Induction of Renoprotective Gene Expression by Cobalt Ameliorates Ischemic Injury of the Kidney in Rats

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Abstract. Hypoxia in the tubulointerstitium has been thought to play pivotal roles in the pathophysiology of acute renal failure and the progression of chronic kidney disease. Pre-induction of hypoxia-inducible and renoprotective gene expression may protect subsequent ischemic injury. This study evaluated the efficacy of cobalt, which inhibits HIF-1 degradation and increases the expression level of hypoxia-related genes, in an acute ischemic tubulointerstitial injury model of rats. Ischemic renal injury was induced by 45-min clamping of renal pedicles with contralateral nephrectomy. Elevation of serum creatinine and morphologic injury after the ischemic insult was observed. Administration of cobalt chloride afforded striking functional improvement (mean ± SEM creatinine in mg/dl: Co treatment group, 2.14 ± 1.21; control, 3.69 ± 1.43; P < 0.05) associated with amelioration of tubulointerstitial damage. Cobalt treatment also reduced macrophage infiltration significantly. In the kidney of rats treated with cobalt, mRNA levels of several genes that serve for tissue protection, such as HO-1, EPO, Glut-1, and VEGF, were increased before ischemic injury. Uprogulation of HO-1 by cobalt was confirmed at the protein level. Subcutaneous injection of cobalt also ameliorated ischemic injury, which was associated with upregulation of renal HIF-1α protein expression. These results suggest that protection against hypoxic tubulointerstitial injury by cobalt administration is mediated by induction of renoprotective gene expression. HIF induction is one possible and attractive explanation for the observed effects.

Hypoxia contributes significantly to the pathophysiology of major categories of human disease, including myocardial and cerebral ischemia, cancer, congenital heart disease, and chronic obstructive pulmonary disease. In the kidney, ischemic injury leads to acute renal failure (ARF). Although renal function after episodes of ARF is thought to be restored, initial ischemic injury is associated with high morbidity and mortality. Furthermore, hypoxia is crucial in progression of chronic renal disease (1). There is growing evidence that reduction of peritubular capillary density coincides with loss of renal function and results in progressive renal failure, implying a role of hypoxia in tubulointerstitial damage, which leads to eventual kidney failure (2).

Improvement of the ability of organs to tolerate ischemic injury would be beneficial in case of hypoxia. An exposure to hypoxic environment is known to stimulate genes that play roles in the adaptation to oxygen deficiency. A category of them includes proteins involved in regulating glucose uptake and glucose metabolism, which allow maintaining energy synthesis under hypoxic condition. The other category includes proteins involved in angiogenesis and erythropoiesis, which increase blood vessel density and blood oxygen-carrying capacity.

Many processes of adaptation to hypoxia are mediated by hypoxia inducible factor (HIF), which is a master regulator of genes activated by low oxygen tension. HIF-1α representing the oxygen-regulated component of HIF-1 is ubiquitinated and then rapidly degraded by proteasomes. The oxygen-sensing pathway involves the oxygen-dependent prolyl hydroxylation of HIF-1α by prolyl-4-hydroxylase (3,4), which serves as a signal for polyubiquitination and proteasomal degradation (reviewed by Wiesener and Eckardt [5] and by Wenger [6]). The prolyl hydroxylation requires iron as a cofactor; thereby iron antagonists such as desferrioxamine and cobalt chloride have “hypoxia-mimetic” effects by inhibiting degradation of HIF-1α (7).

