Central to cellular responses to hypoxic environment is the hypoxia-inducible factor (HIF) transcriptional control system. A role for HIF-2α was investigated in a model of renal ischemia-reperfusion injury (IRI) associated with oxidative stress using HIF-2α knockdown mice. In these mice, HIF-2α expression was approximately one half that of wild-type mice, whereas HIF-1α expression was equivalent. HIF-2α knockdown mice were more susceptible to renal IRI, as indicated by elevated blood urea nitrogen levels and semiquantitative histologic analysis. Immunostaining with markers of oxidative stress showed enhanced oxidative stress in the kidney of HIF-2α knockdown mice, which was associated with peritubular capillary loss. Real-time quantitative PCR analysis showed decreased expression of antioxidative stress genes in the HIF-2α knockdown kidneys. Studies that used small interference RNA confirmed regulation of the antioxidative stress genes in cultured endothelial cells. Although HIF-2α knockdown mice were anemic, serum erythropoietin levels were not significantly increased, reflecting an inappropriate response to anemia as a result of HIF-2α knockdown. Experiments that used hemodiluted mice with renal ischemia demonstrated that anemia of this degree did not affect susceptibility to ischemia. Knockdown of HIF-2α in inflammatory cells by bone marrow transplantation experiments demonstrated that HIF-2α in inflammatory cells did not contribute to susceptibility to renal IRI. Restoration of HIF-2α in endothelium by intercrossing with Tie1-Cre mice ameliorated renal injury by IRI, demonstrating a specific role of endothelial HIF-2α. These results suggest that HIF-2α in the endothelium has a protective role against ischemia of the kidney via amelioration of oxidative stress.

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Received June 20, 2006. Accepted January 26, 2007.

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Published online ahead of print. Publication date available at www.jasn.org.

Materials and Methods

Animals

HIF-2α kd mice were generated as described previously. This mice were viable and fertile, and also our screening did not reveal any pathologic abnormalities as we previously described (14). These mice were back-crossed with C57BL/6j mice for nine generations. Male C57BL/6j wild-type (WT) and homozygous HIF-2α kd mice at the age of 6 to 8 wk were used. To rescue HIF-2α specifically in the endothelium, we used for intercrossing with Tie1-Cre transgenic (Tie1-Cre) mice (15) whose genetic background was C57BL/6j. Mice were housed in a specific pathogen-free facility and were confirmed to be negative for common murine viral pathogens by routine sera analyses. All experiments were conducted in accordance with the Guide for Animal Experimentation at the University of Tokyo.
Induction of Renal IRI

WT and HIF-2α kd mice were anesthetized by intraperitoneal administration of a combination of ketamine and xylazine. Renal ischemia (n = 7 each) was induced by clamping of the bilateral renal pedicles. During the ischemic period, body temperature was maintained by placing the mice on a 37°C heating table. After 20 min, both clamps were released. Sham-treated mice (n = 4 each) had identical surgical procedures without application of clamps. At 24 h after reperfusion, mice were killed, blood was drawn by cardiac puncture, and kidneys were harvested for histologic analysis. In some experiments as described next, kidneys were harvested after 20 min of clamping. Blood urea nitrogen (BUN) levels were measured using a commercial kit (Wako, Osaka, Japan).

Renal Morphology

Methyl Carnoy-fixed, paraffin-embedded sections of 3 μm thickness were stained with the primary antibody. Tubular injury was scored in a blinded manner by estimation of the percentage of tubules in the outer medulla and corticomedullary junction that showed epithelial necrosis or had necrotic debris or cast as follows: Grade 0, no morphologic deformities; grade 1, <25%; grade 2, <50%; grade 3, <75%; grade 4, ≥75% involved. More than 20 consecutive fields were examined at ×400 magnification and averaged per slide.

