The protective effect of prolyl-hydroxylase inhibition against renal ischaemia requires application prior to ischaemia but is superior to EPO treatment

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Abstract

Background. Inhibition of the HIF regulating prolyl hydroxylase domain (PHDs) proteins prior to renal injury (preconditioning) has been shown to protect the kidney via activation of hypoxia-inducible transcription factors (HIF). Application of erythropoietin (EPO), one of the HIF target genes, has also been shown to be nephroprotective, and it remains unclear to what extent the effect of HIF induction is mediated by EPO. It is also unknown whether HIF activation after the onset of ischaemia (postconditioning) is still able to protect the kidney.

Methods. Using a rat model of renal ischaemia–reperfusion injury, animals were treated with the PHD inhibitor (PHD-I) 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido) acetate (ICA), vehicle (Veh) or recombinant human EPO (300 IU/kg) 6 h (ICA or Veh) or 30 min (EPO) prior to ischaemia (preconditioning) or with ICA prior to reperfusion (postconditioning). Renal function was assessed at baseline, 24 h and 72 h. After 72 h, kidneys were processed for histology and morphometric analysis. HIF immunohistochemistry and real-time polymerase chain reaction for HIF target genes, including EPO, were performed to evaluate ICA effects.

Results. ICA treatment resulted in stabilization of HIF-1\textalpha{} and -2\textalpha{} and up-regulation of HIF target genes in a dose-dependent manner. Preconditional activation of HIF by ICA significantly improved serum creatinine levels and renal morphology in comparison to Veh (P < 0.05), while postconditional ICA treatment was ineffective. EPO therapy improved tissue morphology but had no impact on the course of serum creatinine.
Conclusion. These findings are in line with the concept that PHD-Is exert their protective effects through accumulation of HIF target gene products, with time requirements for increased transcription and translation of HIF-dependent genes, and suggest that their renoprotective effect is not predominately mediated by EPO.

Keywords: acute kidney injury; EPO; HIF, hypoxia; protection

Introduction

Acute kidney injury (AKI) is a frequent and severe clinical problem, associated with adverse patient outcomes [1]. While therapeutic options still remain very limited, experimental evidence has accumulated in recent years indicating that activation of hypoxia-inducible transcription factors (HIF) prior to renal injury protects the kidney from AKI [2–6]. HIF, an α/β-heterodimer, plays a central role in the adaptation to low oxygen tensions on the cellular as well as on the systemic level. The β-subunit (HIFβ) is abundantly expressed while the regulation of the two alternative HIFα-isofoms, HIF-1α and HIF-2α, is oxygen dependent. An enzyme family of oxygen- and 2-oxoglutarate (2-OG)-dependent dioxygenases act as oxygen sensors [7, 8]. These enzymes catalyse a hydroxylation reaction, which marks HIFα for ubiquitination and subsequent proteasomal degradation. The three enzymes, which are hydroxylating two specific prolyl-residues of HIFα (Pro402, Pro564), are termed prolyl hydroxylation domain (PHDs) proteins 1–3 [7]. In addition, an asparagyl-hydroxylase termed factor inhibiting HIF (FIH) prevents the interaction of HIF with cofactors required for target gene activation through hydroxylation of an asparagyl residue (Asp803) of the HIFα chain [9]. In the absence of molecular oxygen, the enzymatic activity of the PHDs and FIH is reduced, HIFα accumulates in the cell, forms a heterodimer with HIFβ and translocates into the nucleus, where HIFα/β binds to hypoxia response elements of HIF target genes (reviewed in [10, 11]). To date, far >100 genes induced by HIF have been described, including genes coding for kidney protective gene products like erythropoietin (EPO) [12] or heme oxygenase-1 [13]. Furthermore, HIF regulates a number of genes involved in glucose metabolism [e.g. glucose-transporter-1 (Glut-1), phosphorylase kinase], cell proliferation or cell death [11]. In addition to molecular oxygen, PHDs and FIH require the Krebs cycle intermediate 2-OG as a co-substrate. Application of 2-OG analogues can therefore result in an oxygen-independent competitive inhibition of the PHDs and the FIH with subsequent accumulation of HIFα [14]. Different PHD inhibitors (PHD-Is) have been shown to stabilize HIF in vitro [14] and in vivo [15] with subsequent target gene activation and have been shown to be nephroprotective in different models of AKI [2–5]. These studies have in common that the animals were treated with a PHD-I several hours prior to injury ( preconditioning). While this setting is in line with the assumed mechanism of the protective effect through induction of HIF target genes and translation of protective gene products, efficacy after initiation of renal injury (postconditioning) would greatly facilitate the translation of this approach into clinical practice.

