Activation of Hypoxia-Inducible Factor by Cobalt Is Associated with the Attenuation of Tissue Injury and Apoptosis in Cyclosporine-Induced Nephropathy

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Hypoxia-inducible factor (HIF) is a transcription factor that regulates cellular hypoxic responses. Despite the therapeutic benefits of cyclosporine A (CsA) in organ transplantation, its clinical use is limited due to chronic nephropathy. We investigated whether HIF activation by cobalt could improve CsA-induced nephropathy, and investigated the related mechanism. In animal experiments, rats were kept on a 0.05% low-salt diet and administered CsA subcutaneously for 28 days (15 mg/kg/day). They also received cobalt (10 mg/kg/day) during the entire experimental period. The administration of cobalt significantly increased HIF-1α expression in the kidney. The increased expression of HIF-1α ameliorated CsA-induced afferent arteriolopathy and tubulointerstitial injury in the kidney. Cobalt significantly reduced the infiltration of macrophages/monocytes into the renal tubulointerstitium. In addition, HIF activation by cobalt reduced the number of CsA-induced apoptotic cells in the kidney. Subsequently, HK-2 human renal tubular epithelial cells were used for in vitro experiments. They were pre-treated with 150 µM of cobalt to activate HIF, and then exposed to 10 µM CsA. HIF activation by cobalt decreased the CsA-induced apoptosis in HK-2 cells, as judged by the decreases in the number of apoptotic cells, pro-apoptotic caspase-3 activity, and the expression level of cleaved caspase-3, together with the increase in the expression of anti-apoptotic bcl-2. Cobalt pretreatment also reduced the CsA-induced phosphorylation of NF-κB and the CsA-induced expression of vimentin and α-smooth muscle actin, suggesting the attenuation of inflammation and fibrosis.

In conclusion, the activation of HIF by cobalt may ameliorate the CsA-induced nephropathy by inhibiting apoptosis, inflammation, and fibrosis.

Keywords: apoptosis; cyclosporine; fibrosis; hypoxia-inducible factor 1; inflammation


Despite receiving 20-25% of the cardiac output, the kidneys are susceptible to hypoxia (Schurek et al. 1990; Welch et al. 2001). Hypoxia can affect the expression of various genes, including apoptotic and fibrogenic factors, and is presumed to be a common pathway to chronic kidney disease (CKD) (Nangaku 2006).

Under reduced oxygen availability conditions, there is inhibition of hydroxylation of hypoxia-inducible factor (HIF) by prolyl-hydroxylase domain (PHD) and by factor-inhibiting-HIF (FIH), and HIF accumulates and activates the transcription of target genes such as vascular endothelial growth factor (VEGF) and other secreted factors (Wenger et al. 2005). In addition, HIF activates vascular cells to facilitate angiogenesis and arteriogenesis (Carmeliet 2005; van Weel et al. 2008). HIF also promotes cell survival under anaerobic conditions by activating the expression of lactate dehydrogenase A and, the expression of pyruvate dehydrogenase kinase 1, and mediating a switch from oxidative to glycolytic metabolism (Kim et al. 2006; Papandreou et al. 2006). Several studies reported that HIF has a protective effect in acute kidney injury models (Bernhardt et al. 2006; Weidemann et al. 2008).

Genetic ablation of tubular HIF-1α reduces the occurrence of tubulointerstitial fibrosis in a unilateral ureteral obstruction CKD model (Higgins et al. 2007). The depletion of the Von Hippel-Lindau tumor suppressor gene (VHL), which promotes proteolysis of HIF-1α, significantly increased renal fibrosis in a 5/6 renal ablation model (Kimura et al. 2008). On the other hand, the activation of HIF ameliorated tubulointerstitial injury through angiogenesis at 9 weeks post-subtotal nephrectomy (Tanaka et al. 2005). HIF activation reduced proteinuria, as well as histo-
logical kidney injury, by reducing advanced glycation and oxidative stress in type 2 diabetic rats with nephropathy (Ohtomo et al. 2008). Accordingly, it is unclear whether HIF activation is beneficial in renal fibrosis and CKD.