Immunohistochemistry

Using zinc-fixed, paraffin-embedded sections (3 μm), peritubular capillaries were identified with an anti-CD31 antibody (1:200; BD Pharmingen, San Jose, CA). Subsequently, the slides were treated with a biotinylated anti-rabbit IgG (1:400; Vector), and horseradish peroxidase (HRP)-avidin (1:1000; Vector). For nitrotyrosine staining, methyl Carnoy-fixed, paraffin-embedded sections (3 μm) were probed with anti-nitrotyrosine (1:1000; Sigma-Aldrich, St. Louis, MO), biotinylated anti-rat IgG (1:400; Vector), and HRP-avidin. The slides were then developed with diaminobenzidine (Wako), followed by counterstaining with methyl green. 4-Hydroxynonenal (4-HNE) protein adduct was detected in frozen kidney sections (4 μm) that were fixed with ethanol:acetone (1:1). The sections were stained with monoclonal anti–4-HNE antibody, NA59 (16) (1:100; provided from Dr. J.L. Witztum, University of California San Diego, San Diego, CA) using Histofine mouse stain kit (Nichirei Bioscience, Tokyo, Japan). Color development was made by dianaminobenzidine detection.

Evaluation of Capillaries and Oxidative Injury

Density of peritubular capillaries was quantified using a rarefaction index, as described previously (17). CD31-immunostained sections were examined through 10 × 10 grid under ×400 magnification. Each square within a grid that did not contain CD31-positive cells was scored. At least 10 fields in the cortex and outer medulla were examined and calculated per slide. Oxidative injury was evaluated by counting the number of tubules that were positive for nitrotyrosine and 4-HNE out of more than 15 randomly selected fields for each mouse under ×400 magnification.

Western Blotting Analyses

Nuclear extracts were prepared from harvested whole kidneys using a nuclear extraction kit (Pierce, Rockford, IL), and protein concentrations were measured using a Bio-Rad assay reagent (Bio-Rad, Hercules, CA). Western blot analysis of HIF-1α and HIF-2α was performed as described previously (18). The membranes were incubated with anti–HIF-1α antibody (1:500; Novus Biologic, Littleton, CO) or anti–HIF-2α antibody (1:1000, Novus Biologic), followed by incubation with HRP-conjugated goat anti-mouse Ig (1:10,000; Bio-Rad). The ECL plus kit was used for detection (Amersham Biosciences, Buckinghamshire, UK). Density of bands was analyzed using Image J version 1.34s software (National Institutes of Health, Bethesda, MD).

Total RNA Isolation and Real-Time Quantitative PCR

Total RNA of whole kidneys from each mouse or cultured endothelial cells was isolated with ISOGEN (Nippon Gene, Tokyo, Japan) and reverse-transcribed with Im-PromII reverse transcription kit (Promega, Madison, WI). cDNA was subjected to real-time quantitative PCR using IQ SYBR Green PCR supermix (Bio-Rad) and iCycler PCR system (Bio-Rad). The relative amounts of HIF-1α, HIF-2α, copper/zinc superoxide dismutase (SOD1), manganese superoxide dismutase (SOD2), glutathione peroxidase type 1 (GPX1), catalase, heme-oxgenase-1 (HO-1), and erythropoietin (EPO) were calculated with calibration by β-actin. Corresponding primers have been described in the literature (19,20) except for mouse HO-1 (5′-GCC ACC AAG GAG GTA CAC AT, 5′-GCT TGT TGC GCT CTA CCT CC-3′), human HIF-1α (5′-CCA TTA GAA AGC AGT GCC-3′), 5′-GGG GGT GGA GAT CAT GAT GC-3′, and human HIF-2α (5′-GGG GAT GAC TCC TGG ACC AAC AT-3′, 5′-TGGCCA CTT ACT ACC TGA CCC TT-3′).

Measurement of Hematocrit, EPO, and Hemodilution Procedure

Hematocrit was determined using automatic counter (ERMA, Tokyo, Japan), and serum EPO was measured using EPO ELISA kit (R&D Systems, Minneapolis, MN). Hemodilution was performed by withdrawal of 0.4 ml of whole blood followed by replacement with the equal volume of saline via tail vein. At 1 h after hemodilution, IRI was induced as described above. At 24 h after reperfusion, mice were killed.

Cell Culture and siRNA Transfection Procedures

Human umbilical vein endothelial cells were purchased from Cambrex (Walkersville, MD) and were maintained in endothelial growth medium 2 (Cambrex). The siRNA oligonucleotides were synthesized by TaKaRa Bio (Shiga, Japan). Human umbilical vein endothelial cells were transfected with siRNA at 70% confluence by the use of Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were incubated for an additional 24 h before harvest. The sequence of the HIF-2α siRNA duplex and the scramble HIF-2α control duplex and details of siRNA transfection procedures were described previously (21,22).

