Molecular Mechanisms of Ischemic Preconditioning in the Kidney

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More effective therapeutic strategies for the prevention and treatment of acute kidney injury (AKI) are needed to improve the high morbidity and mortality associated with this frequently encountered clinical condition. Ischemic and/or hypoxic preconditioning attenuates susceptibility to ischemic injury, which results from both oxygen and nutrient deprivation and accounts for most cases of AKI. While multiple signaling pathways have been implicated in renoprotection, this review will focus on oxygen-regulated cellular and molecular responses that enhance the kidney’s tolerance to ischemia and promote renal repair. Central mediators of cellular adaptation to hypoxia are hypoxia-inducible factors (HIFs). HIFs play a crucial role in ischemic/hypoxic preconditioning through the reprogramming of cellular energy metabolism, and by coordinating adenosine and nitric oxide signaling with anti-apoptotic, oxidative stress and immune responses. The therapeutic potential of HIF activation for the treatment and prevention of ischemic injuries will be critically examined in this review.
Introduction

Acute kidney injury (AKI) is defined as a sudden reduction in glomerular filtration rate (GFR) (1, 29, 144). Historically, the etiology of AKI is divided into pre-renal, intrinsic and post-renal causes; ischemia, which limits both oxygen and nutrient supply, being one of the major causes (involved in more than 50% of AKI cases) (97, 144).

Although the care of patients with AKI, which is mainly supportive, has seen significant improvements, the clinical outcome of AKI remains poor and is associated with a mortality of ~40-80% in the intensive care setting (156). While substantial progress has been made towards understanding its pathophysiology, effective therapeutic strategies are urgently needed to lower the increasing prevalence of AKI (3). In addition to the challenges of clinical management, AKI has been shown to accelerate the progression of chronic kidney disease (CKD) to dialysis-dependent end-stage renal disease (ESRD). Recent studies have indicated that ~6% of AKI patients are likely to develop ESRD within 2 years of diagnosis (151). This finding furthermore underlines the pressing need for the development of novel therapies that counteract the pathologic sequelae associated with AKI.

Despite the large volume of blood that is normally directed towards the kidneys (~20% of cardiac output), renal pO₂ is physiologically low, especially in the medulla, where oxygen tensions of as low as 3 mmHg have been measured (103). Most of renal oxygen is utilized to fuel Na/K-ATPase, which drives tubular sodium reabsorption and other transport processes that are critical for the maintenance of physiologic homeostasis.

Since these transport processes are load-dependent, renal oxygen consumption is directly linked to GFR, which in turn is renal blood flow-dependent. Because of this intricate
functional relationship, the kidney operates within a narrow range of relatively constant tissue pO$_2$, rendering it susceptible to hypoxic injury (42). Therefore, alterations in renal perfusion from either hypotension and/or vasoconstriction often lead to clinically significant ischemia, a state of inadequate oxygen and nutrient supply. When the duration of ischemia exceeds a certain threshold, intracellular ATP stores become depleted and cells die from either apoptosis or necrosis (32). While re-establishment of adequate renal perfusion is a main therapeutic goal, rapid re-oxygenation of the ischemic kidney generates additional tissue injury involving multiple mechanisms, such as increased generation of reactive oxygen species (ROS) (72).

Although somewhat counterintuitive, susceptibility to ischemia-reperfusion injury (IRI) can be attenuated by ischemic preconditioning (IPC), which was first reported in 1986 by Murry and colleagues (114). In their study, anesthetized dogs were subjected to four sequential 5-minute-lasting coronary artery occlusions prior to the induction of coronary IRI. This intervention reduced the severity of myocardial infarction significantly, and protected against myocardial cell death. Subsequently, IPC was shown to be effective in other tissues and across several species, suggesting that the molecular mechanisms, which underlie IPC-induced cytoprotection, are evolutionary conserved and independent of cell type (20, 46, 54, 73, 86, 124). In the kidney, the degree of IPC-induced cytoprotection depends on the experimental protocol used, such as the duration of ischemia and reperfusion period (124, 125). By demonstrating that preconditioning with hypoxia is equipotent to preconditioning with ischemia, Shizukuda and colleagues established that acute changes in tissue pO$_2$ alone mediate the cytoprotective effects of IPC (148). Both, the perfusion of canine hearts with severely hypoxic blood and a short
pre-insult period of ischemia resulted in a comparable reduction in infarct size following 60 minutes of complete coronary artery occlusion (148).

While multiple signaling pathways have been implicated in mediating the protective effects of ischemic/hypoxic preconditioning, this review will focus on oxygen-regulated molecular pathways that enhance tolerance to ischemic injury and have therapeutic potential for the prevention and/or reversal of the pathologic sequelae associated with AKI.

Ischemic/hypoxic preconditioning: two phases of protection

Preconditioning is characterized by two phases of protection, an acute or early phase, which immediately follows the preconditioning stimulus and lasts no longer than several hours, and a late phase, which starts ~24h after the preconditioning stimulus and can last for several days (73, 107, 124). Traditionally, the acute phase has been viewed as being mediated by ion channels and signaling molecules produced during ischemia (61), while the late phase has been thought to depend on transcriptional responses that modulate glucose and mitochondrial metabolism, suppress ROS production and inflammation, and inhibit the expression of pro-apoptotic molecules. However, recent studies in a model of cardiac ischemia have challenged this concept and have provided experimental evidence that the acute phase of IPC is also transcription factor-dependent (139). In mammals, transcriptional responses to hypoxia are primarily mediated by hypoxia-inducible factors (HIFs), oxygen-regulated transcription factors that allow cells to respond and adapt to low tissue pO₂.
Oxygen-dependent regulation of HIF