We tested a hypothesis that administration of cobalt may improve ischemic tubulointerstitial injury by stimulating various genes as cellular adaptation to hypoxia. While our ultimate goal is to treat chronic hypoxia in the tubulointerstitium, we employed a well-established acute ischemic model in this study to investigate short-term effects of our approach as the initial step.
Materials and Methods

Experimental Protocol

All experiments were conducted in accordance with the “Guide for the Care and Use of Laboratory Animals” (DHHS Publication No. [NIH] 86-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD). Male SD rats weighing 160 to 180 g were purchased from Nippon Seibutsu Zairei Co. (Saitama, Japan). All rats were housed in individual cages in a temperature- and light-controlled environment in an accredited animal care. Rats in the control group (n = 9) had been drinking tap water, whereas rats in the cobalt group (n = 7) had been drinking water containing 2 mM cobalt chloride from day −10 to day 3. On the basis of the daily water intake, the total dose of cobalt chloride was estimated to be about 80 mg per animal. In some studies, animals (n = 3) were injected with cobalt chloride subcutaneously at dose of 30 mg/kg at day −1 and day 0 with 12-h interval. Twenty-four hours before induction of ischemic injury, at day 0, the right renal pedicle and the right ureter were ligated, and right nephrectomy was performed. Ischemic injury was induced by clamping of left renal artery and vein for 45 min at day 1. Core body temperature was maintained at 37°C using a homeothermic table during surgery. Twenty-four and forty-eight hours after induction of injury, blood samples were obtained via tail vein for determination of serum creatinine levels. Serum creatinine levels were determined enzymatically with a commercial kit that used the creatininase/creatinase/sarcosine oxidase (Kainos Laboratories, Tokyo, Japan). Forty-eight hours after injury, at day 3, rats were euthanized and the kidneys were removed for histologic analysis.

Renal Histologic Analyses

Tissue fixed in methyl Carnoy solution was processed and paraffin-embedded. Four-micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. Vimentin was stained with murine monoclonal IgG antibody V9 (Dako, Carpinteria, CA), renal microvascular endothelial cells were identified with murine monoclonal IgG1 antibody JG-12 (Bender MedSystem, San Bruno, CA), and monocytes/macrophages were identified with murine monoclonal IgG1 antibody ED-1 (Chemicon, Temecula, CA) by an indirect immunoperoxidase method. HIF-1α was identified with monoclonal IgG HIF-1α antibody a67 (Novus Biological, Littleton, CO) at a 1/100 dilution utilizing the Tyramide Signal Amplification Systems (PerkinElmer Life Sciences, Boston, MA). For quantification of morphologic data, more than 15 low-magnification fields including both cortex and outer medulla were randomly selected. Tubular injury in terms of tubular dilation, tubular epithelial injury, debris accumulation, and cast formation was graded with an arbitrary score of 0 to 3: 0, normal; 1, mild; 2, moderate; 3, severe (8). Tubules that are vimentin-positive or are surrounded by vimentin-positive cells were counted in 20 randomly selected cortical fields with a ×20 objective. ED-1–positive cells were counted in 20 randomly selected fields including both cortex and outer medulla with an ×10 objective. Peritubular capillary loss was analyzed using rarefaction index as previously reported (9). Briefly, it was determined by counting the numbers of squares in 10 × 10 grids that did not contain JG-12–positive peritubular capillary staining, in at least ten non-overlapping sequential fields in the outer medulla. All quantification was performed in a blinded manner.

Real-Time Quantitative PCR Analyses

Total RNA was extracted from kidney homogenates with ISOGEN (Nippon gene, Tokyo, Japan). To synthesize cDNA from total RNA, SuperScript II Reverse Transcriptase was used (Life Technologies BRL, Rockville, MD). Renal mRNA levels were assessed by real-time quantitative PCR (real-time PCR) using SYBR Green PCR reagent (QIAGEN, Hilden, Germany) and iCycler PCR system (Bio-Rad Laboratories, Hercules, CA) according to manufacturer’s instructions. Briefly, amplification reactions contained 1 μl of cDNA, 12.5 μl of the Universal 2×PCR mastermix (QIAGEN), and 5 μl of each of the specific primers and the probe. Primer concentrations in the final volume of 25 μl were 500 nM. In control experiments with triplicates, no false positives were detected and the variance between each of the replicates was within 5%. After an initial hold of 15 min at 95°C, the cDNA samples were cycled 40 times at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. All reactions were performed in triplicate. Ct, or threshold cycle, was used for relative quantification of the input target number. The amount of threshold cycle for control sample was considered 1, i.e. 2^ΔCt. The number of threshold cycles for other samples was derived by cycles of control sample and recorded as ΔCt. The amount of amplified molecules at the threshold cycle was given by: 2ΔCt. The mRNA levels of genes were normalized to levels of β-actin. Each genes and PCR primers are as follows; heme oxygenase-1 (HO-1) (5'-TCTATCGTGTCGATGAAC -3', 5'-CAGCTCTT- CAAACAGCTCAA -3'), vascular endothelial growth factor (VEGF) (5’-TTACTCTGCTGATCCCA -3', 5'-ACAGGACCGTGAAGTGA- GATA -3'), erythropoietin (EPO) (5'-TACGTAGCCTCACTTCCAC -3', 5'-ACAGGACCGTGAAGTGA-GATA -3'), glucose transporter-1 (Glut-1) (5'- CAGTTCGGCATATAACCCGGTGC -3', 5'-ATAATGGTGTCCGTACAT -3'), β-actin (5'-TTTCTCTAATGAGCTCGTG -3', 5'-TCATGAGCTGTCAAGT -3').

