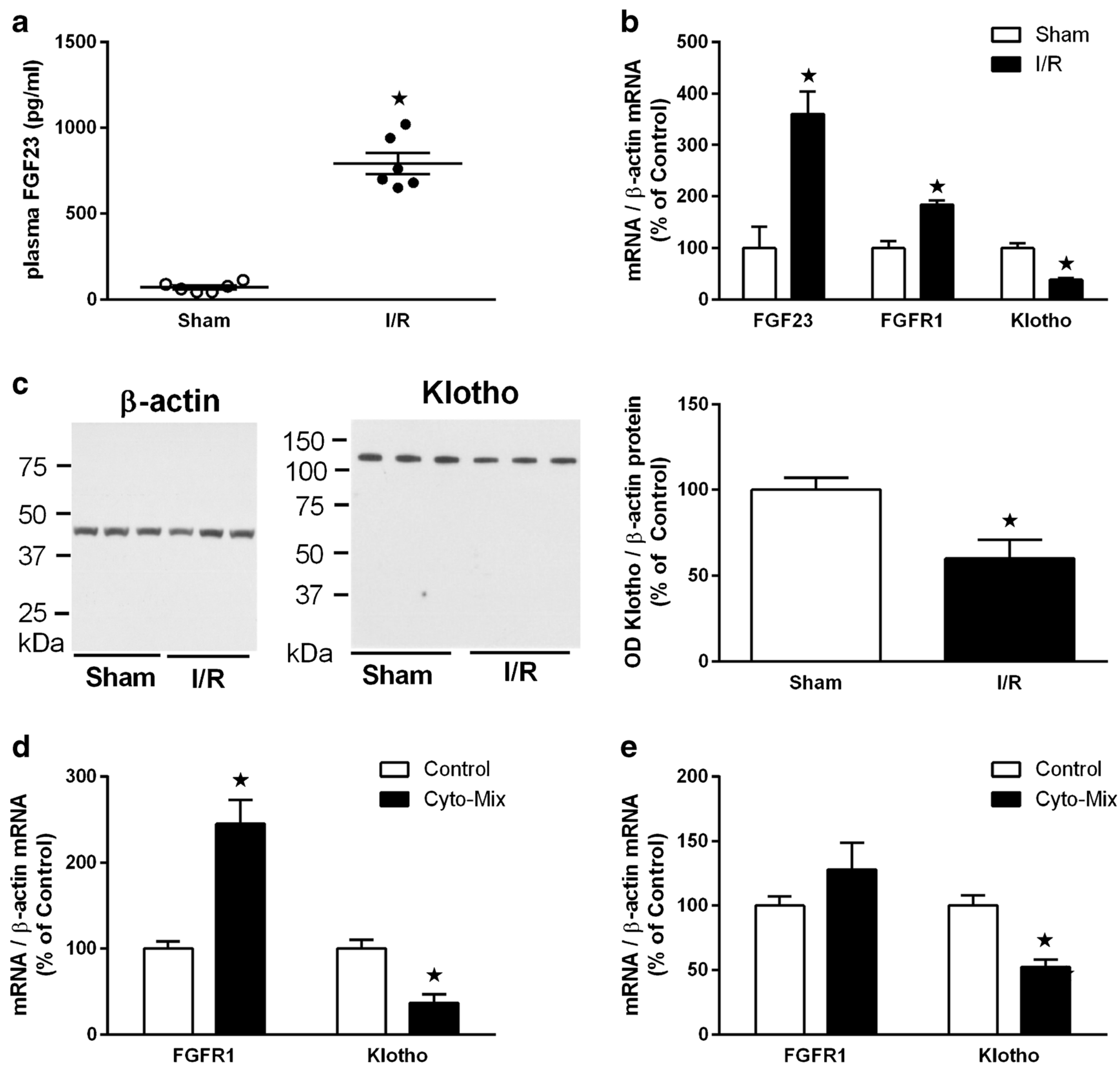

Fig. 2 Ischemia-reperfusion (I/R) injury and cytokines altered FGF23-klotho-signaling in vivo and in vitro. Effect of renal I/R (18 h) on **a** plasma levels fibroblast growth factor 23 (FGF23), **b** renal FGF23, FGFR1, and klotho mRNA expression related to β-actin mRNA expression, and **c** on renal klotho protein expression related to β-actin protein expression. Effect of a cytokine mixture (Cyto-Mix; IL-1β and TNFα; each 100 ng/mL) on FGFR1 and klotho mRNA expression related to β-actin mRNA expression in **d** primary proximal tubule cells and **e** in primary distal tubule cells after 16 h. Shown are representative immunoblots for klotho and β-actin protein. Values are mean ± SEM for six animals or for four independent in vitro experiments. *$P < 0.05$ vs. control

I/R injury alters the expression profiles of genes involved in paracellular transport of Ca^{2+} and Mg^{2+} transport across the thick ascending limb of Henle and the proximal tubule

Next, we examined the renal expression of Ca^{2+} and Mg^{2+} transporters involved in passive, paracellular transport. I/R injury decreased the mRNA expression of Cldn2, Cldn10b, Cldn16, and Cldn19 (Fig. 7a), and increased the mRNA expression of Cldn14. Further, the expression levels of NKCC2 and of the Ca^{2+}-sensing receptor (CaSR) were decreased in kidneys with I/R injury (Fig. 7b). We further found that the protein expression of Cldn16 and Cldn19 was decreased in kidneys with I/R injury (Fig. 7c, d).