HIF-1 and HIF-2 belong to the PAS \{PER/aryl-hydrocarbon-receptor nuclear translocator (ARNT)/single minded\} family of basic helix-loop-helix transcription factors, and consist of an oxygen-sensitive α-subunit and a constitutively expressed β-subunit, also known as ARNT (105, 147). Both transcription factors facilitate oxygen delivery and cellular adaptation to hypoxia by stimulating multiple hypoxia-responses including anaerobic glucose metabolism, adenosine and nitric oxide (NO) metabolism, angiogenesis, erythropoiesis, and iron metabolism (59, 167). In the presence of sufficient oxygen, HIF-1α and HIF-2α are targeted for rapid proteasomal degradation by the von Hippel-Lindau (VHL) tumor suppressor, which functions as the substrate recognition component of an E3-ubiquitin ligase complex (109). VHL-mediated targeting of HIF-α requires oxygen- and iron-dependent hydroxylation of specific proline residues located within the oxygen-dependent degradation domain of HIF-α (Figure 1). HIF prolyl-hydroxylation is carried out by three Fe (II)- and 2-oxoglutarate (2OG)-dependent prolyl-4-hydroxylase domain (PHD) proteins, PHD1, 2 and 3 (also known as EGLN2/HPH3, EGLN1/HPH2, and EGLN3/HPH1 respectively) (65, 76), each displaying different expression profiles and specific subcellular localization patterns \{for a recent review on this topic see (143)\}. PHD enzymes function as oxygen sensors and belong to a large family of 2OG-dependent dioxygenases (~60 family members have been identified in mammals) that utilize molecular oxygen for catalysis and regulate multiple biological processes (76, 101, 115). Under normoxia PHD enzymes are catalytically active and HIF-α is degraded, whereas under hypoxia hydroxylation is inhibited and HIF signaling is activated (Figure 1). PHD catalytic activity is furthermore inhibited by NO and ROS
(76); an important consideration for the study of oxygen sensing mechanisms under conditions where intracellular concentrations of these signaling molecules has changed.

For the purpose of experimental and/or therapeutic IPC, HIF-α stabilization and activation of HIF-dependent signaling pathways can be achieved in several ways: a) exposure to hypoxia, b) genetic inactivation of the VHL-ubiquitylation machinery, c) proteasomal inhibition, and d) inhibition of HIF-PHDs by genetic or pharmacologic means. Pharmacologic inactivation of PHD enzymes can be accomplished by exposing cells and/or tissues to either cobalt chloride (CoCl₂), iron chelators, such as desferrioxamine, or to small molecule compounds that reversibly inhibit PHD catalytic activity, several of which are now in clinical trials for the treatment of renal anemia (91). However, pharmacologic inhibition of HIF-PHDs may also exert HIF-independent effects, as HIF-PHDs have been shown to hydroxylate targets other than HIF-α. Such non-HIF targets include IκB kinase-β (IKKβ), β2- adrenergic receptor (β2AR), sprouty homolog 2 (SPRY2) and pyruvate kinase muscle isoform 2 (PKM2) (170). To what degree HIF-activating PHD inhibitors, especially those that are in clinical trials, inhibit other non-HIF-related 2OG-dependent dioxygenases remains to be investigated.

**Pre-ischemic HIF activation protects from renal ischemia**

In renal cells, the HIF system is activated under conditions of systemic or regional hypoxia, or in response to PHD inhibition. In acute hypoxia, HIF-1α has been detected in glomerular and renal tubular epithelial cells, whereas HIF-2α was detected in glomeruli, peritubular endothelial, and interstitial fibroblast-like cells, the source of renal erythropoietin (EPO) (91, 135). Compared to systemic hypoxia induced by either anemia
or exposure to carbon monoxide (CO) (135), the pattern of HIF stabilization in the ischemic mouse kidney appears to be restricted to cells that are predominantly located at the corticomedullary junction (134). With respect to the reperfused rat kidney, significant HIF-1α accumulation was found in proximal tubular cells and felt to be due to increased HIF-1α translation via enhanced mTOR/AKT activity (28). Consistent with these findings are immunohistochemical studies in human renal post-engraftment biopsies, which were obtained between 25 and 40 min after completion of vascular anastomoses, and which revealed significant variability in the extent of HIF-α stabilization. These studies demonstrated a strong positive correlation between HIF-1α expression and allograft function, suggesting a cytoprotective role for HIF-1 in the setting of acute transplant ischemia (136).

Different experimental strategies have been used to test whether enhancement of HIF expression prior to ischemic kidney injury could afford cytoprotection. Matsumoto and colleagues for example, found that cobalt, a “hypoxia-mimic” and inhibitor of PHD catalysis, conferred significant protection from renal ischemic injury in rats (108). Similarly, preconditional HIF activation in mice via either administration of CO or administration of PHD inhibitors L-mimosine, dimethyloxaloylglycine (DMOG) or FG-4487 protected the kidney from injury (9, 12, 64, 166). In keeping with this notion, HIF-α stabilization with Xenon gas provided morphologic and functional renoprotection in a mouse model of IRI. Interestingly, Xenon did not activate HIF by inhibiting its degradation, but rather by increasing its translation via the mTOR pathway (104). Likewise, treatment of donor rats with FG-4497 improved tissue injury scores, early
mortality rates as well as long-term graft survival in an allogenic Fisher-Lewis rat kidney transplant model (11).

Strong support for a role of HIF signaling in renal IPC also comes from the study of genetic mouse models. Hill and colleagues found exacerbation of ischemic renal injury in mice that were heterozygously deficient for HIF-1α or HIF-2α, and furthermore demonstrated beneficial effects of pre-ischemic HIF activation via the administration of L-mimosine (64). A cytoprotective role for HIF signaling in ischemic kidney injury is also supported by acute or constitutive deletion of Vhl in mice, which phenocopied the cytoprotective effects of hypoxic preconditioning (68, 142).

