Activation of hypoxia-inducible factors ameliorates hypoxic distal tubular injury in the isolated perfused rat kidney

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Abstract

Background. Preconditioning activation of HIF with specific prolyl-hydroxylase inhibitors (PHD-I) attenuates proximal tubular injury, induced by warm ischaemia/reperfusion (Bernhardt, JASN, 2006). Distal tubular damage occurs in humans with acute kidney injury (AKI), in experimental contrast media-induced nephropathy (CIN), as well as in cell-free isolated perfused kidneys (IPKs). Since in the IPK distal tubular damage inversely correlates with HIF activation (Rosenberger, KI, 2005), we explored the potential of PHD-I to improve morpho-functional outcome in this model.

Methods. Male SD rats were randomly given the synthetic PHD-inhibitor FG-4497 (FibroGen®, 50 mg/kg IV) or its vehicle (CTR, n = 10 per group). Six hours later, the right kidney was perfused for 90 min with cell-free oxygenated medium and subsequently perfusion-fixed for morphologic assessment. The left kidney was used for HIF immunostaining.

Results. As compared with CTR kidneys, at 6 h after FG-4497 HIF-α isoforms were markedly up-regulated in all renal zones: HIF-1α in tubules and in papillary interstitial cells (IC), HIF-2α in IC and vascular endothelial cells. FG-4497 treatment resulted in a higher perfusate flow rate (P < 0.04, ANOVA). Tubular injury to medullary thick ascending limbs (mTALs) was significantly attenuated in the treatment versus control group (38.9 ± 6.7% versus 62.7 ± 4.9% of mTALs in the mid-inner stripe (P < 0.02); 23.8 ± 6.8% versus 45.6 ± 7.4% in the innermost zone of the inner stripe (P < 0.05).

Conclusions. These findings illustrate that PHD-I preconditioning attenuates hypoxic distal tubular injury produced in the IPK in the same fashion in which it protects proximal tubules. mTAL conservation may be related to the stabilization of cellular HIF, as well as to preserved endothelial function and microcirculation.

Keywords: HIF-1α; HIF-2α; hypoxia; medullary thick ascending limb; prolyl-hydroxylase

Introduction

Acute kidney injury (AKI), an important cause of morbidity and mortality among critically ill patients, often complicates specific medical, surgical or imaging interventions in quite a predictable pattern. Assessment of risk stratification for the development of AKI has been well established under various clinical setups, such as cardiac surgery [1,2], or radiocontrast administration [3,4]. Since renal parenchymal hypoxia evidently plays an important role in the development of AKI [5], induction of hypoxia adaptation has been proposed as a novel potential preventive/therapeutic approach in such high-risk patients [6,7].

Hypoxia adaptation is mediated by hypoxia-inducible factors (HIFs). HIFs are heterodimers composed of a constitutive β-subunit and one of at least two different oxygen-dependent α-subunits. Regulation of HIF mainly occurs by oxygen-dependent proteolysis of the α-subunit. Under normoxia HIF prolyl hydroxylases (PHD), which may be regarded as cellular oxygen sensors, initiate a cascade of events resulting in HIFα degradation. During hypoxia, PHD activity is blocked, HIFα accumulates, translocates into the nucleus, binds to HIFβ and the HIFαβ-dimers act as transcription factors. Many of the HIF-target genes are cell/tissue-protective [6,7]. HIF activation occurs in the kidney under various hypoxic conditions, such as global or segmental renal artery occlusion, following the induction of functional anaemia [8], the administration of iodinated radiocontrast agents or non-steroidal anti-inflammatory drugs [9]. Renal HIF activation has been shown during experimental AKI, spatially and chronologically in parallel with...
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The distribution of tissue hypoxia [9,10]. It has also been noted during chronic experimental tubulo-interstitial disease [11], in uncontrolled diabetes [12] and in the human transplanted kidney [13]. In various experimental settings, HIF activation proved to be stimulus-specific, cell-specific and inversely correlated to the extent of cell damage [8–10,13].

Activation of the HIF system with use of synthetic PHD inhibitors (PHD-I) has been shown to attenuate proximal tubule damage in vivo induced by renal ischaemia–reperfusion-mediated injury [14,15]. This study was planned to assess the potential of such an inhibitor, FG-4497, to prevent tubular injury in the isolated perfused kidney (IPK)—an ex vivo model of hypoxic AKI, characterized by selective outer medullary distal tubular injury.