Given the broad spectrum of the HIF response, the protection of the kidney is likely to be due to the concerted action of many target genes, but the role of individual genes in this context remains unclear. EPO has been shown to protect the kidney against different forms of acute [16–19] and chronic [20] kidney injury in preclinical settings. If EPO plays a dominant role in HIF-mediated renal protection, the effect of EPO treatment should be comparable to that achieved by HIF activation. However, a direct comparison of HIF activation and EPO treatment in a model of renal injury is not available.

We therefore compared the protective potential of preconditional HIF activation by a PHD-I with postconditional HIF activation and EPO pretreatment in a rat model of acute ischaemic kidney injury.

Materials and methods

Animals

The study was approved by the Institutional Review Board for the care of animal subjects. Male Sprague–Dawley rats (Charles River, Sulzfeld, Germany) weighing between 220 and 260 g were used with free access to water and standard chow.

Induction of renal ischaemia–reperfusion injury

Renal ischaemia–reperfusion was induced essentially as described previously [3]. Animals were anaesthetised with isofluorane (Forene; Abbot, Wiesbaden, Germany) and body temperature was maintained at 37°C using a heated operation table (Heating Controller type 861; Hugo Sachs Elektronik-Harvard Apparatus, March, Germany) with feedback control by a rectal probe. After median laparotomy, the right kidney was removed and processed for analysis and the left renal artery was carefully exposed and clamped with an arterial clamp (#18055-03; Fine Science Tools, Heidelberg, Germany). During the subsequent period of renal ischaemia, the abdomen was covered and animals remained under anaesthesia. After 40 min of ischaemia, the clamp was removed and the abdomen was closed. After 72 h of reperfusion, animals were sacrificed following a left-sided nephrectomy. Blood samples were drawn by tail vein puncture at baseline (0 h), after 24 h and at 72 h. Sham-operated animals underwent the same procedure without clamping of the left renal artery.

Drug description and administration

The inhibitor of the PHDs 2-(1-chloro-4-hydroxyisoquinoline-3-carbox-amido) acetate (ICA) was locally synthesized at the Department of Chemistry and Pharmacy (G.T.) according to the structural formula of the substance ‘bicyclic isoquinolinyl inhibitor’ as published by Stubbs et al. [21] and as further described Tian et al. [22], where ICA is named compound A and a structural formula is provided. ICA predominantly inhibits the PHDs but has only marginal inhibitory effects on FIH [22]. ICA was dissolved in 10% dimethyl sulphoxide (DMSO) and 90% PBS. A total volume of 1 mL was administered containing the weight adapted dosage of 25 mg/kg body weight (b.w.) or 50 mg/kg b.w., respectively. Recombinant human EPO (rhEPO) (Epoetinβ, Roche, Germany) was administered at a dose of 300 IU/kg b.w., diluted in 1 mL of isotonic saline.

Experimental groups

Animals were allocated to six experimental groups:

- **Group 1 (sham):** no clamping of the renal artery. Treatment with 25 mg/kg b.w. ICA intraperitoneally (i.p.) 6 h prior to preparation of the left renal artery (n = 5).
- **Group 2 (Veh):** treatment with 1 mL of vehicle (DMSO+PBS) 6 h prior to ischaemia (n = 8).
- **Group 3 (ICA pre-25):** treatment with 25 mg/kg b.w. ICA s.c. 6 h prior to ischaemia (n = 9).
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- Group 4 (ICA pre-50): treatment with 25 mg/kg b.w. ICA 1 min prior to ischaemia 
  
- Group 5 (ICA post): treatment with 25 mg/kg b.w. ICA 1 min prior to beginning of reperfusion

- Group 6 (EPO): treatment with 300 IU/kg b.w. of EPO intravenously 30 min prior to ischaemia

Tissue preparation

The lower half of the kidneys was snap frozen in liquid nitrogen and then kept at ~80°C for RNA analysis. The upper half was fixed in freshly prepared 4% paraformaldehyde at 4°C and subsequently processed for paraffin embedding.