Cell type-dependent effects of HIF activation

A major challenge in the IPC field is the identification of the cell types that mediate HIF-induced renoprotection. Using a genetic approach, our laboratory has shown recently that endothelial cells are critical cellular mediators of ischemic/hypoxic preconditioning in the kidney. We demonstrated that endothelial HIF-2 plays a key role in renoprotection and suppresses IRI-associated inflammation through the modulation of endothelial vascular cell adhesion molecule 1 (VCAM1) expression and inflammatory cell adhesion (79). Specifically, we found that a) endothelial inactivation of HIF-2α, but not of HIF-1α, was associated with increased expression of renal injury markers and inflammatory cell infiltration in kidneys injured by either ischemia reperfusion or by ureteral obstruction, and b) that the negative effects of HIF-2α deletion were reversed by pharmacologic blockade of VCAM1 and its ligand very late antigen 4 (VLA4). In contrast, activation of HIF by either genetic means or via systemic PHD inhibition
protected from ischemic kidney injury and inflammation (Figure 2). In this context, endothelial HIF-2 activation may be useful for the prevention or amelioration of renal fibrogenesis and CKD progression, which are well-recognized long-term sequelae of AKI. In support of our findings are genetic studies in mice subjected to acute lung injury. In this setting, endothelial HIF-2 was shown to enhance the integrity of adherens junctions and endothelial barrier function (51), supporting the concept that hypoxic signaling in endothelial cells induces a broad cytoprotective response that is directed towards maintaining tissue homeostasis during injury. Therefore, the endothelial PHD/HIF axis represents an attractive therapeutic target for the prevention and treatment of tissue injury associated with ischemia. The renoprotective effects of HIF activation, however, do not appear to be restricted to renal endothelial cells alone, as genetic activation of HIF signaling in other cell types, e.g. epithelial and myeloid cells, has been shown to be renoprotective (43, 87, 142). The role of HIF signaling in immune responses and renal inflammation is discussed in greater detail in a separate section.

While renal epithelial HIF-1 had previously been identified as a profibrotic transcription factor in experimental CKD (63, 84), transient global pre- or post-ischemic HIF stabilization did not promote fibrosis (77). Context, tissue and cell type dependence is expected and conceptually supported by several genetic studies in non-renal injury models. For example, conditional inactivation of Hif1a in neurons resulted in protection from cerebral ischemic injury (62), whereas Baranova and colleagues reported enhanced brain injury in neuron-specific Hif1a^-/- mice following transient focal cerebral ischemia (7). In the heart and kidney, germ line inactivation of one copy of Hif1a worsened both myocardial and renal ischemia (22, 64).
Timing and duration of HIF activation determines clinical outcome

In contrast to the robust renoprotection observed with pre-ischemic administration of PHD inhibitors, post-ischemic HIF-PHD inhibition failed to ameliorate renal injury (77, 164), suggesting that the timing of HIF activation is critical for renoprotection. In contrast, PHD inhibition with DMOG increased HIF-dependent gene expression and reduced injury when administered 30 or 60 minutes post IRI in a model of cerebral ischemia (120). Although tissue-specific changes in HIF-mediated gene expression may explain these differences, the regulation of HIF signaling during the actual ischemic-reperfusion phase remains poorly understood and requires further investigation. Studies performed in our laboratory indicate that transcriptional HIF responses are suppressed in the immediate reperfusion phase, when PHD inhibitors are administered post-injury, which may be due to tissue acidification or the increased production of ROS (77).

In addition to timing, the duration of PHD inhibition/HIF activation appears to be critical for disease outcome and may produce adverse effects on tissue homeostasis. For example, cardiac function in mice with cardiomyocyte-specific Phd2 deletion was significantly improved 3 weeks after myocardial IRI (66), whereas sustained Phd2 ablation or tissue-specific HIF-1α stabilization resulted in cardiomyopathy (113). In the kidney, chronic HIF activation in proximal tubules induced by Vhl gene loss caused tubular microcysts, which showed evidence of dedifferentiation and increased proliferation (133). In addition to renal cysts, inflammatory and fibrotic lesions were observed after inactivation of Vhl in collecting ducts and a subset of distal tubules (129). Furthermore, expression of a non-degradable form of HIF-2α in renal tubules led to cyst formation and fibrosis (140), while expression of a non-degradable form of HIF-1α
resulted in lipid and glycogen deposition and as well as microcyst formation (47),
supporting the notion that chronic activation of the renal epithelial HIF system promotes
inflammation, fibrosis, cell proliferation and dysplasia.

**HIF attenuates ischemia-reperfusion injury through the coordinated activation of renoprotective metabolic and signaling pathways**

Although the molecular and cellular mechanisms underlying HIF–mediated
cytoprotection in renal IRI are complex and only poorly understood, the concerted
transcriptional activation of genes involved in cellular energy metabolism, antioxidant
responses, anti-apoptotic pathways, and other HIF-regulated biological processes is key
to effective and robust cytoprotection (Figure 3).

*a) hypoxia-induced metabolic reprogramming*

Central to achieving hypoxia tolerance is the shift of cellular metabolism from
oxidative phosphorylation to anaerobic glycolysis, which in yeast is referred to as the
Pasteur effect (146). HIF-1 mediates this shift by increasing the expression levels of
several glycolytic pathway enzymes. Among those are hexokinase (HK1),
phosphofructokinase (PFK), aldolase (ALDOA), phosphoglycerate kinase 1 (PGK1),
enolase (ENO1) and lactate dehydrogenase α (LDHα). In addition to increasing
glycolytic flux, HIF-1 blocks the conversion of pyruvate to acetyl-CoA by up-regulating
pyruvate dehydrogenase kinase 1 (PDK1). This suppresses the catalytic activity of
pyruvate dehydrogenase (PDH), which is phosphorylated by PDK1, and results in the
uncoupling of glycolysis from the tricarboxylic acid cycle (83, 123). The importance of
this HIF-1-regulated metabolic response is highlighted by in vitro studies, in which the
absence of HIF-1 during hypoxia led to increased ROS production and apoptosis, both of which can be reversed by forced PDK1 expression (83).

In order to sustain increased glycolytic flux, NAD⁺ must be regenerated through the conversion of pyruvate to lactate. This is facilitated by HIF-1 through the transcriptional up-regulation of LDHα. Inhibition of LDHα in cancer cells results in reduced ATP production, significantly increased oxidative stress and cell death, demonstrating the pro-survival function of LDHα under conditions of increased glycolytic flux (93). To counteract the effects of increased intracellular lactate production and ensuing acidification, HIF-1 facilitates the excretion of lactate and protons by increasing the expression of sodium/hydrogen exchanger (NHE)-1 and monocarboxylic acid transporter (MCT)-4 (126). In fact, intracellular pH is actually kept in a slightly alkaline range as a result of increased expression of membrane-bound ectoenzyme carbonic anhydrase (CA) 9, which is HIF-1-regulated and converts CO₂ to bicarbonate (126).