Western Blot Analyses

Isolation of whole protein from nephrectomized kidney was performed by tissue homogenization in Tris-glycine buffer. Forty micrograms of protein of each animal was resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. Western blot analysis was performed as described previously (10). Briefly, anti HO-1 antibody (StressGen, Victoria, Canada) was used at a 1/2000 dilution; horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) was used at a 1/2000 dilution. The ECL Western blotting systems (Amersham Bioscience, Upsala, Sweden) was used for detection.

Statistical Analyses

Data are reported as mean ± SEM. Statistical analyses were performed using the t test. The relationships between variables were assessed by Pearson correlation analysis. Nonparametric data were analyzed with the Mann-Whitney test when appropriate. Differences with P < 0.05 were considered significant.

Results

Improvement of Ischemic Renal Injury by Treatment with Cobalt Chloride

Cobalt chloride treatment was started at day −10 and continued until the end of the experiments at day 3. A dose of cobalt chloride employed in our experiments was confirmed not to be toxic for rats previously (11). PAS-stained sections obtained at day 0, before ischemic insult, showed no morphologic changes by cobalt administration (data not shown). Cobalt administration to rats significantly prevented an increase in serum creatinine induced by ischemia as compared with that of the rats drinking tap water (Co treatment group, 2.23 ± 0.19 SEM. Statistical analyses were...
mg/dl versus control, 2.95 ± 0.18 mg/dl at 24 h; *P < 0.05; Co treatment group, 2.14 ± 1.21 mg/dl versus control, 3.69 ± 1.43 mg/dl at 48 h; *P < 0.05). To determine the effects of cobalt administration on tubulointerstitial injury, we performed histopathologic analysis. We observed severe tubular damages in terms of tubular dilation, tubular epithelial injury, debris accumulation, and cast formation in our control animals 48 h after the ischemic insult (Figure 1A). Cobalt administration improved tubulointerstitial damage induced by ischemia (Figure 1B). Semiquantitative scoring analysis revealed protective effects of cobalt administration with a statistically marginal significance (Co treatment group, 1.58 ± 0.14; control, 2.18 ± 0.24; *P = 0.08). We observed a good correlation between serum creatinine levels and tubular damages for individual rats (*r² =0.83, *P < 0.001; Figure 1C).

Expression of Vimentin, a Marker of Tubulointerstitial Damage, Was Ameliorated by Cobalt Administration
To confirm the beneficial effects donated by cobalt in the histologic analysis, we performed immunohistochemical studies using a marker of tubulointerstitial injury, vimentin. We observed no vimentin staining in the tubulointerstitium before ischemia in both groups (data not shown). While expression of vimentin in tubular cells or in cells surrounding tubules was obviously increased by ischemia in controls, cobalt administration significantly prevented vimentin expression (Co treatment group, 0.71 ± 0.11 vimentin-positive tubules per field; control, 3.26 ± 0.92; *P < 0.05; Figure 2).