Proinflammatory cytokines activation decrease Cldn16 and Cldn19 mRNA expression in vitro

To examine a possible regulatory effect of proinflammatory cytokines, we incubated primary DTCs and the MKTAL cell line with a combination of TNFα and IL-1β. Addition of the cytokine mixture decreased Cldn16 and Cldn19 mRNA expression in MKTAL cells (Fig. 8a). Incubation of primary DTCs with cytokines did not alter the expression of Mg^{2+} transporters (Fig. 8b). Further, the combination of TNFα with IL-1β did not alter the expression of Trpv5, Ncx1, and Pmca1b mRNA, but increased Trpv6 mRNA abundance (Fig. 8c).

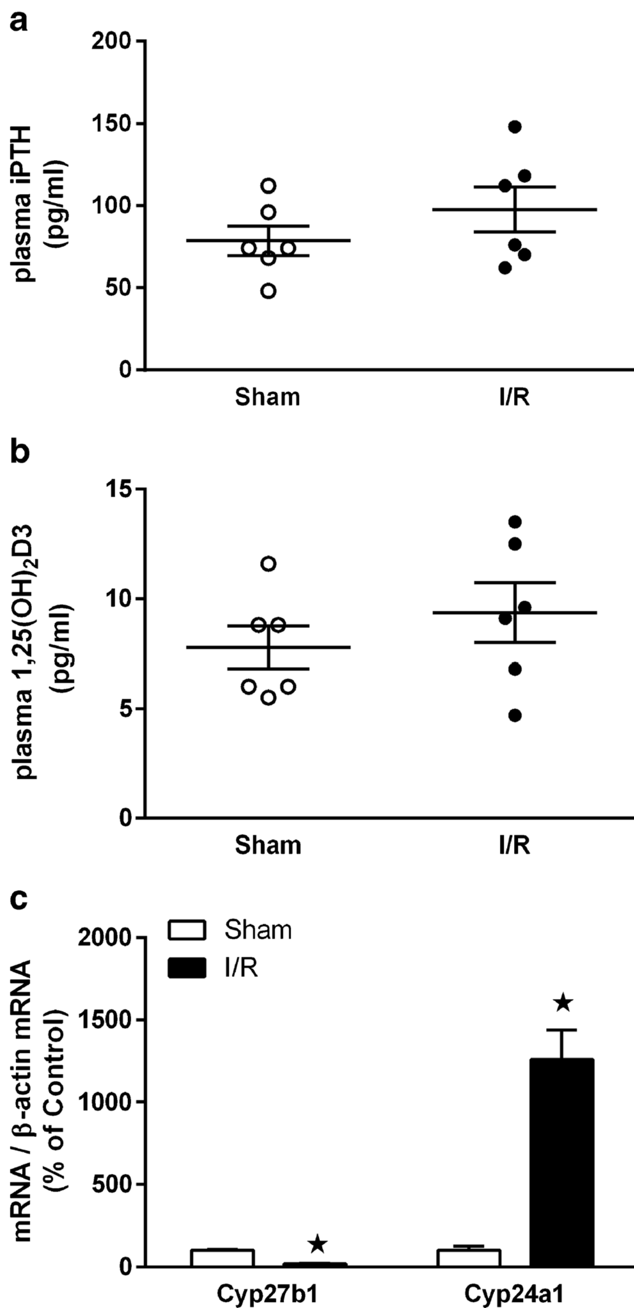

Fig. 3 Ischemia-reperfusion (I/R) injury did not alter plasma levels of PTH and renal vitamin D synthesis. Effect of renal I/R (18 h) on plasma levels of **a** parathyroid hormone (PTH), **b** 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), and **c** renal Cyp27b1 and Cyp24a1 mRNA expression related to β-actin mRNA expression. Values are mean ± SEM for six animals. *$P < 0.05$ vs. control

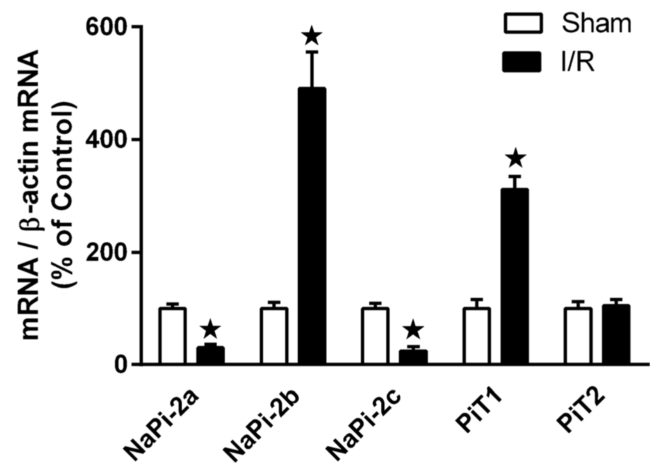

Fig. 4 Ischemia-reperfusion (I/R) injury altered the renal expression of tubular phosphate transporters. Effect of renal I/R (18 h) on renal NaP_i-2a, NaP_i-2b, NaP_i-2c, PiT1, and PiT2 mRNA expression related to β-actin mRNA expression. Values are mean ± SEM for six animals. *$P < 0.05$ vs. control