Cyclosporine A (CsA) has been used to prevent rejection in organ transplantation and to treat various autoimmune diseases. However, the clinical use of CsA is limited due to its nephrotoxicity. CsA induces the activation of the intrarenal renin–angiotensin system (Pérez-Rojas et al. 2007; Bertocchio et al. 2011) that mediates renal vasoconstriction. CsA-induced nephropathy (CIN) causes irreversible renal damages, including arteriolar hyalinosis, tubular atrophy, interstitial fibrosis, and glomerular injuries (Naesens et al. 2009). CsA increases expression of apoptotic genes and promotes apoptosis in renal tubular and interstitial cells, resulting in tubular atrophy (Carlos et al. 2010; Park et al. 2010; Xiao et al. 2010, 2011). CIN also presents inflammatory changes, including macrophage or monocyte infiltration into the injured kidney areas (Carlos et al. 2010; Park et al. 2010). In addition, CsA induces epithelial-mesenchymal transition in the kidney (McMorrow et al. 2005; Park et al. 2010; Hazzan et al. 2011). However, the precise underlying mechanism of CIN remains undefined.

We examined the effects of pharmacologic HIF activation by cobalt (CoCl₂), which is widely used as a PHD inhibitor, on CIN. In the present study, we investigated the molecular mechanism underlying the effects of HIF activation on renal fibrosis, apoptosis, and inflammation in CIN.

Materials and Methods

Cell culture

HK-2 cells (ATCC CRL-2190), which are proximal tubular epithelial cells derived from normal human kidney tissue, were cultured using Renal Epithelial Basal Medium (Lonza Walkersville Inc., Walkersville, MD, USA) with recommended supplements included in the REGM Singlequot Bulletkit. The cells were fed two to three times weekly and subcultivated via trypsinization when near confluence. HK-2 cells between passages 10 and 25 were used for these experiments.

Cell treatment

Cells were grown to 80% confluence before treatment for all experiments. CsA (Sigma, St. Louis, MO, USA) was prepared as a stock solution (4.2 mM) by dissolving 5 mg of the powder in 1 ml of absolute ethanol. This stock was further diluted in growth medium before treatment and added to the main cell growth media. Cobalt (Sigma, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS).

The cells were divided into three groups: control cells, CsA-treated cells, and CsA-treated cells with cobalt pre-treatment (CsA + CoCl₂). CsA concentrations (10 µM) in this study were chosen to approximate the concentration range reached in the kidney in vivo (McMorrow et al. 2005). A dose of cobalt chloride was determined by the pilot experiment. Before the experiments, the cells were incubated in basal medium in the absence of supplements for 24 hours. On the day prior to an experiment, the cells were pre-treated with 150 µM cobalt chloride for 24 hours. The cells were then treated with 10 µM CsA for another 24 hours.

Animal experiments

Seventeen male Sprague-Dawley rats (Orient Bio Inc., Seongnam, Korea) weighing 200 to 250 g were housed in individual cages in a temperature- and light-controlled environment. The rats received a low-salt diet (0.05% sodium; Samtako, Osan, Korea) and were allowed free access to water. This study was approved by, and conducted according to the guidelines of Seoul National University Bundang Hospital Institutional Animal Care and Use Committee. After one week on the low-salt diet, the rats were assigned to experimental groups (N = 5 to 6/group), and osmotic minipumps (model 2ML4; Alzet, Palo Alto, CA, USA) were subcutaneously implanted to deliver cobalt for 4 weeks. Osmotic minipumps are drug delivery systems using an osmotic pressure difference between compartments within the pump. They ensure that constant levels of compounds are maintained at therapeutic levels without the need for repetitive injections. The cobalt was dissolved in PBS. A dose and route of administration of cobalt was determined by the pilot experiments (data not shown). The vehicle consisted of PBS. CsA solution (Novartis, East Hanover, NJ, USA) was diluted in olive oil to a final concentration of 15 mg/ml. The dosage and route of administration for CsA were determined according to the previous study (Yang et al. 2001).