In addition, HIF-1 activation decreases cellular oxygen consumption through its suppressive effects on mitochondrial biogenesis and metabolism. HIF-1 inhibits c-MYC (177) and improves the efficiency by which mitochondria utilize oxygen. The latter occurs through HIF-1-mediated changes in the subunit composition of mitochondrial complex IV (48). Furthermore, HIF-1 controls mitochondrial mass by regulating mitophagy through B-cell lymphoma (BCL)-2 family member BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3) (176). The role of individual dioxygenases in cellular metabolism through either HIF-dependent or HIF-independent mechanisms adds additional layers of complexity to the metabolic regulation of hypoxia tolerance. Aragones and colleagues found that inactivation of PHD1 alone lowers oxygen
consumption in skeletal muscle by reprogramming glucose metabolism from oxidative to anaerobic ATP production through the activation of peroxisome proliferator-activated receptor alpha (PPARA). This metabolic shift, which was HIF-2-dependent, reduced oxidative stress and preserved viability of ischemic myofibers (4). Therefore, activation of the PHD/HIF axis, through its effects on cellular energy metabolism, enhances the capacity of tissues to generate ATP in hypoxic environments, which is predicted to increase resistance to ischemic injury.

b) oxidative stress responses

Intracellular ROS play a key role in the pathophysiology of ischemic renal injury, as signaling pathways that scavenge ROS and/or prevent their formation, promote cell survival and limit post-ischemic damage. HIF-2 has been implicated in the regulation of cellular oxidative stress responses, as HIF-2α-deficient mice exhibit impaired ROS clearance and develop multiple organ pathologies (145). HIF-2 plays a crucial role in this regard, as it stimulates the transcription of primary antioxidant enzymes such as catalase (CAT), glutathione peroxidase type 1 (GPX1), copper/zinc superoxide dismutase (SOD1) and manganese superoxide dismutase (SOD2) (145). In support of this notion are studies in mice homozygous for a Hif2a hypomorphic allele, which indicated a protective role for endothelial HIF-2 in renal IRI via regulation of ROS scavenging (89). Further support for a key role of HIF-2 in IPC comes from a study where transient ureteral obstruction was used as a preconditioning maneuver and protected from renal IRI in a HIF-2-dependent manner. The investigators of this study proposed that this occurred via enhanced recovery of intrarenal microvascular perfusion (178).
The hypoxic regulation of renal oxidative stress responses also involves HIF-1. Heme oxygenase 1 (HMOX1), for instance, is HIF-1-regulated, degrades heme and generates CO and bilirubin, both of which have antioxidant and/or anti-inflammatory effects (137). Induction or overexpression of HMOX1 confers protection from renal IRI, while genetic inactivation of HMOX1 results in exacerbation of injury (14, 117, 128, 169).

c) adenosine signaling

Adenosine, an endogenous purine nucleoside involved in cellular adaptation to hypoxia, is considered by many investigators to be a critical mediator of IPC. The relationship between HIF signaling and adenosine became evident when administration of adenosine rescued Hif1a+/− hearts from IRI injury (22). This finding indicated that the production of adenosine in IPC required intact HIF-1 signaling. Extracellular adenosine is mainly derived from the enzymatic phosphohydrolysis of ATP and ADP, the first step of this reaction being catalyzed by ectonucleoside triphosphate diphosphohydrolase 1 (CD39), and the final step being the dephosphorylation of AMP, which is catalyzed by 5′-ectonucleotidase (CD73).

A mechanism by which IPC enhances adenosine production involves the hypoxic induction of CD39 and CD73 genes. While HIF-1 binds directly to the CD73 promoter, the hypoxic regulation of CD39 involves the Sp1 transcription factor (41, 152). Both enzymes have been studied in the context of renal IRI and IPC. The role of CD39 was investigated using a hanging weight model to induce ischemia in mice (56). Remarkably, pharmacologic inhibition of CD39 eliminated the protection normally conferred by IPC. Similarly, renoprotection was abolished in Cd39−/− mice. Administration of soluble
apyrase, an adenosine diphosphatase derived from *Solanum tuberosum* recapitulated the protective effects conferred by IPC. In contrast to CD39, the protective role of CD73 in renal IRI and IPC is more controversial. While Grenz and colleagues demonstrated a renoprotective function using similar experimental protocols (55), others reported that CD73 promoted tissue injury in a model of unilateral renal IRI, which was attributed to excessive accumulation of AMP (102). In addition to increasing adenosine production, HIF also modulates the clearance of extracellular adenosine. Specifically, HIF-1 diminishes uptake of adenosine via transcriptional repression of the equilibrative nucleoside transporter 1 (ENT1) and thereby contributes to the accumulation of extracellular adenosine (40). Accordingly, *Ent1*<sup>−/−</sup> mice displayed protection from ischemic AKI via improved restoration of post-ischemic blood flow (53).

Adenosine signals through its receptors A1, A2a, A2b, and A3, which are G protein–coupled receptors that use cyclic AMP as their second messenger. Adenosine A2a receptor (ADORA2A) and adenosine A2b receptor (ADORA2B) are regulated by HIF-2 and HIF-1 respectively, adding an additional layer of complexity to the hypoxic control of adenosine signaling (2, 90). While all four adenosine receptors appear to have a role in the pathogenesis of AKI, Grenz and colleagues used global knockout mice to investigate the contribution of each individual receptor to IPC-induced renoprotection. In contrast to *Adora1*<sup>−/−</sup>, *Adora2a*<sup>−/−</sup>, or *Adora3*<sup>−/−</sup> mice, IPC failed to induce protective responses in *Adora2b*<sup>−/−</sup> mice, which displayed increased renal inflammation following IRI (54). The results from these genetic studies are in concordance with the pharmacologic inhibition of ADORA2B with PSB1115, which abrogated IPC-induced renoprotection, while treatment with a selective ADORA2B agonist induced protection.
and phenocopied the renoprotective effects of IPC, which appeared to be specifically mediated by renal endothelial ADORA2B (54). Furthermore, intact adenosine signaling through endothelial ADORA2B was required for the restoration of post-ischemic renal blood flow induced by ENT1 blockade (53). Additional support for a central role of ADORA2B in HIF-mediated cytoprotection comes from studies of myocardial ischemia, where the protective effects of pharmacologic HIF activation were abrogated by genetic Adora2b deficiency (37).