Macrophage Infiltration Was Reduced by Cobalt Treatment
Ischemic injury was associated with macrophage infiltration in the tubulointerstitium as determined by an increase in ED-1-positive cells. The number of infiltrating macrophages was significantly reduced in animals treated with cobalt (Co treatment group, 65.4 ± 11.2 ED-1 positive cells per field; control, 160.9 ± 62.4; *P < 0.05; Figure 3).

Cobalt Chloride Induced Renoprotective Gene Expression
We speculated that the beneficial effects of cobalt treatment might be mediated by increasing the expression level of genes

Figure 1. Cobalt chloride treatment conferred functional and morphological protection against ischemic tubulointerstitial injury. (A) Tubular injury, tubular dilation, tubular epithelial injury, debris accumulation, and cast formation were apparent in controls. (B) Damage was less severe in cobalt-treated rats. Periodic acid-Schiff staining. Magnification, ×200. (C) There was a significant positive correlation between the morphological severity and serum creatinine levels.

Figure 2. Immunohistochemical analysis of markers of tubulointerstitial injury, vimentin, in controls (A) and cobalt-treated (B) rats was induced after ischemic injury. While photomicrographs of controls demonstrated expression of vimentin intermediate filaments in tubular cells and in cells surrounding tubules, vimentin-positive cells disappeared in cobalt-treated rats. Magnification, ×200.
that work as an adaptive mechanism against hypoxia. To determine expression of renoprotective genes stimulated by cobalt administration, we performed real-time quantitative PCR using kidneys obtained before and after ischemic insult.

Real-time PCR analysis allowed us to perform relative quantification based on the difference in threshold cycles (ΔCt) of target and control samples (ΔCt) in cobalt and control groups, respectively. The variance between each of the replicates was within 5%. Cobalt administration increased mRNA levels of HO-1, EPO, Glut-1, and VEGF before ischemic insult. Fold increases of each mRNA expression compared with control were 9.43-, 6.93-, 2.77-, and 1.46-fold, respectively (Figure 4A). Even after ischemic surgery, mRNA levels of HO-1, Glut-1, and VEGF remained higher in the cobalt-treated group compared with the control at the same time point. The fold increases were 2.47-, 1.31-, and 1.36-fold respectively (Figure 4B). On the other hand, EPO expression dropped down to undetectable level after ischemia in both cobalt-treated and control groups.

To confirm upregulation of protein products of renoprotective genes, we performed Western blot analysis of HO-1. The expression level of HO-1 protein in kidneys was not detectable in a control group, but it was clearly increased in a cobalt-treated group (Figure 4C).

**Cobalt Administration Prevented Peritubular Capillary Loss Induced by Ischemic Insult**

The increase in VEGF expression in cobalt-treated kidneys raised a possibility that protection of the kidneys against ischemic insult may be mediated by structural changes of the microvasculature. However, before ischemic insult, at day 0, capillary endothelial cells stained with JG-12 antibody were well maintained without any changes of the density of peritubular capillaries by cobalt administration (Rarefaction index score: Co treatment group [n = 2], 1.06 ± 0.44%; control [n = 3], 1.68 ± 0.16%). Cobalt administration into rats significantly prevented peritubular capillary loss by ischemia compared with that of the rats drinking tap water (rarefaction index score: Co treatment group, 9.34 ± 1.30% versus control, 17.2 ± 2.17%; P < 0.05; Figure 5).

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**Figure 3.** Immunohistochemical analysis of markers of monocytes/macrophages, ED-1. In controls (A), ischemic injury caused massive macrophages infiltration. In contrast, fewer ED-1-positive cells were recruited in the kidneys of cobalt-administrated animals (B). Magnification, ×200.