Methods

General

The PHD-I, FG-4497, was provided by FibroGen Co. This small molecule inhibitor of PHD enzymes was previously disclosed in patent filings US20040254215A1. It was reported to induce HIF activity in HeLa and 1G6 cells, to increase plasma erythropoietin (EPO) (100- to 150-fold within 4–6 h) and haemoglobin in rodents [16,17], to enhance the induction of HbF in erythroid progenitors [17] and to ameliorate mucosal damage in experimental murine colitis [16]. All other chemicals were purchased from Sigma Co. Male Sprague Dawley rats (383 ± 6 g), fed on regular chow and allowed free excess to water, were used for all experiments. Studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Treatment groups and experimental design

Rats, randomized to the treatment (PHD-I) or control (CTR) groups, were anaesthetized with ketamine (100 mg/kg IP). Freshly prepared FG-4497 50 mg/kg dissolved in alkaline 5% dextrose (10 mg/dL, pH 8.7), or its vehicle, respectively (n = 10 per group), was injected to the femoral vein and the rats were allowed to recover. Six hours later, the animals were anaesthetized with Inactin (100 mg/kg IP). As previously described [10], the right kidney was prepared for isolated perfusion: following mid-abdominal incision the ureter was cannulated and a glass cannula was inserted into the renal artery through the superior mesenteric artery and the aorta. Renal perfusion flow was uninterrupted, with instantaneous substitution of blood flow with perfusate flow, and immediate removal of the kidney into the perfusion system. The left non-perfused kidney was subsequently hastily removed, cut, immersed in 3% paraformaldehyde in phosphate-buffered saline (pH 7.4), stored for 4 h at 4°C and then transferred into cooled phosphate-buffered saline until paraffin embedding for immunohistochemistry.

The dosage of FG-4497 and the time chosen for isolated perfusion following its administration were aimed to achieve marked up-regulation of HIF-target genes. Indeed, under these settings, a 150-fold increase was noted in plasma EPO in mice [16].

The IPK system

IPK was performed according to the method by Ross et al. [18]. Perfusion media consisted of a Krebs-Ringer-Henseleit solution containing (in mM) Na 143; K 4.5; bicarbonate 24; Ca 2.5; Mg 1.2; phosphate 1.2 and bovine serum albumin at a concentration of 6.7 g/dL (pH 7.4). The perfusate was supplemented with a mixture of 20 amino acids, as previously detailed [19].

All perfusions, gassed with 5% CO2 and 95% O2, were carried out in a temperature-controlled chamber (38°C) for 90 min at a constant pressure of 100 mmHg at the catheter tip. The rate of perfusate flow was monitored by a Brooks flow meter in line. Sequential urine collection and perfusate samples were obtained for clearance measurements every 10 min after a 20-min period of stabilization. Glomerular filtration rate (GFR) was estimated from the clearance of 3H-inulin. Filtration fraction (FF) was extrapolated from the perfusate flow and GFR. Fractional sodium reabsorption (TRNa) and potassium excretion (FEK) were calculated from their concentrations in the perfusate and urine, and the inulin clearance. At the conclusion of the experiments kidneys were perfusion-fixed with 1.25% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for the assessment of renal morphology.

Determination of renal morphology

Slices of the IPK were postfixed in buffered 2% OsO4, dehydrated and embedded in an Araldite-EM bed 812 mixture. Large sections were cut perpendicular to the renal capsule, containing cortex and medulla. The sections (1 µm) were analysed in a blinded fashion for morphologic alterations.

Cortical damage and papillary necrosis were absent in these studies, and injury was confined to the outer medulla. As previously detailed [20], tubular necrosis at this region was determined separately for S3 proximal tubules in the outer stripe and for medullary thick limbs (mTALs) in the outer, mid- and inner zones (A, B and C zones, respectively) of the inner stripe of the outer medulla. The extent of damage was expressed as the percentage of necrotic tubules out of total tubules counted.

Immunohistochemistry

Immunostaining for HIF-1α and HIF-2α was performed in 3 µm paraffin sections from the immersion-fixed left kidney, using primary antibodies as previously reported [8–13]: mouse anti-human HIF-1α (α 67, Novus Biologicals, Littleton, CO, USA, 1:10 000), and rabbit anti-mouse HIF-2α (PM9, gift from Patrick Maxwell, Wellcome Trust Centre for Human Genetics, Oxford, UK, 1:10 000). Glut-1 was detected using polyclonal rabbit anti-human Glut-1 (1:10 000; Biotrend, Golden, CO, USA) as previously detailed [8].

Immunostaining for HIF-α isoforms was assessed semiquantitatively in a blinded fashion, separately for cortical, outer- and inner medullary structures: HIF-1α in glomeruli and in cortical tubules, in CDs and S3 segments in the outer stripe, in CDs and mTALs in the inner stripe and in papillary CDs and interstitial cells (IC); HIF-2α in vascular...
endothelial- and interstitial-cells in the cortical labyrinth, within glomeruli, in the outer and inner stripes and in the papilla.