Bone Marrow Transplantation

Bone marrow transplantation (BMT) was performed as described previously (23). Briefly, male recipient mice aged 7 wk were lethally irradiated with a total dose of 940 rad (MBR-1520RB; Hitachi, Tokyo, Japan). Bone marrow cells were harvested from male donor aged 7 to 9 wk. Six hours after irradiation, the recipient mice received bone marrow cells (1 × 10^7) by tail-vein injection. Replacement of the peripheral blood was confirmed as described previously (23).

Statistical Analyses

Values were presented as means ± SD. Statistical comparisons were analyzed with the t test. Nonparametric data were analyzed with the Mann-Whitney test. P < 0.05 was considered statistically significant.

Results

Ischemia Activated the HIF Pathway in Clamped Kidneys

To investigate a pathophysiologic role of HIF-2α, we used the well-established IRI model of the kidney. Twenty minutes of
ischemia induced activation of the HIF pathway in both WT and HIF-2α kd mice (Figure 1A). In HIF-2α kd mice, HIF-2α mRNA expression was approximately 50% compared with WT mice as described previously (14). HIF-2α kd ischemic kidney had reduced HIF-2α protein levels (65%) relative to WT ischemic kidney. However, we found no significant difference of HIF-1α expression levels between WT and HIF-2α kd.

**HIF-2α Kd Mice Were More Susceptible to Renal Ischemia**

To investigate whether partial deficiency of HIF-2α could render the kidney more susceptible to ischemia, we examined renal function as assessed by BUN levels. Whereas 20 min of ischemia impaired renal function in both WT and HIF-2α kd mice, HIF-2α kd mice had a significantly higher BUN compared with WT mice (Figure 1B). Impairment of renal function was corroborated by histologic evidence. WT mice that were subjected to IRI had relatively mild tubular injury (Figure 2A). In contrast, we observed severe tubular damage in terms of tubular dilation, tubular epithelial injury, debris accumulation, and cast formation in the HIF-2α kd kidney sections (Figure 2A). This was confirmed by semiquantitative analysis (tubular injury score: WT 0.72 ± 0.21; kd 1.15 ± 0.31; n = 7 each; P < 0.01).

Sham-operated mice had no tubular damage (Figure 2B). Tubular damage as estimated by periodic acid-Schiff staining for individual mice showed a good correlation with BUN levels (Figure 2C).

**Peritubular Capillary Loss Was Aggravated in HIF-2α Kd Mice**

Because HIF-2α in the kidney is localized in endothelial cells or peritubular fibroblasts (9), we evaluated structural changes of the microvasculature (Figure 3A). Capillary endothelial cells were well maintained in both sham-operated groups (rarefaction index score: WT 0.63 ± 0.21%; kd 0.83 ± 0.19%; n = 4 each). Kidneys of HIF-2α kd mice showed aggravation of peritubular capillary loss after ischemia compared with WT kidneys (rarefaction index score: WT 5.00 ± 2.93%; kd 8.20 ± 2.36%; n = 7 each; P < 0.05).
HIF-2α Kd Mice Had More Oxidative Stress by IRI

To evaluate oxidative stress in the kidney by ischemia-reperfusion, we performed immunohistochemical staining of nitrotyrosine, which predominantly localized on the surface of exfoliated epithelial cells inside the tubular lumen (Figure 3B). The number of nitrotyrosine-positive tubules significantly increased in HIF-2α kd mice 24 h after reperfusion (positive tubules/field: WT 3.50 ± 2.04; kd 11.36 ± 2.90; n = 7 each; P < 0.001). Accumulation of another oxidative protein product, 4-HNE, was also observed in the desquamated tubular epithelial cells in ischemic kidneys (Figure 3C). More severe accumulation of 4-HNE in HIF-2α kd mice was confirmed by the quantitative analysis (positive tubules/field: WT 6.94 ± 4.78; kd 13.67 ± 2.26; n = 7 each; P < 0.05). Staining of nitrotyrosine and 4-HNE was negative in WT and HIF-2α kd mice without IRI (data not shown).