The exact mechanisms that underlie adenosine-induced cytoprotection in the context of IPC are unclear. While mechanistic insights into adenosine’s role in renal IRI are limited, studies in other tissues have suggested that adenosine acts as a critical modulator of post-ischemic inflammation (8). For example, Khoury and colleagues suggested that stimulation of the ADORA2B receptor suppresses NF-κB activity through de-neddylation of Cul-1 (80). Although not studied specifically in the context of IPC, stimulation of ADORA2A also confers beneficial immune-modulatory effects in renal IRI, which include the regulation of natural killer T cell (NKT) activation (96, 158). In addition to the suppression of inflammation, adenosine signaling activates the serine/threonine kinase AKT and reduces glycogen synthase kinase (GSK)-3β activity, which prevents mitochondrial leakage, swelling and cell death (74, 150). The latter is consistent with studies in heart, kidney and other tissues, which demonstrated that inhibition of GSK-3β mimics the cytoprotective effects of IPC (118, 163).

d) NO signaling, ROS and the regulation of oxygen sensing

NO has diverse biological effects, it possesses anti-apoptotic, anti-inflammatory and vasodilative activity, it can both promote and prevent cell injury, and it has been
implicated as a mediator of IPC in multiple organs including the kidney (73, 95, 112, 119). Endogenous NO is synthesized by NO synthase (NOS), an enzyme that converts L-arginine to L-citrulline and NO. Three different NOS isoforms have been identified: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3).

With regard to the HIF pathway, NO has been shown to inhibit the catalytic activity of PHDs and FIH resulting in HIF-α stabilization. NO-mediated HIF activation is therefore likely to contribute to the cytoprotective effects of NO in IPC (110). Furthermore, HIF-1 induces iNOS levels and thus increases NO production (75, 153), creating a positive feedback loop between HIF and NO-dependent signaling that is predicted to enhance cytoprotection. In support of this notion is the finding that the infarct-limiting effect of CoCl$_2$, an inhibitor of HIF-prolyl-4 hydroxylation, was absent in $\text{Nos2}^{-/-}$ mice (171).

Another mechanism by which NO is likely to mediate IPC involves the regulation of mitochondrial respiration and ROS generation, as NO has been shown to inhibit the activity of mitochondrial complex IV (121). A certain sublethal level of ROS, however, seems to be required for IPC to take effect and produce cytoprotection in the setting of cardiac ischemia {for a detailed discussion the reader is referred to (21)}. This concept is supported by findings in the kidney, where pharmacologic ROS scavenging with manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin or N-acetylcysteine abrogated cytoprotection that was induced by a single event of ischemia and reperfusion (81). In particular, mitochondrial ROS, via modulation of PHD catalytic activity, has been shown to be required for the hypoxic stabilization of HIF-α subunits (19, 23, 58, 106).
An important question in the field concerns the role of individual NOS isoforms in IPC-induced cytoprotection. Using 30 minutes of bilateral renal ischemia for preconditioning, Park and colleagues identified iNOS as a key contributor to the protective effects of IPC (up to 12 weeks of protection), whereas the role of eNOS in IPC was negligible (124). Either genetic deletion of iNOS or pharmacologic inhibition of NOS enzymes abolished IPC-induced renoprotection, whereas genetic deletion of eNOS had no effect (124). In contrast to these studies, eNOS-mediated NO production was found to be important in a study by Yamasowa and colleagues, who applied three 2-min cycles of ischemia followed by 5-min of reperfusion prior to 45-min ischemia in uninephrectomized mice. Using this model, Yamasowa and colleagues found that eNOS-deficiency abrogated IPC-mediated cytoprotection (173). The discrepant results reported by these studies could be due to the differences in experimental protocols used. Nevertheless, further investigation is required to reconcile these findings and to better understand the role of NO signaling in IPC-induced cytoprotection.

e) anti-apoptotic pathway regulation

The degree of programmed cell death in the kidney correlates directly with the severity of histologic injury and functional impairment in AKI. While many studies have shown that experimental up-regulation of HIF decreases apoptosis of renal cells in AKI, only few studies have provided evidence for a direct HIF-dependent regulation of apoptotic and/or anti-apoptotic signaling pathways in the IPC setting. Yang and colleagues employed a repetitive hypoxic preconditioning protocol, which up-regulated HIF-1α transcription and protein levels, subsequently evoking resistance against renal IRI-induced apoptosis (174). The authors attributed this anti-apoptotic response to a
HIF-1-dependent induction of BCL-2, which was associated with inhibition of BCL2-associated X protein (BAX) and cytochrome c translocation in the mitochondria. In another study, repetitive hypoxic preconditioning inhibited renal tubular cell apoptosis and autophagy due to activation of HIF-1-dependent HSP70 signaling (175).