1. Vehicle (VH): The rats received a subcutaneous (SC) injection of olive oil 1 ml/kg/day plus a continuous infusion of PBS via osmotic minipump.
2. CsA group: The rats received a SC injection of CsA 15 mg/kg/day plus a continuous infusion of PBS via osmotic minipump.
3. CsA + CoCl₂ group: The rats received a SC injection of CsA 15 mg/kg/day plus a continuous infusion of CoCl₂ (10 mg/kg/day) via osmotic minipump.

After 4 weeks, the rats were anesthetized with zolazepam and xylazine, their blood was sampled, and their kidneys were collected. The left kidney from each rat was fixed in 10% phosphate-buffered formalin for morphologic and immunohistochemical analyses. The right kidney was snap-frozen in liquid nitrogen and stored at −80°C for protein extraction.

Physiologic measurements

At the end of experiments, the rats were weighed and placed in metabolic cages, and urine was collected for 24 hours. The urine volume was measured, and the protein concentration was determined by spectrophotometric assay (modified by Lowry using bicinchoninic acid reagent (Thermo Fisher Scientific, Rockford, IL, USA)). Urinary protein excretion was calculated in terms of milligrams of protein per 24 hours. Creatinine levels in the serum and urine were measured using an automatic analyzer (ADVAIA 1650, Siemens). Creatinine clearance was calculated using a standard formula and factored for body weight. CsA blood level was measured with a direct CsA radioimmunoassay kit (Immunotech, Czech Republic).

Renal histologic and immunohistochemical analyses

The tissue samples used for light microscopy and immunoperoxidase staining were fixed in formalin and embedded in paraffin. Three-micrometer sections were stained with periodic acid-Schiff (PAS) or Masson Trichrome (MT). Indirect immunoperoxidase staining was performed, as previously described (Song et al. 2010), with anti-CD68 antibody, ED-1 (Serootech, Oxford, UK). ED-1 recognizes a
cell-specific marker for monocytes/macrophages. The histologic findings were subdivided into two categories: tubulointerstitial injury and arteriolopathy. Tubulointerstitial injury consisted of matrix expansion with tubular distension, collapse and basement membranes thickening. More than 20 consecutive fields were examined under X200 magnification and the results were averaged: 0 = normal interstitium, 0.5 = ≤ 5% of areas injured, 1 = 5 to 15%, 1.5 = 16 to 25%, 2 = 26 to 35%, 2.5 = 36 to 45%, and 3 = ≥ 45%. Arteriolopathy was determined by counting at least 100 glomeruli. During this counting, arteriolopathy was recorded as present or absent. Arteriolopathy consisted of the hyalinization and destruction of afferent arterioles. The results are expressed as the percentage of juxtaglomerular affected arterioles over total number of arterioles: 0 = no arterioles injured; 0.5 = ≤ 15%; 1 = 15 to 30%; 1.5 = 31 to 45%; 2 = 46 to 60%; 2.5 = 61 to 75%; and 3 = ≥ 75%. The mean numbers of infiltrating interstitial macrophages (ED-1 positive cells) were calculated by averaging the total numbers of positive cells in more than 20 consecutive fields under X200 magnification.

Western blot analysis

Western blotting was performed as previously described (Song et al. 2010). Whole kidneys were homogenized, and nuclear extracts were prepared using a nuclear extraction kit (Sigma, St Louis, MO, USA). HK-2 cell lysates were prepared. Protein concentrations were measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). The samples were run on SDS-polyacrylamide mini-gels (Bio-Rad Mini Protein III). The proteins were transferred to nitrocellulose membranes by electroelution. Antibodies against HIF-1α (BD Bioscience, Franklin Lakes, NJ, USA), (Novus Biologicals, Littleton, CO, USA), phosphorylated NF-κB p65 (p-NF-κB p65) (Ser276) (Cell Signaling Technology, Danvers, MA), NF-κB p65 (Santa Cruz Biotech, Santa Cruz, CA, USA), caspase-3 (Cell Signaling Technology, Danvers, MA), bcl-2 (Cell Signaling Technology, Danvers, MA), β-actin (Santa Cruz Biotech, Santa Cruz, CA), vimentin, and α-smooth muscle actin (α-SMA) (Aabcam, Cambridge, UK) were used for this study. Incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA) was followed by band visualization using an enhanced chemiluminescence substrate (Thermo Fisher Scientific, Rockford, IL, USA). The band densities were quantified by densitometry (GS-700 Imaging Densitometry, Bio-Rad, Hercules, CA, USA). To facilitate comparisons, the densitometry values were normalized to a control, thereby defining the mean for the control group as one.