Immunohistochemistry

Immunostaining of HIF-1α and HIF-2α was performed as described previously [23]. In brief, paraffin sections (2 μm) were dewaxed in xylene and rehydrated by descending concentrations of ethanol. A mouse monoclonal anti-HIF-1α antibody (clone sc67; Novus Biologicals, Littleton, CO) and a rabbit polyclonal non-commercial anti-HIF-2α antibody (PM-9) were used. For antigen retrieval, specimens were cooked for 7 min in target retrieval solution (Dako, Hamburg, Germany) using a pressure cooker. For immunostaining, a catalysed signal amplification system (CSA-Kit; Dako) based on a modified streptavidin–biotin method was used according to the manufacturer’s instructions. Between incubations, slides were washed two to three times in TBST-buffer [50 mM Tris–HCl, 300 mM NaCl and 0.1% Tween-20 (pH 7.6)]. Peroxidase–diaminobenzidine was used as chromogen. Slides were cover slipped with an aqueous mounting media (Aquamount; Merck, Darmstadt, Germany).

Morphometric analysis

Haematoxylin and eosin-stained sections of left kidneys were analysed in a blinded manner by a nephropathologist (K.A.) as described [3]. The results were semi-quantified using a scoring of 0–4; 0 = no abnormality; 1 = changes affecting up to 25% of the specimen; 2 = changes affecting up to 50% of the specimen; 3 = changes affecting up to 75% of the specimen; 4 = changes affecting >75% of the specimen. The renal damage on every sample was indicated with the average scoring of ten ×200 magnification fields.

Real-time polymerase chain reaction (PCR)

The messenger RNA (mRNA) levels of the HIF target genes EPO and Glut-1 were assessed by real-time polymerase chain reaction (PCR) (n = 3–5 per group) in the right kidneys removed before onset of ischaemia. Complementary DNA (cDNA) was synthesized using 1 μg of total RNA from rat kidneys. Random hexamer primers and the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany) were used. Real-time PCR was performed on ABI Prism 7000 thermocycler with Power SYBR Green Master Mix (Applied Biosystems, Darmstadt, Germany). Conditions were 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 60 s at 60°C. Data were analysed using the 2−ΔΔCT method. Relative expression values were calculated, using 18S as the reference transcript. Equal PCR efficiencies were documented using serial dilutions of cDNA and specificity of PCR products was verified by gel electrophoresis and melting curve analysis. Primers for rat EPO and Glut-1 and 18S were generated with ABI primer express software: EPO forward (+) 5′-GACACGGAGGCCAGAAAATGC-3′; EPO reverse (−) 5′-CACCTTCATCTTCTTTCCAAGCC-3′; Glut-1 forward (+) 5′-GGTGTCACGACGGCTTGTGTA-3′, Glut-1 reverse (−) 5′-GACCAAGCAGCAACCAGAGTC-3′; 18S forward 5′-TGGTATAGTCCCTGGCCTTGGT-3′, 18S reverse 5′-CGATCGAGGCCCCACTCT-3′.

Plasma EPO levels

Rat EPO levels of ICA and Veh-treated animals were determined using the Quantikine Mouse/Rat EPO ELISA kit (R&D Systems) according to manufacturer’s protocol at the time points 0, 24 and 72 h (n = 3–6 per time point). EPO concentrations >3000 pg/mL were indicated as ‘>Max’ by the ELISA reader and were set at 3000 pg/mL.

Plasma EPO levels after administration of 300 IU/kg rhEPO were estimated according to the pharmacokinetic data of Emmanouel et al. [24]. The mean half-life of human EPO in the rat is ~4 h, 90–93% of the initial dose remain in the plasma after a short period of administration. A rat of 200 g has an estimated blood volume of 12 mL, thus, a plasma volume of 7 mL. A dose of 300 IU/kg would be an absolute dose of 60 IU, equating to 857 000 pg of human EPO. Of these 857 000 pg, 90% (771 000 pg) appear in the plasma, which are distributed in 7 mL of plasma resulting in a concentration of 110 000 pg/mL, which disappears from circulation in a multiplexponential fashion [24] and returns to baseline after ~8 h. With an initial peak concentration of 110 000 pg/mL, EPO concentration would be ~55 000 pg/mL after 4 h. Based on these estimates, a concentration curve has been calculated and included into Figure 2B in order to compare EPO levels between groups.