HIF-2α Kd Mice Had Lower Baseline Expression Levels of Antioxidant Enzyme Genes

Because histologic studies showed greater oxidative stress by IRI in HIF-2α kd mice, we examined baseline expression of primary antioxidant enzyme genes in the kidney. Expression of SOD1, SOD2, and GPX1 genes was significantly lower in HIF-2α kd kidney (Figure 4). Conversely, expression of HO-1, a target gene of HIF-1α, was not different between WT and HIF-2α kd kidneys.

Anemia in HIF-2α Kd Mice Did Not Contribute to Susceptibility to Renal IRI

HIF-2α kd mice showed persistent anemia with an approximately 20% decrease in hematocrit levels compared with WT mice (WT 45.9 ± 1.9%; kd 37.1 ± 1.6%; n = 4 each; P < 0.001). However, we did not observe a significant difference in serum EPO levels (WT 149.2 ± 37.1 pg/ml; kd 124.4 ± 40.7 pg/ml; n = 8 each; P = 0.24) and the baseline expression of EPO mRNA in the kidney (WT 1.00 ± 0.38; kd 0.74 ± 0.16; n = 4 each; P = 0.17) between the two groups. Next, to determine whether this decreased hematocrit contributed to susceptibility to renal ischemia, we performed hemodilution of WT mice, followed by IRI. After the hemodilution, hematocrit levels of WT mice remained equivalent to those of HIF-2α kd mice up to 24 h, whereas hemodilution itself did not change BUN levels in WT mice (Table 1). Furthermore, when IRI was induced in control WT and hemodiluted WT mice, we did not observe a significant difference in BUN levels between the two groups (Table 2).
Table 1. Hematocrit and BUN levels in the hemodilution procedure

<table>
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<tr>
<th>Hemodilution</th>
<th>Hematocrit (%)</th>
<th>BUN (mg/dl)</th>
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<tr>
<td>Before</td>
<td>45.9 ± 1.9</td>
<td>27.1 ± 3.1</td>
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<tr>
<td>After 1 h</td>
<td>35.7 ± 1.7b</td>
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<tr>
<td>After 24 h</td>
<td>36.6 ± 2.4b</td>
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Table 2. Hematocrit levels of the hemodilution group were significantly lower than those in the control group. IShamia-reperfusion injury (IRI) impaired renal function in both control wild-type (WT) and hemodiluted WT mice at 24 h after reperfusion, but there was no significant difference in BUN levels between the two groups. Data are means ± SD. *p < 0.001 versus before hemodilution.

Knockdown of HIF-2α Suppressed Antioxidant Enzyme Gene Expression in Cultured Endothelial Cells

To elucidate whether HIF-2α is directly involved in the expression of antioxidative genes, we used the siRNA strategy to inhibit HIF-2α expression. We treated human cultured endothelial cells with two independent siRNA, because HIF-2α is expressed mainly in endothelial cells in vivo. HIF-2α mRNA expression was suppressed to approximately 40 and 20% by the siRNA compared with scramble control of HIF-2α, whereas knockdown of HIF-2α did not affect HIF-1α mRNA levels (Figure 5A). Knockdown of HIF-2α attenuated the expression of SOD1, SOD2, and catalase mRNA significantly (Figure 5B). Scramble control siRNA had no effect on the expression of these genes (data not shown).

Knockdown of HIF-2α in Inflammatory Cells Did Not Contribute to Susceptibility to Renal IRI

HIF-2α can be found not only in endothelial cells but also in renal interstitial cells, including inflammatory cells. To investigate whether the expression of HIF-2α on inflammatory cells is involved in renal IRI, we performed BMT followed by renal IRI at 4 wk after BMT. BUN levels 24 h after IRI are shown in Figure 6A. Importantly, no significant difference was observed between the two groups, BMTHIF-2αkd::Tie1-Cre mice and control Tie1-Cre mice (n = 4 each). This result showed that the knockdown of HIF-2α in inflammatory cells was not involved in susceptibility to renal IRI. Tubular injury score was also equivalent between the two groups (BMTWT→WT, 0.84 ± 0.20; BMTHIF-2αkd−→WT, 0.88 ± 0.25; P = 0.86).