**Statistics**

Data are presented as the mean ± SEM. Two-way analysis of variance (ANOVA) with the post hoc Newman Keuls test was carried out for between-group comparisons of functional parameters. The non-paired t-test was applied for comparisons of medullary tubular damage and HIF expression in the two experimental groups. Statistical significance was set at $P < 0.05$.

**Results**

**HIF and Glut-1 immunostaining**

As opposed to previous data from our laboratory in perfusion-fixed kidneys, which all had been negative for HIFα, the immersion-fixed kidneys investigated in the present study occasionally were positive for HIFα (Figures 1–3). FG-4497 induced extensive HIFα expression at all renal zones. HIF-1α immunostaining was principally found in tubular segments (Figure 2B, D, F and H), but glomerular (Figure 2B) and papillary IC (Figure 2H) stained as well. The intensity of tubular HIF immunostaining was strong in the labyrinth (Figure 2B) and in CDs in all renal zones (Figure 2B, D, F and H), less intense in S3 segments (Figure 2D) and weak in mTALs (asterisks in Figure 2F). HIF-2α immunostaining was restricted to glomerular (Figure 3B), endothelial cells and IC (Figure 3B, D, F and H).
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Fig. 3. Comparison of HIF-2α immunostaining in non-perfused CTR and PHD-I-treated kidneys. Abbreviations: G = glomerulus and VB = vascular bundles. Whereas control kidneys are near-negative, HIF-2α is markedly expressed in PHD-I-treated kidneys at all renal regions. Original magnification: 120×.

In the inner stripe of the outer medulla, the renal zone with most prominent injury in this experimental setting, Glut-1 immunostaining was detected in mTALs and to a lesser extent in CDs. FG-4497 markedly intensified Glut-1 expression at the same distribution pattern (Figure 4).

Kidney function
As shown in Figure 5, renal perfusate flow was larger in PHD-I-treated rats, as compared with CTR (P < 0.04, ANOVA), and urine volume tended to be lower (P = 0.11). GFR, TRNa, FF and FEK were all comparable in the two experimental groups. Average absolute sodium reabsorbed during the last 30 min of perfusion was 52.2 ± 6.7 µmol/min and 41.9 ± 5.5 µmol/min for the PHD-I and CTR groups, respectively (non-significant difference).

Renal morphology
As repeatedly reported by our group [21], widespread moderate degree of hypoxic damage (mitochondrial swelling and nuclear pyknosis, without cell membrane disruption) developed in CTR kidneys over 90 min of perfusion under the conditions specified above (Figure 6). A small fraction of affected tubules displayed severe (irreversible) injury, characterized by cell membrane disruption and cell fragmentation [21].

Outer medullary hypoxic injury was significantly attenuated in the PHD-I versus CTR group. As shown in Figure 7, it was noted in 38.9 ± 7.4% versus 62.7 ± 4.9% of mTALs in the mid-inner stripe (Zone B, P < 0.02) and in 23.8 ± 6.8% versus 45.6 ± 7.4% of mTALs the innermost zone of the inner stripe (Zone C, P < 0.05). Tubular injury among S3 segments of proximal tubules in the outer stripe was 8.3 ± 4.1% versus 16.4 ± 5.4% (non-significant difference).
Fig. 5. Functional parameters in the two experimental groups. While perfusate flow is significantly higher in the PHD-I group ($P < 0.04$, ANOVA) and urine volume tends to be lower ($P = 0.11$), the two experimental groups display comparable glomerular filtration (GFR), filtration fraction, tubular sodium reabsorption (TRNa) and fractional potassium excretion (FEK).

Discussion

Hypoxic preconditioning may be an alternative to ischaemic preconditioning, which improves tolerance to experimental AKI [22,23], but is impractical in clinical settings. Most likely, hypoxic preconditioning is conferred through HIF target genes, among which EPO and haem-oxygenase-1 clearly have cell/tissue protective properties [24–27]. Pharmacological up-regulation of HIF by PHD-I is a novel strategy in the prevention of AKI. Indeed, PHD-I were found to attenuate experimental AKI, induced by warm ischaemia and reperfusion [14,15]. While this model is characterized by predominant proximal tubular injury, in human AKI both proximal and distal tubular injuries have been reported [28]. To our knowledge the present study is the first to demonstrate that PHD-I preconditioning protects distal tubules, namely mTALs, in experimental AKI. This finding might therefore explain the better clinical outcome following PHD-I pre-treatment in experimental transplantation following cold-ischaemia in rats [29], an AKI setup characterized by predominant mTAL damage [30].