Statistical analysis

Statistical analysis was performed using repeated measures analysis of variance, Student’s t-Test and Kruskal–Wallis test using SPSS Statistics software (Version 18; SPSS Inc., Chicago, IL). A P-value <0.05 was considered significant. Data are shown as mean ± SD.

Results

HIF accumulation in kidneys after ICA treatment

Two dosages of ICA, 25 mg/kg b.w. used in ICA pre-25 (Group 3) and sham (Group 1) and 50 mg/kg b.w. used in ICA pre-50 (Group 4) stabilized both HIF-α isoforms in vivo (Figure 1). As described previously, HIF-1α was found in nuclei of tubular epithelial cells (Figure 1, left panel) and HIF-2α in interstitial and endothelial cells (Figure 1, right panel) [3, 23]. Thus, ICA is able to activate HIF in the rat kidney, which is prerequisite for testing its potential protective effect in a rat model of ischaemic AKI. Using immunohistochemistry, there was no apparent difference in the distribution or intensity of HIF signalling between both doses. In right kidneys of animals receiving no treatment prior to nephrectomy (ICA post; Group 5) or a vehicle injection (Group 2) or EPO (Group 6), HIF-α was not detectable (Figure 1 and data not shown).

Effect of ICA on target gene expression

In order to evaluate the transcripational response at the time of initiation of ischaemia 6 h after ICA treatment, the mRNA levels of the HIF target genes EPO and Glut-1 were quantified in the right kidney, which was removed immediately prior to ischaemia of the left kidney. EPO mRNA was strongly up-regulated in the groups pretreated with ICA (ICA pre-50 and ICA pre-25), as compared to untreated or Veh-treated animals (Figure 2A). The increase of EPO mRNA was dose dependent [37.7±34-fold in ICA pre-25 (Group 3) compared 124.8±77-fold in ICA pre-50 (Group 4); P < 0.05]. In addition, 50 mg/kg of ICA significantly increased Glut-1 compared to Veh (P = 0.016), while the increase in Glut-1 following 25 mg/kg did not reach statistical significance (P = 0.123) (Figure 2A), thus also pointing to a dose-dependent effect of ICA. However, the comparison of Glut-1 mRNA levels in the two treated groups did not reveal a significant difference (P = 0.289). In comparison to Veh, plasma EPO levels (Figure 2B) increased after ICA application with a significantly higher increase in the ICA pre-50 group than in the ICA pre-25 group at 24 h (P = 0.02). At 72 h, the EPO levels in both groups were comparable (Figure 2B).
Effects of preconditional HIF activation as compared to postconditional activation and EPO treatment on renal function

No differences in baseline creatinine levels were observed between the different experimental groups (Figure 3). Following ischaemia–reperfusion, serum creatinine rose significantly in vehicle-treated animals (Group 2, $P < 0.01$ versus sham) but increased only marginally in sham-operated animals (Group 1) at 24 and 72 h, respectively (Figure 3).

Preconditional HIF activation with both ICA doses (Groups 3 and 4) significantly reduced the rise in serum creatinine at 24 h ($P = 0.001$ for Group 3 and $P = 0.044$ for Group 4, respectively) and 72 h ($P = 0.001$ for Group 3 and $P = 0.003$ for Group 4, respectively) (Figure 3A), while no difference in creatinine was evident between ICA pre-25 (Group 3) and ICA pre-50 (Group 4) at both time points (Figure 3A; $P = 0.121$ at 24 h, $P = 0.711$ at 72 h).

ICA application at the beginning of reperfusion (post-conditioning) (Group 5) had no significant effect on serum

*Fig. 1.* HIF-1α and HIF-2α are stabilized by ICA in vivo. Six hours after treatment with both dosages (25 mg/kg b.w. (sham, ICA pre-25) and 50 mg/kg b.w. (ICA pre-50) of ICA), HIF-1α accumulated in tubular epithelial cells of the removed right kidneys, and HIF-2α was detectable in interstitial and endothelial cells. Kidneys of vehicle-treated animals (Veh) stained negative for HIF-α. (Immunohistochemistry, magnification ×400).
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Injection of 300 IU/kg rhEPO were estimated based on the previously determined pharmacokinetics of human EPO in the rat [24]. At the time of ischaemia, the EPO levels are presumably 30- to 50-fold higher than after ICA treatment (Figure 2B) and will return to baseline after ~8 h (Figure 2B).