**Figure 4.** Cobalt chloride increased expression levels of renoprotective genes. Expression of mRNA for each gene in the whole kidney of rats drinking cobalt chloride or controls drinking tap water at day 0 (A) and at day 3 (B). Three controls and two cobalt-treated animals were analyzed. mRNA expression was determined by real-time quantitative reverse transcription-PCR. Expression was normalized to the housekeeping β-actin gene and was shown as a ratio to control animals. One percent of the total cDNA was analyzed in triplicate; comparative controls without reverse transcription were negative for each gene. (C) Western blot analysis of HO-1. In controls (n = 2 different animals), no signal was detected. HO-1 protein was induced by cobalt administration (n = 2 different animals). Molecular weight markers are shown on the left (in kD).
Short-Term Cobalt Administration also Ameliorated Ischemic Insult as well as Long-Term Administration

To verify whether the efficacy observed in 13-d-administered animals was due to the primary effect of cobalt, we employed short-term cobalt administration against ischemic injury. Subcutaneous cobalt chloride injection at a dose of 30 mg/kg twice diminished the rise of serum creatinine by ischemia as well as the long-term administration (Co treatment group, 1.54 ± 1.08 mg/dl; control, 3.12 ± 0.20 mg/dl at day 3). The improvement of renal function was associated with less severe damage shown in PAS-stained sections (Figure 6, A and B). To test our hypothesis that a protective effect of cobalt is attributed to hypoxia-related genes induced via HIF-1α stabilization, immunohistochemical study using anti–HIF-1α antibody was performed. Staining for HIF-1α was observed in some tubules of the medulla after 6 h of the last subcutaneous injection of cobalt. In contrast, no staining was seen in control animals (Figure 6, C and D).

Discussion

In this study, we demonstrated that cobalt administration protected kidneys from ischemic injury. Our histologic analysis and estimation of renal function showed improvement of tubulointerstitial injury due to ischemia by treatment with cobalt. The improvement of renal morphology was paralleled with serum creatinine levels. Amelioration of tubulointerstitial injury in cobalt-administered rats was confirmed by immunohistochemical analysis to detect macrophages and vimentin as a marker of tubulointerstitial injury (12). To our knowledge, this is the first study to demonstrate effects of cobalt against ischemic injury in the kidney.

The most severe damage resulting from ischemic insult spread from the outer medulla to the medullary ray of the cortex, which is consistent with previous observations showing that proximal S3 segment and the thick ascending limb of the nephron are susceptible to ischemic injury. Cobalt treatment ameliorated overall tubular damage assessed by PAS, and this was supported by reduction of infiltration of monocyte/macrophages and the number of vimentin-positive tubules.

As an attempt to reveal a mechanism of renoprotection by cobalt, we demonstrated that cobalt increased expression level of hypoxia-related genes such as HO-1, EPO, Glut-1, and VEGF. Administration of cobalt was started before ischemic injury so that protective mechanisms were ready when the kidneys undergo ischemic insult. Transient ischemic insult before severe ischemia is shown to be protective in various organs and is called preconditioning. Mechanisms of beneficial effects by ischemic preconditioning are multifactorial in kidney (reviewed by Bonventre [13]). Recent studies reported that cobalt administration induced HIF-1 expression in renal tubu-
lar cells (14) and several HIF-1–regulated genes in various organs, i.e., glucose transporters both in retina and brain (15), VEGF in heart, and EPO in liver (16). We speculate that cobalt treatment would work as chemical preconditioning and ameliorated ischemic kidney injury via multiple mechanisms, including upregulation of expression of HO-1, EPO, Glut-1, and VEGF as shown in this study.

HO-1, which catalyzes the conversion of heme, was implicated in a cytoprotective mechanism to prevent cells and tissues from oxidative injury. Postischemic damage is, at least in part, considered to be related to the generation of free oxygen radicals. Hence, biliverdin and bilirubin, which are end products of heme degradation and are reported to possess potent antioxidant properties, may play a protective role against ischemia reperfusion injury.

EPO increases oxygen delivery to tissue and thus could reduce injury due to hypoxia in the long term. Furthermore, there are accumulating reports that EPO exhibited tissue protection mediated by activation signaling via EPO receptor. Grimm et al. (17) showed that EPO acted as preconditioning against photochemical retinal injury, which was accounted by anti-apoptotic effects. Chong et al. (18) demonstrated that EPO offered cytoprotection during ischemic vascular injury through direct modulation of Akt1 phosphorylation. Although the mechanism is not fully appreciated, EPO could also contribute to renal protection through direct effects on the vasculature.