Detection of apoptosis

Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick-end labeling (TUNEL), following which the apoptotic cells were counted. Apoptotic cells were defined by chromatin condensation or nuclear fragmentation (apoptotic bodies). Apoptosis was detected in the specimens using the In Situ Cell Death Detection Fluoroscence Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s protocol. The same slides were stained with 4′,6′-diamidino-2-phenyindole (DAPI) in phosphate-buffered saline to reveal total nuclei. For apoptotic nuclei counting, cells from at least 10 consecutive fields under X400 magnification were counted. The final count was expressed as the percentage of total cells counted by fluorescence microscopy (Carl Zeiss, Jena, Germany). In addition, TUNEL-positive cells were counted in the cortical tubular cells in 10 consecutive fields under X400 magnification. This assay was performed in both kidney sections and HK-2 cells.

Caspase-3 activity assayed through the use of the Caspase-3/CPP32 Fluorometric Assay Kit (BioVision, Mountain View, CO, USA). Cells were incubated in cell lysis buffer and centrifuged at 14,000 rpm, and the supernatants were incubated with DEVD-AFC (a specific substrate for caspase-3) at 37°C for 1 hour. Subsequently, the activity was assayed through the use of a fluorescence microplate reader ( Molecular Devices, Sunnyvale, CA, USA).

Statistical analyses

The results are presented as the mean ± standard deviation of mean. The statistical analyses were performed using SPSS (version 18.0. for Windows; SPSS Inc., Chicago, IL, USA). The comparisons between groups were conducted with an analysis of variance followed by a Tukey’s test, a Student’s t-test, or a Mann-Whitney test. The level of statistical significance was set as \( P < 0.05 \).

Results

Administration of cobalt increased HIF-1α expression in vivo and in vitro

We examined whether continuous infusion of cobalt for 4 weeks would successfully increase the expression of HIF-1α. As shown in Fig. 1, the continuous infusion of cobalt with CsA markedly increased the amount of HIF-1α protein in the kidney compared to CsA-only-treated rats \( (P < 0.01) \) (Fig. 1A, B). In addition, pre-treatment with cobalt for 24 hours prior to the administration of CsA significantly increased HIF-1α expression in HK-2 cells \( (P < 0.05) \) (Fig. 1C, D).

Increased expression of HIF-1α ameliorated CsA-induced arteriolaropathy and tubulointerstitial injury

Increased expression of HIF-1α did not improve the physiologic parameters in CIN (Table 1). CsA-treated animals presented a significant increase in serum creatinine and a decline in creatinine clearance and body weight \( (P < 0.05) \). However, there were no significant differences in body weight, serum creatinine, creatinine clearance, and CsA blood levels between the CsA and CsA + CoCl2 groups. In addition, 24-hour urine protein excretion was not significantly different among the groups.

However, marked histologic differences were observed among groups. VH-treated rats displayed a normal renal histology (Fig. 2A, 3A). In contrast, CsA-treated rats developed a significant arteriolar hyalinosis (Fig. 2B), extensive tubulointerstitial injury and tubular atrophy (Fig. 3B). The concurrent administration of cobalt with CsA improved the afferent arteriolopathy \( (P < 0.01) \) and the tubulointerstitial injury \( (P < 0.01) \) compared to the no-cobalt administered CsA-treated rats (Fig. 2C, 3C). The semiquantitative scores for the VH, CsA, and CsA + CoCl2 groups for arteriolar hyalinosis were 0.50 ± 0.00, 1.38 ± 0.25, and 0.75 ± 0.29, respectively, whereas the respective tubulointerstitial injury scores were 0.50 ± 0.00, 2.83 ± 0.17, and 2.00 ± 0.00 (Fig. 2D, 3D), respectively.
There was a significant difference in the number of ED-1 positive cells that indicate macrophages/monocytes. Very few ED-1 positive cells were noted in the VH-treated rats (0.4 ± 0.2 ED-1 positive cells/field, Fig. 4A). In contrast, in the CsA-treated rats, there were large numbers of macrophages or monocytes with most infiltrating the tubulointerstitium (12.9 ± 3.5 cells/field, Fig. 4B). The animals treated with CsA and CoCl2 displayed the infiltration of a few macrophages or monocytes (4.6 ± 1.7 cells/field) (P < 0.01) (Fig. 4C). Very few ED-1 positive cells were identified in the glomeruli of all the rats.