Discussion

In the present study, we characterized the impact of I/R injury on systemic and local renal factors involved in renal Ca^{2+}, Mg^{2+}, and P_i homeostasis. Our data reveal that I/R injury-induced AKI deregulates electrolyte homeostasis in mice, which was paralleled by an increased or decreased expression of renal Ca^{2+}, Mg^{2+}, and P_i transporting proteins, respectively.

Renal I/R injury clearly induced clinical sings of AKI, as reflected by the increase in plasma urea and creatinine concentration, an elevated renal expression of the tubular injury markers NGAL and Kim-1 [56], and an altered renal morphology [30, 31]. Further, renal I/R injury clearly disturbed P_i homeostasis, a common hallmark observed in patients with AKI [28], as reflected by increased P_i blood levels despite an increased urinary excretion of P_i. A decrease in renal function is the most common cause for hyperphosphatemia [5, 20]. Therefore, the decline in GFR in response to I/R injury could likely account for the development of hyperphosphatemia [32]. In addition, I/R injury disturbed Mg^{2+} homeostasis, as reflected by hypermagnesemia and a decreased urinary Mg^{2+} excretion despite an increase in FE Mg^{2+}. Because the kidneys play a central role in the control of Mg^{2+} homeostasis, the impairment of renal function could also be the cause for the increase in Mg^{2+} blood levels [20]. Taken together, the deterioration of renal function in response to I/R injury may likely account for the observed hyperphosphatemia and hypermagnesemia in our study. However, it should be noted that this study was not designed to specifically address the cause of I/R injury-induced hypermagnesemia and hyperphosphatemia.

Up to now, the functional importance of the FGF23-klotho-vitamin D axis in AKI is not well-defined [45]. It has been found, for example, that the rapid rise in FGF23 blood levels in response to folic acid-induced AKI is due to an increased expression of FGF23 in multiple organs including the bone, thymus, spleen, and heart [19]. The rapid increase in FGF23 blood levels is independent of the action of PTH and $1,25(OH)_2D_3$ [10], two well-known stimulators for FGF23 production [33, 64]. Consistent with this finding, I/R injury

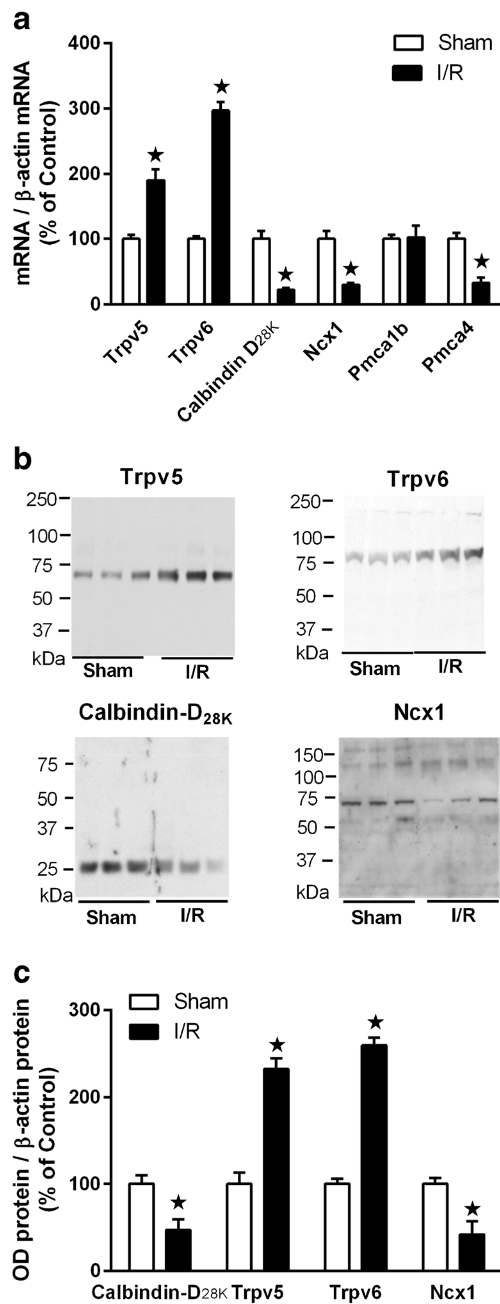

Fig. 5 Ischemia-reperfusion (I/R) injury altered the renal mRNA expression of tubular calcium transporters. Effect of renal I/R (18 h) on **a** renal Trpv5, Trpv6, calbindin-D_{28K}, Ncx1, Pmca1b, and Pmca4 mRNA expression related to β-actin mRNA expression. **b**, **c** Effect of renal I/R (18 h) on renal Trpv5, Trpv6, Ncx1, and calbindin-D_{28K} protein expression related to β-actin protein expression. Shown are representative immunoblots. Values are mean ± SEM for six animals. *$P < 0.05$ vs. control