Restoration of HIF-2α in Endothelium Ameliorated Renal Injury by IRI

Because knockdown of HIF-2α gene was achieved by inserted neomycin gene sandwiched between two loxP sequences, HIF-2α can be restored to WT function upon Cre-mediated recombination (14). To investigate whether the susceptibility to renal ischemia is due to the reduced expression of HIF-2α in endothelium, we restored the expression of HIF-2α in endothelium by intercrossing HIF-2α kd mice with Tie1-Cre mice that express the Cre protein specifically in endothelium. Then we subjected HIF-2αkd::Tie1-Cre mice and control Tie1-Cre mice (n = 4 each), both of which were heterozygous for Tie1-Cre transgene and from the same litter, to renal IRI. Renal IRI impaired renal function in both Tie1-Cre and HIF-2α kd::Tie1-Cre mice, but susceptibility to renal ischemia that HIF-2α kd mice had was reduced in HIF-2α kd::Tie1-Cre mice (Figure 6B). This was supported by histologic evidence (tubular injury score: Tie1-Cre 0.75 ± 0.21; HIF-2α kd::Tie1-Cre 0.94 ± 0.26; P = 0.30).
Discussion

Many studies including ours previously demonstrated that activation of HIF by treatment with cobalt ameliorated IRI (24–27). Furthermore, transgenic mice that expressed dominant negative HIF with the endothelium-specific promoter showed multiple cardiovascular defects and died at approximately embryonic day 11.5 (28). Our previous studies using in vivo gene transfer of dominant negative HIF and constitutively active HIF also demonstrated involvement of HIF in IRI of the kidney (29). However, it remained to be determined whether activation of HIF-1α or HIF-2α was responsible in these studies. Recent studies that used a lentiviral vector system to express HIF-1α and HIF-2α identified novel genes that were activated by a combination of HIF-2α overexpression and hypoxic insult, suggesting a critical role for HIF-2α in mediating protective responses against ischemic injury (30). To our knowledge, this is the first report to clarify direct evidence of a protective role of HIF-2α against an exogenous insult in vivo. Our results showed that reduction in HIF-2α makes the kidney more susceptible to IRI. Elucidation of a protective mechanism against IRI is important because IRI plays a critical role in a wide range of disorders, including renal failure, organ transplantation failure, myocardial infarction, and cerebrovascular diseases.

Predominant localization of HIF-2α in the endothelium suggests that one possible benefit by HIF-2α against IRI is mediated via protection of the endothelium. We showed that peritubular capillaries were preserved by sufficient HIF-2α. Furthermore, we demonstrated that restoration of HIF-2α to wild levels specifically in endothelial cells ameliorated renal injury that was seen in HIF-2α kd mice. Inflammatory cells can infiltrate into the kidney and participate in renal IRI (31), but we excluded the contribution of HIF-2α in circulating inflammatory cells by BMT experiments. The endothelium is not an inert, single-cell lining that covers the internal surface of blood vessels but in fact plays a crucial role in regulating vascular permeability, inflammation, and hemodynamics (32–34). HIF-2α upregulates expression of various genes that are involved in erythropoiesis, glycolysis, and angiogenesis by binding to the hypoxia responsive element (HRE) in the promoter of these genes. Among these are EPO and vascular endothelial growth factor, which are known to serve as endothelial survival factors. Some glycolytic enzymes, such as Glut-1 and aldolase A, are also regulated by the HIF-HRE system and promote survival of cells under hypoxic conditions. HIF-2α specifically regulates gene expression of endothelial receptors for angiogenic factors such as Tie2 (35) and vascular endothelial growth factor receptor-2 (Flk-1) (36). Furthermore, HIF-2α may be responsible for expression of hypoxia-induced mitogenic factor, a newly found cytokine with angiogenic properties (37). Although our immunohistochemical analysis of oxidative stress markers accentuated accumulation of nitrotyrosine and 4-HNE in damaged tubules, this may be explained by a larger volume occupied by tubules than endothelial cells, and we speculate that aggravation of tubular injury may be secondary to insufficient antioxidative defense in the endothelium.

Previous studies that emphasized an important role of HIF-2α in vascular development support a crucial role of HIF-2α in the vasculature. Peng et al. (11) reported vascular defects in HIF-2α knockout mice, and this defect was rescued by endothelium-specific expression of HIF-2α (38). In contrast, the endothelial-specific ablation of HIF-1α did not interfere with normal vascular development (39). Our previous studies that used cultured endothelial cells demonstrated that HIF-2α mediates migration and network formation of endothelial cells (26). All of these findings support our current finding that HIF-2α serves as a defensive mechanism against IRI via protection of endothelium.