Effects of preconditional HIF activation as compared to postconditional activation and EPO treatment on renal morphology

As expected, renal ischaemia–reperfusion resulted in marked changes in kidney morphology, comprising brush border loss, interstitial fibrosis, flattening of the tubular epithelium, acute tubular necrosis and leucocyte infiltration. In contrast, the kidneys of sham-operated animals (Group 1) showed only minimal morphologic changes as compared to those of non-treated, normal animals.

In line with the functional data, the kidneys of the animals pretreated with either of the two doses of ICA (Group 3 and Group 4) showed significantly preserved renal morphology compared to vehicle-treated animals (Group 2) (Figure 4; P < 0.05), but there was no difference between the two groups (P = 0.742). Histology between animals with postconditional HIF activation (Group 5) and vehicle treatment (Group 2) was not different (Figure 4, P = 0.678). In contrast to the lack of effect on serum creatinine values, EPO treatment (Group 6) resulted in a significant improvement of renal histology as compared to vehicle treatment (Group 2) (Figure 4, P = 0.037). In fact, despite the significant differences in renal function, no differences were observed in the histomorphological scoring of renal injury at 72 h between preconditional HIF activation (Group 3 and Group 4) and EPO treatment (Group 6) (Figure 4; P = 0.742), suggesting that EPO has some tissue-protective properties, which differ, however, from those of PHD inhibition and do not equally translate into a preservation of renal function.

Discussion

The present study was performed to better characterize the nephroprotective effect of inhibiting enzymes that initiate the degradation and reduce the transcriptional activity of HIF. Our results confirm that this approach results in protection of the kidney against ischaemia–reperfusion injury and show that it is superior to the administration of EPO but requires intervention prior to injury.

In our experiments, the 2-oxoglutarate analogue ICA, which predominantly acts on PHDs and not on FIH [22], yielded similar effects as described previously for other 2-oxoglutarate analogues, including t-Mimosin, dimethylloxalylglycin [5], FG-4487 [3] and FG-4497 [2, 4]. ICA resulted in accumulation of both HIF isoforms and a dose-dependent induction of known HIF target genes in the kidney. Although the net effect associated with renal HIF activation prior to AKI is protective, the importance of the two HIF isoforms, of HIF induction in different target cells and of the relative role of different genes induced by HIF are unknown and difficult to define.
Particular interest has arisen in the role of EPO [25, 26]. EPO is produced by cortical peritubular cells [27, 28] and primarily regulated by HIF-2α [29–31]. PHD-Is used in this and previous studies have been shown to induce EPO in rodents [3], non-human primates [32] and humans.

Fig. 3. Only preconditional HIF activation improved renal function after ischaemic AKI. Application of ICA 6 h prior to injury (preconditioning) resulted in significantly lower serum creatinine levels compared to vehicle (Veh) after 24 and 72 h, respectively (P < 0.05), with no difference between the higher and the lower dosage of ICA (A). Comparison of ICA treatment after injury (ICA post) with vehicle did not reveal a difference in serum creatinine at any time point (B) and compared to preconditioning (ICA pre-25 or ICA pre-50), serum creatinine levels were significantly higher at 72 h (P < 0.05), albeit not at 24 h (P > 0.05) (C). After application of EPO serum creatinine levels were comparable to vehicle (veh) and postconditional ICA administration (ICA post) (P > 0.05, respectively) (C). The difference in creatinine between EPO and preconditional HIF activation reached significance after 72 h (P < 0.05), but not at 24 h (P > 0.05) (C). Serum creatinine in the sham group was significantly lower than in all other groups at 24 and 72 h (P < 0.05) (A–C). The baseline creatinine (0 h) did not differ between groups (P > 0.05). (Data are presented as mean ± SD, #P < 0.05 versus Veh; *P < 0.05 versus ICA pre-25).

Fig. 4. Preconditional HIF activation and EPO application improved renal morphology. (A) Scoring for acute tubular necrosis 72 h after reperfusion revealed a better preserved renal morphology after preconditional ICA (ICA pre-50, ICA pre-25) and EPO treatment compared to vehicle (Veh) and postconditional ICA administration (ICA post). (Data are presented as mean ± SD, #P < 0.05 versus Veh). (B) Representative haematoxylin and eosin-stained renal cross-sections of animals of the respective experimental group (magnification×400).