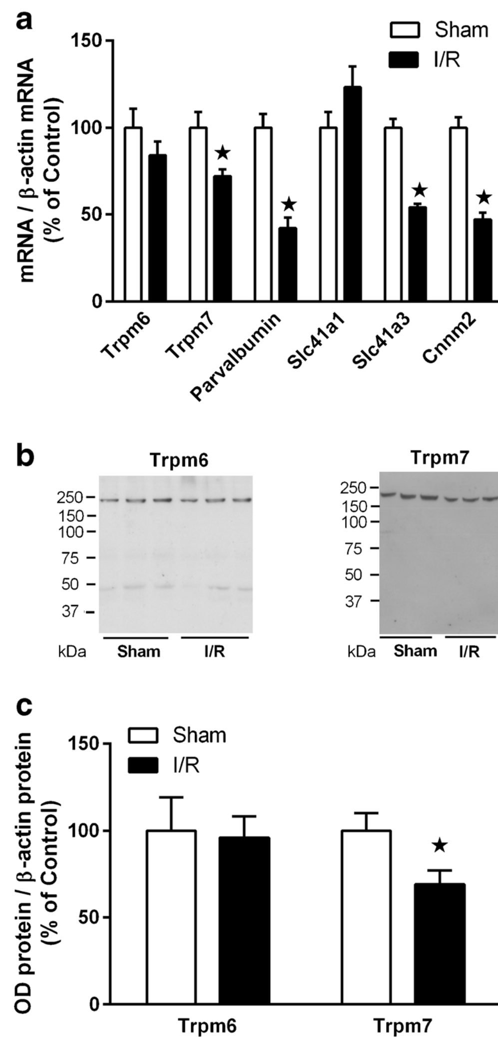

Fig. 6 Ischemia-reperfusion (I/R) injury altered the renal expression of tubular magnesium transporters. Effect of renal I/R (18 h) on **a** renal Trpm6, Trpm7, parvalbumin, Slc4a1, Slc41a3, and Cnnm2 mRNA expression related to β-actin mRNA expression, and **b** effect of renal I/R injury (18 h) on Trpm6 and Trpm7 protein expression related to β-actin protein expression. Inset shows representative immunoblot. Values are mean ± SEM for six animals. *$P < 0.05$ vs. control

was associated with increased FGF23 blood levels and unchanged levels of PTH and 1,25(OH)$_2$D$_3$. Therefore, other mechanisms and regulators of FGF23 are responsible for the observed increase in FGF23 blood levels in our study. Beside increased P_i blood levels [45], the observed increase in proinflammatory cytokines could likely account for the increase in FGF23 blood levels and renal tissue expression, as well as for the decrease in renal klotho expression in response to I/R injury [16, 26, 44, 49, 51]. Renal expression of FGF23 has been reported in animal models of acute and chronic kidney disease [19, 38, 59, 60, 67]. However, the intrarenal localization of FGF23 is still controversial. Zanchi et al., for example, found focal expression of FGF23 in both proximal and distal tubulus cells in a model of diabetic nephropathy [67]. In contrast, we did not clearly detect FGF23 mRNA in primary PT and DTCs. Therefore, our data rather support a more recent study, where FGF23 mRNA was found to be expressed exclusively in cells within the interstitial space [38]. Nevertheless, the observed increase in FGFR1 mRNA favors a possible paracrine signaling of renal-derived FGF23 in response to I/R injury. The observed downregulation of klotho, which may