The hypoxia-vulnerable mTAL has a particularly low capacity to generate HIF [9,10], perhaps since it generates large amounts of reactive oxygen species [31], considered as inhibitors of hypoxic HIF activation [32]. Therefore, the current study was aimed to specifically address the potential protective capacity of PHD-I in the prevention of mTAL hypoxic injury. We used the IPK model, since it enables a strict control over the degree of renal oxygenation, allows for the consistent generation of distal tubular hypoxic injury when perfused with cell-free oxygenated medium [33] and as the spatial distribution of intrarenal oxygen gradients and inherent HIF generation in this model have been well characterized [10].

Our immunohistochemical findings confirm that PHD-I very effectively activate HIF in a cell-specific way in all renal zones, including mTALs. Furthermore, though mTAL is the nephron segment showing the least expression of HIF following PHD-I, the extent of subsequent hypoxic mTAL
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**Fig. 7.** Quantification of outer medullary hypoxic damage. S3 injury in the outer stripe (OS) and mTAL damage in the superficial-, mid- and innermost layers of the inner stripe (IS, Zones A, B and C, respectively) are expressed as the percentage of injured tubules out of all tubules counted in this region. The severity of damage is defined as moderate (mitochondrial swelling, nuclear pyknosis, represented as open/hatched bars) or severe (fragmented cell membrane, represented as black bars). The total amount of tubular damage, illustrated by the bar heights, consists of the summation of both moderate and severe injury. PHD-I treatment significantly attenuates the extent of mTAL injury in B and C zones ($P < 0.02$ and $<0.05$, respectively, versus CTR, for both moderate injury and for the combined moderate + severe damage, non-paired t-test).

Injury is significantly attenuated by PHD-I pre-treatment. Furthermore, enhanced Glut-1 expression in mTALs illustrates that HIF activation leads to transactivation of target genes with potential cell-protective action.

The IPK model provides a snapshot of an ongoing hypoxic insult, which on continuation would invariably lead to organ loss. Under such conditions, the finding of partial mTAL preservation by PHD-I is remarkable. Up to now only the increase in perfusate oxygen availability (by adding red blood cells, or blood substitutes with oxygen carrying capacity) or the reduction of mTAL transport activity (by reduction of glomerular filtration or with the inhibition of mTAL salt reabsorption) has been able to confer structural protection in this model [10,20,34].

Attenuation of mTAL cell injury by PHD-I reported herein can be attributed to at least two mechanisms: direct up-regulation of HIF in the mTAL cells, or due to HIF-2α activation in neighbouring interstitial/endothelial cells, resulting in a better endothelial cell function (possibly with enhanced generation of nitric oxide) and subsequently improved medullary oxygenation. PHD-I-associated lower vascular resistance with unchanged GFR indicates reduced post-glomerular resistance, and hence improved tubular oxygen supply. It is noteworthy that the cell free-IPK is characterized by high perfusate flow and low resistance, related to a large extent to reduced viscosity and denervation. The 17% further increase in renal perfusate flow in the PHD-I group conceivably reflects attenuated endothelial dysfunction, allowing for a better renal oxygen supply.

The mTAL structural preservation in the PHD-I group was not associated with statistically significantly improved kidney function, though absolute sodium reabsorption at the last 30 min period of renal perfusion, a robust counterpart of mTAL integrity [10], was 29% higher in the PHD-I group. This lack of statistical significance may be attributed to the rather moderate attenuation of structural tubular damage. In previous experiments using IPKs, functional improvement (higher GFR) was noted only when structural damage was almost completely prevented by the total cessation of mTAL ion transport activity [10,19].

Finally, immersion-fixed CTR kidneys occasionally showed some HIFα signals unlike our so far negative findings following in vivo perfusion fixation of intact kidneys. Possibly, delayed kidney fixation during immersion in formalin would cause artificial hypoxia, but this possibility needs further investigations.

In conclusion, the novel findings in this work include (a) the determination of the extent and wide distribution of renal PHD-I-stimulated HIF expression, involving all cell types at all renal regions; (b) confirmation that HIF-1α and HIF-dependent genes can be up-regulated by PHD-I specifically in mTALs, nephron segments prone to hypoxic damage that normally hardly express HIF; and (c) most importantly, our current findings illustrate that PHD-I preconditioning improves renal blood flow and attenuates mTAL injury in the functioning hypoxic kidney.

Thus, PHD-I pre-treatment can attenuate hypoxic distal tubular injury as it protects the proximal tubule. mTAL conservation in the cell-free IPK system may be related to the appearance of cellular HIF, as well as to preserved endothelial function, microcirculation and tissue oxygenation.

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