Increased expression of HIF-1α protein in CoCl2-treated rat kidneys and renal tubular cells. (A) Western blot analysis of HIF-1α molecules in the nuclear extracts of whole kidney homogenates from experimental rats. Representative blots of two animals from each group were shown. (B) Arbitrary units of HIF-1α abundance in immunoblot expressed as densitometric ratios as fold-change over the control group in rats. (C) Western blot analysis of HIF-1α molecules in HK2 cells. A representative set of three independent experiments is shown. (D) Arbitrary units of HIF-1α abundance in immunoblot expressed as densitometric ratios as fold-change over the control group in HK-2 cells. The data are the means ± s.d. *P < 0.05 vs. control, †P < 0.05 vs. CsA.

Table 1. Physiologic data.

<table>
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<tr>
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<th>Control (N = 6)</th>
<th>CsA (N = 5)</th>
<th>CsA + CoCl2 (N = 6)</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>317.3 ± 24.2</td>
<td>245.0 ± 22.5*</td>
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<tr>
<td>Cr (mg/dl)</td>
<td>0.6 ± 0.5</td>
<td>1.3 ± 0.2*</td>
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</tr>
<tr>
<td>CCr (ml/min/100 g)</td>
<td>0.094 ± 0.014</td>
<td>0.040 ± 0.009*</td>
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<tr>
<td>24-hr urine protein (mg/day)</td>
<td>97.1 ± 13.7</td>
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<td>2,838.0 ± 360.1*</td>
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We analyzed kidney sections after having detected DNA fragmentations with the in situ TUNEL assay to detect apoptosis. The large numbers of scattered and bright nuclei associated with TUNEL staining were detected in the kidneys from CsA-treated rats; however, a significantly lower numbers were observed in the CoCl2-treated rats. The percentage of apoptotic cells was 4.6 ± 0.8, 69.5 ± 7.8, and 20.6 ± 10.2 in the VH-, CsA-, and CsA + CoCl2 groups, respectively (P < 0.01, Fig. 5).

We also determined CsA-induced apoptosis in vitro. In HK-2 cells, the number of apoptotic cells were significantly increased by CsA treatment, and pre-treatment with cobalt markedly reduced CsA-induced apoptosis (P < 0.01, Fig. 6A). Pro-apoptotic caspase-3 activity was significantly increased by CsA treatment. However, pre-treatment with cobalt significantly reduced caspase-3 activity in CsA-treated HK-2 cells (P < 0.01, Fig. 6B). The expression levels of anti-apoptotic bcl-2 and pro-apoptotic caspase-3 were analyzed by western blotting (Fig. 6C). The administration of CsA significantly reduced the abundance of bcl-2 (P < 0.05) and increased that of cleaved caspase-3 (P < 0.01). Pre-treatment with cobalt reversed these changes in the

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Fig. 2. Afferent arteriolopathy in CsA-treated rats. (A) Vehicle group, (B) CsA group, and (C) CsA + CoCl₂ group (PAS stain, × 200). The arrow indicates arteriolopathy. (D) Semiquantitative scoring of arteriolopathy according to mean percentage of afferent arterioles injured, which was estimated in at least 100 preglomerular afferent arterioles from the (A), (B), and (C) groups. Compared with the CsA group, CoCl₂-treated rats displayed improved afferent arteriolopathy. The data are the means ± s.d. *P < 0.05 vs. control, †P < 0.05 vs. CsA.