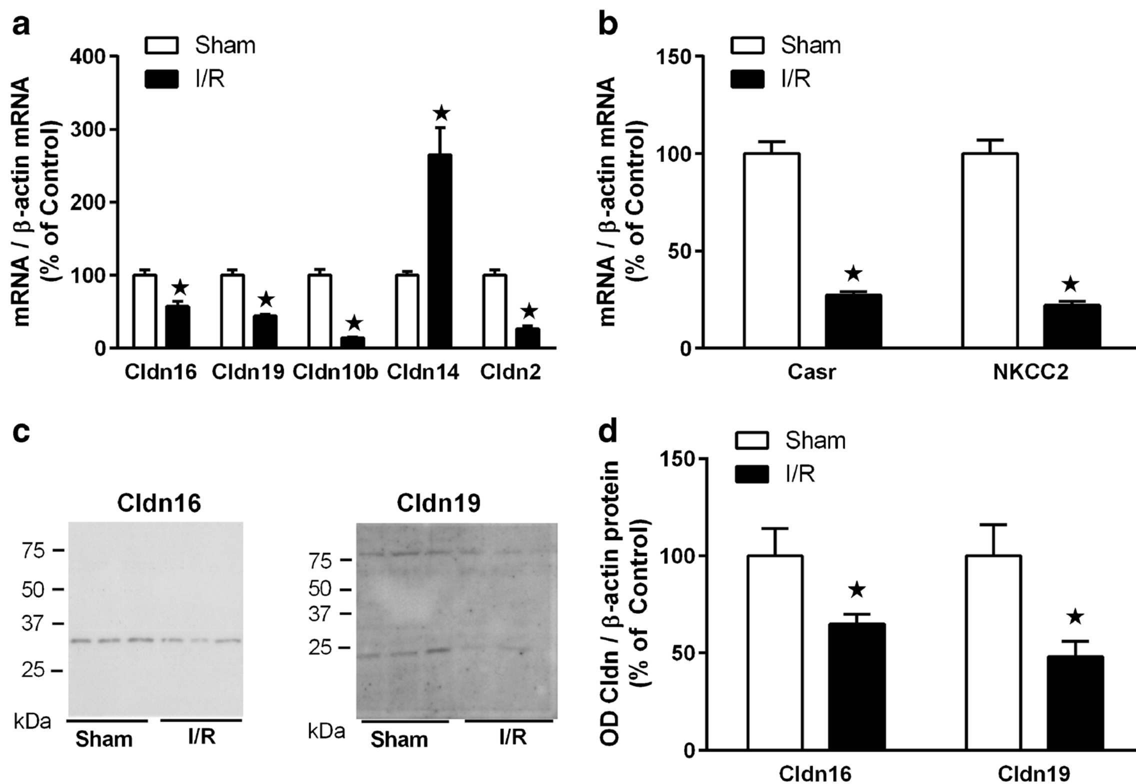

Fig. 7 mRNA expression profiles of genes involved in renal tubular paracellular transport of Ca^{2+} and Mg^{2+}. Effect of renal I/R (18 h) on renal mRNA abundance of **a** Claudin (Cldn) 2, Cldn10b, Cldn14 Cldn16, and Cldn19 related to β-actin mRNA expression, **b** renal sodium-potassium-chloride cotransporter (NKCC2) and Ca^{2+}-sensing receptor (Casr) mRNA expression related to β-actin mRNA expression, and **c**, **d** effect of renal I/R injury (18 h) on Cldn16 and Cldn19 protein expression related to β-actin protein expression. Inset shows representative immunoblot. Values are mean ± SEM for six animals. $*P < 0.05$ vs. control

theoretically lead to FGF23 resistance and thus elevated FGF23 levels, may contribute to the FGF23 elevation in response to I/R injury [45]. However, our findings rather suggest that during the acute phase of I/R injury FGF23 resistance seems not to be of major relevance, because the inhibitory effect of FGF23 on the formation of $1,25(OH)_2D_3$ via downregulation of Cyp27b1 expression and upregulation of Cyp24a1 expression was still present [58]. Confirming a human study in patients with AKI [68], but in contrast to other experimental models of AKI, like folic acid- or endotoxin-induced AKI, for example [16, 42], PTH levels were not altered in response to I/R injury in our study. Beside ionized blood Ca^{2+} levels, PTH secretion is also modulated by P_i, FGF23, and $1,25(OH)_2D_3$ blood levels [35]. Therefore, the inhibitory effect of FGF23 on PTH release could likely account for unchanged PTH blood levels despite an increase in P_i blood levels. In addition, systemic administration of folic acid and/or endotoxin may elicit additional stimulatory effects on PTH secretion, like a fall in free plasma Ca^{2+} levels, which is the main physiological stimulus for PTH secretion [42]. Therefore, our data may also indicate that acute I/R injury-induced AKI does not cause hypocalcemia. Because FGF23 is filtered by the kidneys, a decrease in GFR could also contribute to increased blood levels of FGF23 in response to I/R injury. However, previous studies suggest that an increased formation rather than decreased excretion accounts for the increase in FGF23 levels in AKI [10].

Taken together, inflammatory stimuli, the decrease in renal kotho expression as well as the decline in renal function are likely responsible for high circulating FGF23 levels, which in turn may inhibit the formation and/or secretion of PTH and $1,25(OH)_2D_3$.

To examine renal adaptive mechanisms of tubular electrolyte regulation in response to I/R injury, the renal expression of major Ca^{2+}, Mg^{2+}, and P_i transporters was investigated. In line with previous reports and with the phosphaturic effect of FGF23, renal I/R injury decreased the renal expression of NaP_i-2a and NaP_i-2c [22, 32], the principal renal type II sodium-dependent P_i transporters [63]. This downregulation occurred despite a decrease in renal klotho expression, and is likely mediated via activation of FGFR1 [22]. In contrast, NaP_i-2b mRNA abundance was clearly increased in response to I/R injury. A low basal expression of NaP_i-2b has been reported at the basolateral side of renal epithelial cells and administration of a high phosphate diet strongly increased the NaP_i-2b expression. Therefore, it has been assumed that the increased expression of NaP_i-2b may further increase tubular phosphate excretion [61]. In line with the observed