It is likely that hypoxic preconditioning regulates anti-apoptotic signaling pathways independently of HIF. A recent example is the anti-apoptotic protein IAP-2, which is induced by hypoxia in a HIF-1-independent manner and confers protection to cells subjected to apoptosis-inducing agents, such as staurosporine (36). In the context of pharmacologic HIF-PHD inhibition, it has been shown that PHDs may also contribute to anti-apoptotic responses independently of HIF. For instance, genetic loss of PHD1 decreases epithelial cell apoptosis in a model of inflammatory bowel disease induced by dextran sulfate sodium (DSS), potentially via derepression of nuclear factor-κB (NF-κB) signaling, as PHD1 been shown to hydroxylate and thus negatively regulate IκB kinase (IKK) (30, 154).

f) erythropoietin in IPC

The kidney responds to changes in tissue oxygenation with increased production of erythropoietin (EPO), a glycoprotein hormone that prevents apoptosis of erythroid progenitor cells and thus stimulates red blood cell production. While studies in Hep3B cells initially identified HIF-1 as the transcription factor that regulates EPO, ours and other laboratories have established that HIF-2 and not HIF-1 mediates the hypoxic induction of renal and hepatic EPO in vivo (57, 78, 132). The EPO receptor (EPOR), which is also hypoxia-inducible, is expressed in multiple non-hematopoietic tissues, including the brain, cardiovascular tissues, liver and kidney (71), and lacks intrinsic
enzymatic function. EPOR homo-dimers undergo conformational changes upon ligand binding (100) and are associated with the tyrosine kinase janus kinase 2 (JAK2), which phosphorylates EPOR at multiple positions, thus providing docking sites for signal-transducing molecules that contain SRC homology 2 domains. EPOR homo-dimers signal through multiple pathways, including the signal transduction and activator of transcription (STAT) 5 pathway, the phosphatidylinositol 3-kinase/protein kinase B (PI-3K/AKT) and the mitogen-activated protein kinase/extracellular signal–regulated kinase (MAPK/ERK) pathways, and protein kinase C (PKC) (71). A low-affinity heterodimeric EPO receptor is also present on the surface of multiple cell types and consists of EPOR and the β-common receptor (βcR), a signal transduction subunit that is shared between the interleukin-3 (IL-3), -5 (IL-5), and granulocyte macrophage colony-stimulating factor receptors (GMCSF) (17).

While EPO is mainly known for its role in hematopoiesis, it is well established that it has multiple non-hematopoietic functions (71). For example, EPO administration reduced ischemic neuronal damage and neurological dysfunction in experimental stroke models, suggesting a role in the regulation of cytoprotective and anti-apoptotic pathways (16, 18, 149). These pathways are likely controlled by EPO signaling through the EPOR/βcR hetero-dimer (17) and involve anti-apoptotic proteins of the BCL-2 family, extracellular signal-regulated kinases and PI3-kinase/AKT, and JAK-2 regulation of NF-κB signaling and proangiogenic signaling (33, 52, 149). Subsequently, several studies have investigated the potential protective effects of EPO in other injury models, including renal IRI and cisplatin-induced nephrotoxicity (10, 24, 45).
In renal injury, EPO-induced cytoprotection was associated with a reduction in apoptosis and inflammation, and an increase in tubular cell proliferation (157). These effects could not be attributed to increased erythropoiesis since the experimental protocols employed did not affect hemoglobin concentrations. While the molecular mechanisms by which EPO exerts cytoprotection in the kidney are ill-defined, they are likely to involve multiple cell types as the EPOR is expressed in endothelial and mesangial cells and in cells of the proximal and distal renal tubule and collecting duct (168). Aside from regulating anti-apoptotic and cytoprotective signaling pathways, EPO has been shown to mobilize endothelial progenitor cells (5) and to modulate inflammation and ischemia-induced neovascularization, indicating a critical role for EPO in the regulation of renal repair (6). However, when administration of EPO alone was compared to preconditional HIF activation with a PHD inhibitor, cytoprotection was reported to be inferior (164), supporting the concept that the concerted activation of HIF-regulated signaling pathways provides more potent and robust renoprotection than the activation of individual HIF target genes or signaling pathways alone.

The potential use of recombinant human EPO (rhEPO) in the prevention of renal IRI in high-risk patients has recently been studied in the critical care setting. In contrast to preclinical studies in animals and clinical pilot trials in humans, the predicted beneficial effects of perioperative administration of rhEPO to patients who underwent cardiac surgery and who were at high risk for the development of AKI could not be demonstrated (82). This was also the case in patients undergoing kidney transplantation, who were studied in a prospective, randomized, double-blind, placebo-controlled trial to assess the effects of high-dose rhEPO treatment on short- and long-term graft function (60). In
patients with myocardial infarction single intravenous bolus injection of epoetin alfa did not reduce infarct size within 4 hours of percutaneous coronary intervention (REVEAL trial), whereas subgroup analyses showed an increase in infarct size among older patients (116). These concerns for adverse cardiovascular events in the setting of rhEPO administration are reinforced by the TREAT trial, a randomized, double-blind, placebo-controlled trial of darbepoetin alfa in diabetic patients with renal anemia, which reported an increase in stroke rate (127). Interestingly, in contrast to the beneficial effects of rhEPO in patients with acute stroke initially reported in a proof-of-concept, pre-thrombolysis era clinical trial (38), the German Multicenter EPO Stroke Trial was a negative intent-to-treat study and reported increased death rates particularly in patients receiving systemic thrombolysis (39).

**Differential activation of stress related kinases**

Park and colleagues have evaluated the effect of IPC induced by 30 minutes of bilateral renal ischemia on the activation pattern of stress related kinases (125). They found that the ischemia-related activation of jun N-terminal kinase (JNK) and p38 were markedly diminished by IPC, whereas an effect on post-ischemic activation of extracellular-signal-regulated kinases 1/2 (ERK1/2) was not observed. Similarly, the phosphorylation of MKK7, MKK4 and MKK3/6, upstream activators of JNK and p38, was markedly reduced. Given the involvement of JNK and p38 in the expression of adhesion molecules and cytokine production, the authors speculated that these alterations may lessen leucocyte-endothelial interactions and therefore explain the remarkable IPC-induced reduction in outer medullary cellular congestion. A link between enhanced cell
survival, decreased JNK and increased ERK1/2 activation was shown in an in vitro model of endoplasmic reticulum stress preconditioning (67). Joo and colleagues, using a different IPC protocol, found rapid phosphorylation of both ERK and AKT, but only the inhibition of PI3K-AKT pathway was able to blunt renoprotection afforded by IPC (73). Since AKT regulates several cellular survival pathways, it is likely that AKT acts as an important mediator of IPC-induced cytoprotection. This concept is supported by recent pharmacologic studies, in which inhibition of AKT signaling with PI3K inhibitor wortmannin abrogated cytoprotection induced by single ischemia reperfusion preconditioning (69).