Induction of glucose transporter expression such as Glut-1 should be important to allow for continued energy generation in hypoxic environments. Cobalt administration did not change the density of peritubular capillaries in the intact kidney but prevented peritubular capillary loss induced by ischemia. Hence, VEGF might act on endothelial cells as a survival factor rather than as an angiogenic factor, at least this time point.

We also demonstrated that mRNA levels of HO-1, Glut-1, and VEGF were still higher in cobalt-treated kidneys 48 h after ischemic insult. Accordingly, these genes could play roles not only in preconditioning but also in recovery from ischemic injury. Our observation that EPO was diminished in postischemic kidney was consistent with the previous studies (19).

Oral and long-term administration has the advantage, especially in consideration of application to patients with chronic progressive kidney disease, in which hypoxia is believed to be involved in progression. In long-term administration, however, we cannot deny a possibility that the protective effect was not due to induction of hypoxia-related genes themselves but was mediated by other factors indirectly derived from cobalt treatment. To eliminate this possibility, we employed short-term and relatively low-dose cobalt treatment. The analysis of renal function and histologic damage in this experiment confirmed the efficacy of cobalt even by a short period of treatment.

Recently renal hypoxia has been well mentioned to play...
pivotal roles in progression of chronic kidney disease. To maintain the microvasculature and support oxygenation, several angiogenic, survival, and proliferative factors to endothelial cell are crucial. VEGF treatment ameliorates disease manifestations in several models, such as glomerulonephritis (20), remnant kidney (21), and TMA (9), probably via protection of endothelial cell and subsequent improvement in oxygenation in the tubulointerstitium. HIF-1 is considered as a key regulator of oxygen homeostasis (22), and stimulation of HIF-1 signaling can be theoretically more effective in ischemic states because it can induce expression of a variety of oxygen-regulated and renoprotective genes including VEGF. Vincent et al. (23) employed a strategy of constitutively-active HIF-1 hybrid transcription factor as an angiogenic therapy. William et al. (24) showed an accelerated angiogenic response by peptide blockade of HIF-1α degradation. In short-term cobalt administration, we showed that the tissue protection was associated with expression of HIF-1α protein. This result suggested that HIF-1, at least partially, participated in induction of hypoxia-related genes and tissue protection by cobalt. Since HIF-2α is degraded in the presence of oxygen and cobalt can stabilize renal HIF-2α protein (14), HIF-2 could also contribute to the tissue protection. However, the possibility that mechanisms other than HIF induction may be involved in this protection cannot be excluded, which needs to be investigated in future work.

Recent studies reported that cobalt administration ameliorated ischemic injury of the heart (15,25) and the liver (26–28). In the kidney, Tullius et al. (29) showed that treatment with cobalt protoporphyrin improved chronic graft survival of transplant kidneys via induction of heme oxygenase-1. While they attributed improvement in graft survival to prevention of ischemic injury, no detailed information about acute phase of ischemic injury in the kidney was provided, and it remains unclear whether other mechanisms participated in their study. We employed relatively high amount of cobalt chloride in comparison with the dose of cobalt protoporphyrin used in their studies. It was based on the difference of pharmacokinetics that noncomplexed cobalt exhibited a much faster elimination rate than protoporphyrin chelate of the metal (30). The total dose injected in the current study was within the range that has been used to activate HIF-1α (14,31) and induce hypoxia-related genes (16).

In summary, although the mechanisms are not fully elucidated, cobalt administration improved ischemic renal injury. HIF induction is one possible and attractive explanation for the observed effects, which needs to be confirmed in future work. Our findings give a clue to development of a novel therapeutic strategy against hypoxic kidney damage. While there are obviously some differences in the pathomechanisms between acute and chronic injury in the kidney, we hope we can extend our observations in the acute model to chronic hypoxia in progressive kidney disease. Studies to investigate therapeutic effects of cobalt in chronic hypoxia in progressive renal disease are currently ongoing.

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