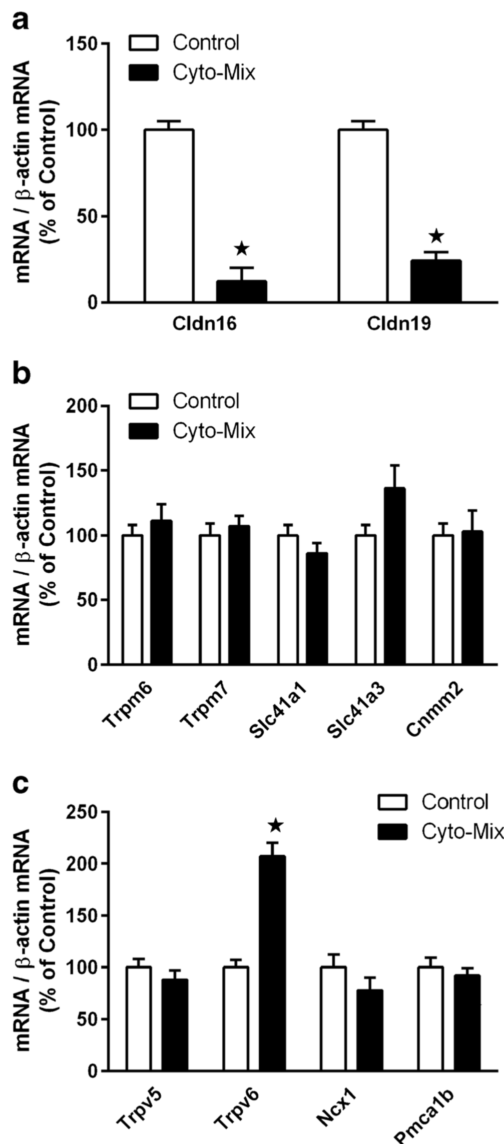

Fig. 8 Cytokines altered the expression of important renal transporters for magnesium and calcium transport in vitro. Effect of a cytokine mixture (Cyto-Mix; IL-1β and TNFα; each 100 ng/mL) on **a** claudin (Cldn) 16 and Cldn19 mRNA expression related to β-actin mRNA expression in a mouse kidney thick ascending limb cell line after 16 h, **b** on transient receptor potential melastatin (Trpm) 6, Trpm7, Na^{+}-Mg^{2+}-exchanger solute carrier family (Slc) 41a1, Slc41a3, and ancient conserved domain protein 2/cyclin M2 (Cnnm) 2 mRNA expression related to β-actin mRNA expression in primary distal tubule cells, and **c** on Trpv5, Trpv6, Ncx1, and Pmca1b mRNA expression related to β-actin mRNA expression in primary distal tubule cells. Values are mean ± SEM of four independent experiments. *$P < 0.05$ vs. control

regulation of NaP_i-2 transporters, FE P_i and the urinary excretion of Pi were increased. The increase in PiT1 mRNA expression may be a compensatory mechanism [46], and argues against a global decrease in tubular apical membrane carriers and ion channels in response to I/R injury. However, because I/R injury is associated with renal tubular injury, we cannot completely exclude that the observed changes are also a result of cellular damage [30, 31].

The expression of the Ca^{2+} transporters Trpv5, the major apical Ca^{2+} channel in the late distal convoluted and connecting tubule for the initiation of transcellular Ca^{2+} reabsorption, and Trpv6 was elevated in response to I/R injury. Increased levels of FGF23 as well as proinflammatory cytokines may likely account for this induction [3, 27, 42, 51]. Despite the increase in Trpv5, the intracellular Ca^{2+} binding protein calbindin-D_{28K} and the basolateral calcium extrusion proteins Pmca4 and Ncx1 were decreased in response to I/R injury, suggesting that the downregulation limits the overall tubular transport of Ca^{2+}. The increase of Trpv5 and the downregulation of calbindin-D_{28K} are peculiar as these two proteins are usually regulated in parallel. However, a very similar regulation has been recently reported for klotho-deficient mice, TRPV5 S682P mutant, adenine-fed mice, and endotoxemic mice [2, 37, 42, 51], suggesting that the decline of klotho and/or other unknown stimuli is involved in the different regulation of renal Ca^{2+} transport proteins. Further, our in vitro study indicates that proinflammatory cytokines directly increase Trpv6 expression, but have no effect on other Ca^{2+} transporters. Taken together, FGF23 as well as inflammatory cytokines may influence the expression of Ca^{2+} transporting proteins. It should be noted that this study was not designed to specifically address the cause of the different regulation of renal Ca^{2+} transporting proteins. The exact mechanism and signaling pathways have to be addressed in further studies.

The final urinary Mg^{2+} concentration is determined by an active, transcellular transport in the DCT. Although it has recently been found that the renal expression of Trpm6, the major apical Mg^{2+} channel in the early distal convoluted tubule for the initiation of transcellular Mg^{2+} reabsorption, is decreased in response to renal ischemia [17], Trpm6 was not altered in our study confirming a previous report [15]. The effect of I/R injury on the renal expression of Trpm7 is unclear, because increased as well as decreased or unaltered expression levels have been reported [15, 17, 36]. Increased Trpm7 expression has been associated with increased tissue damage [36, 40]. The reason for the variable effect of I/R injury on Trpm7 expression is unclear, but may be due to species differences and different ischemia periods, for example. Further, the intracellular Mg^{2+} binding protein parvalbumin and the putative basolateral Mg^{2+} extrusion proteins Cnnm2 and Slc41a3 were decreased in response to I/R injury, suggesting that the downregulation limits the overall tubular transport of Mg^{2+}. Further, the transcellular Mg^{2+} transport process in the DCT is an electrogenic process [13]. Therefore, the well-described downregulation of the thiazide-sensitive sodium chloride cotransporter (NCC) in response to I/R injury may diminish the driving force for the entry of Mg^{2+} across the apical membrane, thereby inhibiting the absorption of Mg^{2+} in the DCT [24].