Fig. 3. Tubulointerstitial injury in CsA-treated rats. (A) Vehicle group, (B) CsA group, and (C) CsA + CoCl₂ group (MT stain, magnification × 200). (D) Semiquantitative scoring of tubulointerstitial injury according to mean percentage of injured area obtained in at least 20 fields from the (A), (B), and (C) groups. Compared with CsA group, CoCl₂-treated rats displayed improved tubulointerstitial injury. The data are the means ± s.d. *P < 0.05 vs. control, †P < 0.05 vs. CsA.
Fig. 4. Effects of cobalt on macrophage infiltration in CsA-induced nephropathy. (A) Vehicle group, (B) CsA group, and (C) CsA + CoCl\(_2\) group (magnification × 200). (D) Absolute count of ED-1 positive cells obtained in at least 20 fields from the (A), (B), and (C) groups. The data are the means ± s.d. *\(P < 0.05\) vs. control, †\(P < 0.05\) vs. CsA.

Fig. 5. Effects of cobalt on apoptosis in CsA-induced nephropathy. Representative TUNEL staining of renal cortex in CsA-induced nephropathy. (A) Vehicle group, (B) CsA group, and (C) CsA + CoCl\(_2\) group (magnification × 400). Arrows indicate TUNEL-positive cells. (D) The percentage of apoptotic cells (TUNEL-positive cells) obtained in at least 10 fields from the (A), (B), and (C) groups. The data are the means ± s.d. *\(P < 0.05\) vs. control, †\(P < 0.05\) vs. CsA.
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CsA-treated HK-2 cells (Fig. 6D, E).

Increased expression of HIF-1α reduced CsA-induced phosphorylation of NF-κB and the expression of α-SMA and vimentin in vitro

Since NF-κB regulates inflammatory responses, we examined the activation of NF-κB in response to CsA treatment and HIF activation. CsA treatment activated NF-κB by increasing the densitometric ratio of p-NF-κB p65 to total NF-κB p65 approximately twofold. In contrast, pretreatment with cobalt markedly reduced the ratio of phosphorylated to total NF-κB below the level of the control cells (Fig. 7).

We further determined the role of HIF-1α in CsA-induced cell transdifferentiation. CsA increased the expression of vimentin, a marker of cell transdifferentiation, in HK-2 cells. However, the activation of HIF-1α by cobalt reduced the CsA-induced expression of vimentin ($P < 0.01$). We also examined α-SMA, a marker for fibrosis. CsA-treated HK-2 cells displayed a slight increase in the amount of α-SMA, but pre-treatment with cobalt markedly reduced it ($P < 0.05$, Fig. 8).

**Discussion**

The activation of HIF by cobalt had a protective effect on CIN via the inhibition of apoptosis, inflammation, and fibrosis. Although HIF activation could not improve the renal dysfunction caused by CIN, it ameliorated histologic changes such as tubulointerstitial injury and afferent arteriopathy and decreased the infiltration of inflammatory cells. HIF activation reduced CsA-induced apoptosis both in vivo and in vitro. In addition, it reduced CsA-induced activation of NF-κB and reduced the expression of vimentin and α-SMA in renal tubular epithelial cells.
The apoptosis of renal tubular cells leads to tubular atrophy and dilatation in chronic kidney injury (Böttinger and Bitzer 2002). Two main intracellular pathways for apoptosis have been identified; an intrinsic pathway involving intracellular organelles and an extrinsic pathway leading to the assembly of multi-molecular complex including Fas-Associated protein with Death Domain (FADD) and caspase-8 (Sanz et al. 2008). The CsA-induced apoptosis appears to be associated with the intrinsic pathway because CsA does not affect FasL-induced apoptosis and caspase-8 activity (Justo et al. 2003). CsA promotes Bax aggregation and translocation to mitochondria, inducing permeabilization of the outer mitochondrial membrane, release of cytochrome C, and activation of caspase-9 (Sanz et al. 2008). Cleaved caspase-9 activates the effector caspase, caspase-3. In addition, Bcl-2 binds and sequesters Bax and inhibits the activation of caspase-9. In this study, TUNEL-positive apoptotic cells were increased in CsA-treated rat kidneys and HK-2 cells. Accordingly, CsA increased the expression of cleaved caspase-3 and caspase-3 activity, in contrast, it downregulated the expression of Bcl-2. These changes were recovered by cobalt treatment, suggesting that HIF activation attenuated CsA-induced apoptosis through the intrinsic pathway.