microRNAs in IPC

microRNAs (miRs) are small non-coding RNA molecules that in their mature form regulate gene expression, primarily through binding to the 3′-untranslated region (UTR) of target mRNAs leading either to their degradation or to translational inhibition. Maturation and binding to 3′-UTRs involves several enzyme complexes. Dicer cleaves miR precursors and generates a double-stranded RNA product, which then in association with argonaute endonuclease proteins becomes part of the RNA-induced silencing complex (RISC) that directly binds to target RNAs (13). A subset of miRs is hypoxia-regulated and is differentially expressed in ischemic tissues, where they modulate cell survival and tissue repair, and either positively or negatively impact the clinical outcome of IRI. In the heart for example, miR-499 inhibited apoptosis and improved injury in animal models of myocardial infarction (162), while inhibition of miR-92a promoted angiogenesis and functional recovery from limb and myocardial IRI (15). Interestingly,
some micro-RNAs seem to play a dual role in the recovery from IRI, which appears to be cell type-dependent. miR-24 for example inhibits cardiomyocyte apoptosis through the repression of pro-apoptotic molecule Bcl-2 interacting mediator of cell death (BIM) (130), while it also promotes apoptosis of cardiac endothelial cells (44).

The relevance of miRs to the pathophysiology of renal IRI is underscored by the proximal renal tubule-specific deletion of dicer, which suppressed miR maturation and made kidneys resistant to IRI (165). Several miRs are differentially expressed following warm IRI (50). These include miR20a, miR-21, miR-192, miR-194, miR-214 and others. Of those miR21 is well characterized and has been shown to be required for the late phase of IPC in the kidney (172) and IPC in the heart (26). miR-21 is expressed in multiple cell types and is hypoxia-inducible in a HIF-1-dependent manner (99); its expression increases after IRI (35, 50, 99). miR-21 reduces the expression of programmed cell death 4 (PDCD4), phosphatase and tensin homolog (PTEN) and other proteins, and has been shown to have anti-apoptotic properties (35, 50, 98, 99).

However, whether miR21 represents a suitable therapeutic target for the prevention of ischemic injuries is unclear, as it regulates other signaling pathways that may promote inflammation and fibrosis (179). Several reports indicate the involvement of multiple miRs in IPC of other organs (94, 138); to what degree these miRs contribute to renal IPC is currently under investigation.

While their role as single therapeutic agents for the prevention or treatment of IRI is uncertain, it is clear that miRs regulate and fine-tune cellular hypoxia responses. There are numerous examples of miRs that are hypoxia-regulated, either positively or negatively. A prototypical example is miR-210, which is HIF-inducible and was initially
identified in cancer cells (92). More recently, miR 210 was shown to function as a negative regulator of HIF-1α in T-cells (161), illustrating that hypoxia- and also non-hypoxia-regulated miRs operate in complex feedback loops that can amplify or suppress HIF-controlled pathways (31), and thus have the potential to modulate IPC. For example, miR-199a is suppressed under hypoxic conditions and modulates HIF-1-induced preconditioning in cardiomyocytes by directly targeting HIF-1α and SIRT1-induced suppression of PHD2 (131).

The role of immune responses in IPC

Immune cells are key players in tissue injury and repair, and recent studies have established their crucial role in renal IPC. Burne-Taney and colleagues transferred immune cells from ischemic and sham-operated mice into T cell-deficient mice, which then underwent IRI (20). The investigators found that T cell-deficient mice, which received leukocytes from mice with prior exposure to ischemia, had reduced injury compared to mice, which received leukocytes from sham-operated animals. Furthermore, experiments using iNOS-deficient leukocytes demonstrated that immune cell-mediated IPC was not dependent on iNOS. Among lymphocytes, regulatory T cells (Tregs) have been identified as mediators of IPC. Tregs are CD4⁺CD25<sup>high</sup>Foxp3<sup>+</sup> lymphocytes that are recruited into areas of inflammation and have immune-suppressive functions (111). Kinsey and colleagues found that an ischemic insult caused a significant increase in CD4⁺CD25<sup>high</sup>FoxP3<sup>+</sup> Tregs in the kidney at day 7 post IRI, which was furthermore associated with an absolute increase in the number of IL-10-expressing Tregs (86). Treatment of preconditioned mice with a Treg-depleting anti-CD25 antibody blunted the
The protective effects of IPC with regard to preserving renal function and morphology and reducing neutrophil infiltration. Conversely, adoptive transfer of Tregs to naive mice prior to IRI produced cytoprotection and mimicked the protective and anti-inflammatory effects of IPC in the kidney (86). Remarkably, in a follow-up study the same investigators showed that adenosine generation by CD73 and adenosine signaling through ADORA2A were essential in Treg-mediated renoprotection (85). Because CD73 and ADORA2A are both hypoxia-regulated (Figure 1), enhanced adenosine signaling may be central to Treg-mediated IPC. Additional support for a crucial role of immune responses in IPC comes from a study, where systemic CD11c⁺ macrophage/dendritic cell depletion with clodronate diminished renoprotection associated with IPC (27). However, in a separate study a role for infiltrating macrophages could not be established (70), suggesting that the role of macrophages in IPC may depend on the conditions of the experimental strategies used.