The observed increase in FE Ca^{2+} and FE Mg^{2+} fits with a reduced reuptake of Ca^{2+} and Mg^{2+}. However, fractional excretion is a parameter that reflects whole tubular handling of

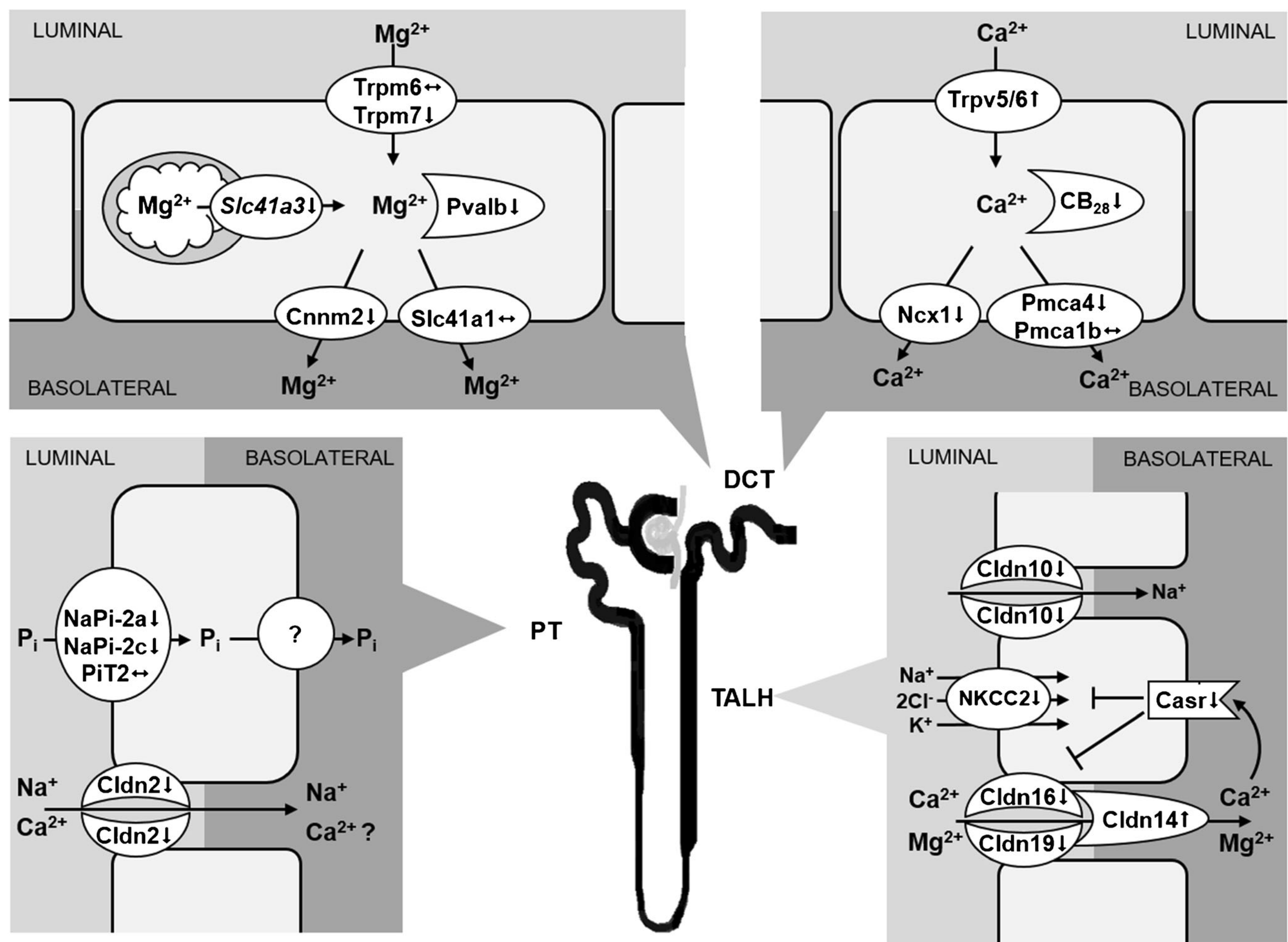

Fig. 9 Simplified summarization of the effects of I/R injury on the renal expression of specific Ca^{2+}, Mg^{2+}, and P_i transporters investigated in this study. Proximal tubule (PT): P_i enters the cell at the apical side through type II sodium-dependent P_i co-transporters (NaPi-2) and type III sodium-dependent phosphate transporter (PiT). The precise renal localization and/or function of PiT1 and NaPi-2b is unknown. Ca^{2+} and Mg^{2+} are mainly reabsorbed paracellularly. Whether Cldn2 participates in PT Ca^{2+} and/or Mg^{2+} permeability is not entirely clear. Thick ascending limb of Henle (TALH): Ca^{2+} and Mg^{2+} are reabsorbed by controlled paracellular pathways involving claudin (Cldn) 16, Cldn19, and Cldn14. The driving force is initiated by sodium reabsorption via the sodium-potassium-chloride cotransporter (NKCC2 or Slc12a1). Activation of the basolateral Ca^{2+}-sensing receptor (Casr) reduces NKCC2 activity and modulates paracellular Ca^{2+} permeability in response to increased interstitial Ca^{2+} concentrations. Distal convoluted tubule (DCT): Luminal Mg^{2+} uptake from the pro-urine is mediated by transient receptor potential channel melastatin (Trpm) 6/7 channels. Subsequently, Mg^{2+} binds to intracellular parvalbumin (Pvalb) and exits the cell at the basolateral side via the Na^+-Mg^{2+}-exchanger solute carrier family (Slc) 41a1 and the ancient conserved domain protein 2/cyclin M2 (Cnnm2). Mitochondria serve as intracellular Mg^{2+} stores. Intramitochondrial Mg^{2+} concentration is determined by Slc41a3. Ca^{2+} enters the cell at the apical side through transient receptor potential channel vanilloid (Trpv) 5/6 channels, subsequently binds to intracellular calbindin-D_{28K} (CB_{28}), and exits the cell at the basolateral side via the Na^+/Ca^{2+}-exchanger 1 (Ncx1) and the Ca^{2+} ATPases Pmca1b and Pmca4. Arrows indicate increased (↑), decreased (↓), or unaltered (↔) expression in response to I/R injury

Ca^{2+} and Mg^{2+}, and the major portions of Ca^{2+} and Mg^{2+} are reabsorbed in the PT and in the thick ascending limb of Henle (TALH) by paracellular pathways. About 60–70% of the glomerular filtered Ca^{2+} is reabsorbed in the PT. Although the majority of PT Ca^{2+} reabsorption occurs via a passive paracellular process, driven by active solute and subsequent water reabsorption, a significant unknown transcellular transport of Ca^{2+} (~30% of the total reabsorption) contributes to PT Ca^{2+} reabsorption [1]. In contrast to Ca^{2+}, only 10–20% of the filtered Mg^{2+} load is reabsorbed in the PT via a passive paracellular