The infiltration of inflammatory cells in CIN has been repeatedly demonstrated (Mizuiri et al. 2004; Carlos et al. 2010; Park et al. 2010). Activated macrophages may injure renal tissue and lead to fibrosis by the production of inflammatory cytokine and pro-fibrotic molecules (Carlos et al. 2010; Park et al. 2010). In this study, cobalt reduced the monocyte/macrophage infiltration induced by CsA, confirming that HIF activation has an anti-inflammatory effect in CIN. NF-κB is a transcription factor that regulates the induction and resolution of inflammation. After stimuli, NF-κB is released and translocated into the nucleus, where it induces transcriptional activation of the target genes leading to inflammation (Sanz et al. 2010). NF-κB activates macrophages in renal tubules and glomeruli, and its activity correlates with the severity of various diseases, including ischemia-reperfusion injury, diabetic nephropathy and glomerulonephritis (Mezzano et al. 2001; Loverre et al. 2004; Starkey et al. 2006). The activation of NF-κB in the kidney has also been reported in the treatment with CsA (Mizuiri et al. 2004; Buffoli et al. 2005; Carlos et al. 2010; Park et al. 2010). We found that CsA treatment increased the phosphorylation of p65 NF-κB subunit and cobalt attenuated it, suggesting that HIF activation exerts its anti-inflammatory effects in CIN through the NF-κB signaling pathway.

Epithelial-mesenchymal transition (EMT) is a process in which fully differentiated epithelial cells lose their epithelial phenotype and acquire new features of the fibroblast. It has emerged as an essential part of tissue fibrogenesis after renal injury (Liu 2010). EMT is manifested by the loss of epithelial cell adhesion and by acquisition of new mesenchymal markers such as vimentin, α-SMA, interstitial matrix component type I collagen, and fibronectin (Strutz 2009; Liu 2010). The administration of CsA has been reported to induce EMT in rat kidneys and in renal tubular cells (Mc Morrow et al. 2005; Park et al. 2010; Hazzan et al. 2011). In this study, CsA induced significant tubulointerstitial fibrosis in rat kidneys. The activation of HIF by cobalt significantly reduced the fibrosis. In HK-2 cells, CsA increased the expression of vimentin and α-SMA, and cobalt reduced it. This suggests that HIF is associated with EMT in renal tubular cells, a key event in the pathogenesis of renal interstitial fibrosis.

Our study has a couple of limitations. First, despite the marked improvement of histologic injuries in CIN mediated by HIF activation, we could not demonstrate the improvement of physiologic parameters. Additional studies using diverse experimental settings and different methods of HIF activation are required. Second, we only investigated the extent of NF-κB phosphorylation in this study. To find more direct evidence that NF-κB is involved in HIF activation of CIN, an electrophoretic mobility shift assay (EMSA) or studies using NF-κB inhibitors is required.

In summary, HIF activation by treatment with cobalt demonstrated a protective effect on CIN through anti-apoptotic, anti-inflammatory, and anti-fibrotic actions.
Clinically, we have little knowledge of when tissue hypoxia, HIF activation, chronic kidney injury, or fibrosis begin in various kidney diseases. The activation of HIF could be a target for protecting against the progression of renal injury, but an understanding of HIF expression in individual disease needed to facilitate such a potential therapy.

Acknowledgments

This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (Grant No. 2009-0071488) and Grant No. 03-2010-010 from the SNUBH Research Fund.

Conflict of Interest

There is no conflict of interest.

References


Loverre, A., Dittono, P., Crovace, A., Gesualdo, L., Ranieri, E.,
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