While the molecular mechanisms regulating immune cell function in the context of IPC are not well studied, it is likely that the PHD/HIF axis is critically involved, as it controls metabolic and functional adaptation of immune cells to hypoxic microenvironments (122). For example, inflammatory functions and lifespan of neutrophils under hypoxic conditions are promoted by HIF-1-dependent NF-κB activity, and also require PHD3 expression (159, 160), while HIF-2 regulates neutrophilic apoptosis impacting on the resolution of inflammation (155). The impact of HIF-1-dependent metabolic reprogramming on innate immunity (shift towards anaerobic glycolysis) is exemplified by a recent elegant study showing that myeloid HIF-1 is critical in β-glycan-derived trained immunity responses against bacterial sepsis (25).
However, whether IPC has the potential to trigger trained immunity via HIF, and to what degree this impacts renoprotection is not known. It is also unclear whether IPC in the kidney signals to resident immune cells only or whether renal IPC can also signal and condition immune cells in remote locations such as bone marrow or spleen. Nevertheless, genetic deletion of Hif1a in myeloid cells prevented functional recovery after renal IRI injury (141), a finding, which supports the notion that myeloid cell-derived hypoxia responses may have significant positive impact on injury outcomes in the setting of IPC. Accordingly, a protective role for myeloid HIF-1 was shown in a model of renal fibrosis (87). The differential effects of HIF-homologs on immune cell function add additional complexity to understanding IPC-induced renoprotection. For instance, in response to different cytokines, HIF-1 and HIF-2 act antagonistically in the polarization of macrophages via the induction of either iNOS or arginase 1 expression respectively, regulating NO levels differentially (153). With regard to adaptive immune responses, HIF controls T cell activation, the function of Tregs, the balance between Tregs and T helper 17 cells, and enhances the effector responses of CD8+ T cells via modulating the expression of transcription factors, effector molecules and co-stimulatory receptors (34, 122). Furthermore, both B cell development and hypoxia-induced cell cycle arrest require intact HIF-1 signaling (49, 88). Given the increasing evidence that hypoxic signaling plays a pivotal role in the regulation of immune cell function (122), additional studies are needed that investigate the contribution of innate and adaptive immune responses to IPC.
Conclusions

Recent advances in understanding the molecular and cellular basis of renal hypoxia responses have identified the PHD/HIF axis as a pivotal oxygen-sensing mechanism that mediates IPC through the coordinated activation of renoprotective signaling pathways. Pre-ischemic activation of these pathways through pharmacologic HIF stabilization mimics normal hypoxia responses and would represent a comprehensive physiologic approach to maintaining renal tissue homeostasis, protecting from ischemia and promoting renal tissue repair. Effective HIF-activating compounds are currently being investigated in clinical trials for renal anemia and would be readily available for pilot studies in patients at high risk for development of AKI. However, the physiologic consequences of systemic HIF activation in humans are only incompletely understood, and more investigations are needed to establish treatment protocols that permit safe and effective targeting of the HIF oxygen-sensing pathway for therapy of ischemic kidney diseases.
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DISCLOSURES

VHH has received honoraria from AstraZeneca and Daiichi Sankyo, and serves on the Scientific Advisory Board of Akebia Therapeutics, a company that develops prolyl-4-hydroxylase inhibitors for the treatment of renal anemia.
REFERENCES


inducible factor 2α regulates key neutrophil functions in humans, mice, and zebrafish.


FIGURE LEGENDS

Figure 1. Overview of canonical hypoxic signaling via the PHD/HIF axis. Under normoxic conditions, PHD enzymes hydroxylate specific proline residues located within the oxygen-dependent degradation domain of HIF-α subunits. Prolyl-hydroxylation is required for binding to the VHL-E3 ubiquitin ligase complex, which ubiquitylates HIF-α, triggering its proteasomal degradation. When prolyl-4-hydroxylation is inhibited, e.g. in the absence of molecular oxygen or ferrous iron, HIF-α escapes degradation and translocates to the nucleus, where it dimerizes with ARNT, the constitutively expressed HIF-β subunit. HIF-α/ARNT hetero-dimers bind to hypoxia response element (HRE)-containing regulatory DNA sequences and increase the transcription of oxygen-regulated genes (e.g. EPO, PDK1, LDHα) through recruitment of transcriptional coactivators such as p300/CBP. Factor-inhibiting HIF (FIH), another oxygen- and iron-dependent dioxygenase, hydroxylates a specific asparagine residue located within the HIF-α COOH-terminal transactivation domain thereby inhibiting transcriptional cofactor recruitment. HIF-α stabilization results in the activation of multiple transcriptional programs, including erythropoiesis, iron metabolism, vascular remodeling, cellular metabolism, and others.

Figure 2. Schematic depicting the role of the endothelial PHD/HIF-2 axis in renoprotection. Pharmacologic HIF activation through HIF prolyl-4-hydroxylase inhibition (PHI) promotes recovery from renal ischemic injury via activation of endothelial HIF-2-dependent signaling and suppression of VCAM1.
Figure 3. Molecular mechanisms implicated in PHD/HIF-mediated renoprotection induced by ischemic/hypoxic preconditioning. HIF attenuates IRI through coordinated activation of cytoprotective signaling pathways. Shown are oxygen-regulated biological processes and signaling pathways with key roles in PHD/HIF-mediated renoprotection.
Figure 1

HIF-α

Normoxia

HIF-α

Hypoxia

Target Genes

Adaptation to Hypoxia

EPO, Ceruloplasmin, Transferrin, Transferrin R, VEGF, VEGF-R, iNOS, GLUT1 and -3, PGK1, LDHα, CA9, PDK1, HMOX1, Cyclin G2, IG2, ITF, MDR1, CD73, ADORA2A, ADORA2B, CTGF, PAI1.

Transcription factors:

ETS1, DEC1/STRA13

Erythropoiesis / Iron metabolism

Angiogenesis / Vasculogenesis

Metabolic adaptation / Glycolysis

Mitochondrial function

Proliferation / Cell survival

Cellular barrier function

Extracellular matrix production

Cell migration
Figure 2

PHI ➔ PHD ➔ Endothelial Cell ➔ HIF-2 ➔ VCAM1 ➔ Inflammation, Tissue Damage
Figure 3

- Ischemic/Hypoxic Preconditioning
- PHD/HIF axis

- Metabolic Reprogramming
- Inflammatory Responses
- Innate/Adaptive Immunity
- Oxidative Stress Responses
- Anti-apoptotic Effects
- Cytoprotective Factors: EPO, Adenosine, NO

- Cell Survival
- Repair
- Apoptosis
- Inflammation

Ischemic Injury