process [1]. Paracellular Ca^{2+} and Mg^{2+} reabsorption in the PT occurs likely via Cldn2, which depends on active, transcellular Na^+ reabsorption, driven by the NHE3 and Na^+/K^+-ATPase. In the TALH, Ca^{2+} and Mg^{2+} reabsorption via Cldn16 and Cldn19 requires a lumen-positive transepithelial voltage gradient, which drives paracellular reabsorption of Ca^{2+} and Mg^{2+}. The driving force for Ca^{2+} and Mg^{2+} reabsorption in the TALH is established by sodium absorption via NKCC2 and the activity of the Na^+/K^+-ATPase. Therefore, an impaired Na^+ reabsorption due to decreased levels of NHE3, NKCC2, and Na^+/K^+-ATPase abundance has to be considered for the increase in FE Ca^{2+} and FE

Mg^{2+} [24, 30, 32, 54]. Therefore, the I/R-induced downregulation of Cldn16 and Cldn19 in our study likely accounts for the increase in FE Ca^{2+} and FE Mg^{2+}. The increase in Cldn14 mRNA expression, which blocks Ca^{2+} reabsorption in the TALH [14], may be a compensatory mechanism, and argues against a global decrease of transporters and channels in the TALH in response to I/R injury. In addition, it should be noted that deletion of Cldn10 rescues Cldn-16-deficient mice from hypomagnesemia and hypercalciuria [8]. Therefore, the decrease in Cldn10b may counteract the decreased passive reabsorption of Ca^{2+} and Mg^{2+} due to the decrease in Cldn16 in our study. Further, our in vitro study indicates that proinflammatory cytokines directly decrease Cldn16 and Cldn19 mRNA expression. In addition, an increased concentration of Mg^{2+} in the pro-urine due to a decreased proximal tubular reabsorption of Mg^{2+} could also be involved in the downregulation of Cldn16 expression [18]. Despite the increase in FE Ca^{2+} and FE Mg^{2+}, total urinary excretion of Ca^{2+} and Mg^{2+} was decreased in response to I/R injury. It should be noted that FE and total excretion are integrated functions of the whole nephron together with GFR and renal blood flow. Therefore a decline in GFR and an altered renal perfusion, for example, should be taken into account for the observed changes in FE Ca^{2+} and FE Mg^{2+} and in the urinary excretion of Ca^{2+} and Mg^{2+}.

In summary, we provide evidence that acute renal I/R injury is associated with hyperphosphatemia and hypermagnesemia. We found a disturbed FGF23-klotho-vitamin D axis leading to an increased or decreased expression of specific renal Ca^{2+}, Mg^{2+}, and P_i transporters, respectively (Fig. 9). Hormonal as well as inflammatory stimuli mediate this dysregulation via direct and indirect effects.

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Compliance with ethical standards

All animal experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local animal protection committee.

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