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imply major challenges for the possible translation of the protective effect of PHD-Is into clinical application. Nevertheless, their application could be useful in a variety of clinical situations. Firstly, there are situations, in which AKI occurs predictably, so that intervention prior to injury is feasible. We have recently shown in a rat model of kidney transplantation that donor treatment a few hours prior to nephrectomy improves early and long-term graft function [2]. Other situations that might justify preventive treatment with a PHD-I include radiocontrast application or complex surgery. Secondly, AKI in the clinical setting is not always a ‘one-hit event’ but frequently results from repetitive injury and our results do not exclude that in such situations HIF induction beyond the endogenous response could prove beneficial. Finally, since we have only tested a single-dose treatment prior to reperfusion, we can also not exclude potential effects of HIF stabilization by repetitive application of a PHD-I on the regenerative capacity of tubular cells during the post-ischaemia period. With regard to preconditioning, repetitive application of Dimethylolglycine prior to renal ischaemia in mice was not superior to a preconditioning protocol with application of a single dose of the PHD-I l-mimosine 6 h prior to injury [3], suggesting that pretreatment with a single dose of a PHD-I is sufficient to protect the kidney from ischaemic injury.

In summary, the study confirms the marked tissue-protective effect of oxoglutamate analogues in a model of ischaemia–reperfusion injury. Our findings strengthen the concept that this protective effect is mediated through concerted induction of HIF target genes and indicate that EPO alone is not sufficient to mimic this protection.

Conflict of interest statement. All of the authors have read the manuscript and approved its submission. The manuscript has not been published previously and is not being considered for publication elsewhere, either in whole or in part, except as an abstract. W.M.B. and K.-U.E. received a research grant from Roche for a different project. W.M.B., K.-U.E. and C.W. received lecture fees from Roche. K.-U.E. received consultancy honorarium from Roche. Z.W., G.S., T.G. and K.U.A. declare no conflict of interest.

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[33] and there is an interest in exploring the potential of PHD-Is for the treatment of anaemia [34–36]. Moreover, experiments reported by several investigators have indicated that comparatively high doses of EPO have tissue-protective properties and can protect kidneys against ischaemia–reperfusion injury when administered prior to ischaemia [16–18, 37–39]. In addition, some authors also reported protective effects of high doses of EPO on isolated tubular cells, suggesting that these cells have EPO receptors [40]. It is therefore tempting to speculate that the protective effect of PHD inhibition may to a large extent be mediated through induction of the EPO gene in the renal cortex. When testing this hypothesis through a direct comparison of HIF stabilization by PHD inhibition and direct administration of a high dose of recombinant EPO prior to injury, we found that only PHD inhibition improved the course of serum creatinine. However, blinded analysis of tissue morphology did indicate that EPO administration was tissue protective. The reason for the discrepancy between functional and morphological assessment in EPO-treated animals remains unclear. However, the extent of the protective effect of EPO reported in previous studies is also variable and some studies reported no effect on renal function after injury [41]. Differences between different studies may be related to the different animal species investigated, differences in the protocol as well as differences in the preparation of recombinant EPO. The dose of 300 IU/kg b.w. given 30 min prior to injury in our experiments was adopted from Sharples et al. [17], who reported improved function and morphology 24 h after bilateral clamping of the renal pedicle for 45 min in Wistar rats. Interestingly, recent attempts to protect human kidney transplants against ischaemia–reperfusion injury with a similar dose of rhEPO (related to b.w.) were not successful [42, 43], also indicating that the protective effect of EPO is complex and not entirely robust. Whatever the discrepancies between changes in morphology and function may be due to the direct parallel group comparison with PHD inhibition suggests that the latter is far more effective in terms of preservation of renal function. This difference was observed although the serum EPO concentrations achieved after application of rhEPO were presumably much higher than those achieved through stimulation of endogenous EPO production. It remains a limitation that a direct comparison of tissue EPO concentrations in the kidney is impossible. Nevertheless, the data suggest that the effect of PHD inhibition is not predominately mediated by renal EPO induction, although we can certainly not exclude that EPO may contribute to the protective effect.

The second main finding of our experiments is that the PHD-I has to be administered prior to injury to achieve renal protection. This is important since this class of compounds could theoretically interfere with pathways other than HIF and exert acute protection against reperfusion injury through mechanisms other than HIF induction. The fact that such a protective effect was not observed strengthens the concept that tissue protection is achieved through a cascade of HIF stabilization, increased target gene transcription and translation of the respective gene products, processes, which in aggregate require a few hours to achieve sufficient efficacy [2]. Obviously, these findings

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