Oxidative stress alters many functions of the endothelium (40,41) and likely mediates many of the deleterious effects of IRI (42,43). Endothelial exposure to oxidative stress induces cell dysfunction and apoptosis (44,45). Oxidative stress also accelerates the onset of senescence endothelial cells and impairs angiogenesis (46). All of these are likely to contribute peritubular capillary loss. Our studies showed aggravation of oxidative markers such as nitrotyrosine and 4-HNE in ischemic kidneys of HIF-2α kd mice. Scortegagna et al. (19) suggested that multiorgan damage that was observed in their HIF-2α

Figure 6. (A) Contribution of HIF-α in inflammatory cells against renal IRI. Bone marrow transplantation was performed followed by renal IRI at 4 wk after the procedure. No significant difference of BUN levels after renal IRI was observed between the two groups (BMTWT→WT 57.4 ± 24.2 mg/dl; BMT HIF-2α kd→WT 62.4 ± 25.9 mg/dl; n = 7 each; P = 0.72). BMT HIF-2α kd→WT, for example, represents WT transplanted with bone marrow of HIF-2α kd mice. (B) Effect of restoration of HIF-2α in endothelium against renal IRI. HIF-2α kd; Tie1-Cre mice, whose expression of HIF-2α was restored in endothelial cells, were not more susceptible to renal ischemia, as indicated by elevated BUN levels 24 h after IRI than control Tie1-Cre mice (Tie1-Cre 60.7 ± 23.6 mg/dl; HIF-2α kd; Tie1-Cre 72.0 ± 23.7 mg/dl; n = 4 each; P = 0.53).
knockout mice could be ascribed to enhanced generation of reactive oxygen species. It should be noted that their results may not be appropriate to be generalized because they analyzed precious few surviving knockout F1 mice that were generated by hybrid (129S6/SvEvTac-HIF-2α+/− × C57BL/6j-HIF-2α+/−) matings. However, their observations are consistent with our data. Higher oxidative stress in HIF-2α−/− mice can be ascribed to lower expression of some antioxidative enzyme genes that are responsible for reactive oxygen species elimination. For supporting this notion, in vitro studies using siRNA specific for HIF-2α also showed suppression of these antioxidative genes in association with knockdown of HIF-2α in cultured endothelial cells.

HIF-2α participates in regulation of EPO expression (14,47), although HIF-1α was originally identified as a factor to bind to an enhancer element located 3′ to the human EPO gene (48). Our HIF-2α−/− mice were slightly anemic, suggesting a physiologic role of HIF-2α in EPO. Recent studies emphasized a cytoprotective effect of EPO, and administration of EPO attenuated ischemic renal injury directly (49,50). Our measurement of serum EPO levels demonstrated no difference between WT and HIF-2α−/− mice, suggesting that changes in serum EPO did not affect different susceptibility to renal ischemia in our study. Although red blood cells contain high levels of antioxidants, our “hemodilution” experiments also ruled out a possibility that anemia itself was responsible for enhanced susceptibility to renal ischemia in HIF-2α−/− mice.

In addition to clarifying a pathophysiologic role of HIF-2α in protection of endothelium against ischemia and oxidative stress, our studies implicated an insight of future therapeutic approaches in a wide range of diseases. Stimulation of the HIF-HRE system is a promising approach against various ischemic diseases because it induces coordinated responses against hypoxia. However, activation of this system is a two-edged sword, because the HIF-HRE system, especially HIF-1α, may also be associated with tumorigenesis. From this point of view, specific activation of HIF-2α may benefit from its more restricted localization against a wide range of diseases that are associated with ischemia and oxidative stress compared with ubiquitously expressed HIF-1α.

Conclusion

We have shown that HIF-2α−/− mice were highly susceptible to IRI of the kidney compared with WT mice. It is likely that the amelioration of the tissue injury by IRI was mediated by protection of endothelium via alleviation of oxidative stress. Induction of HIF-2α would be a possible and attractive therapeutic strategy against a wide range of diseases that are associated with ischemia and oxidative stress in the future.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (17390246).

We thank Nobuaki Eto and Takamoto Ohse for helpful discussions, Toshiko Yashiro and Yukari Kaneda for technical assistance, and Koei Yamada for technical advice.

Disclosures

None.

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Renoprotective Role of HIF-2α


