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Induction of protective genes by cobalt ameliorates tubulointerstitial injury in the progressive Thy1 nephritis

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Induction of renoprotective genes by cobalt ameliorates tubulointerstitial injury in the progressive Thy1 nephritis model.

Background. We previously demonstrated that chronic hypoxia has pivotal roles in the progression of tubulointerstitial injury from the early stage of the uninephrectomized Thy1 nephritis model. We have also shown that pretreatment of cobalt confers renoprotection in the ischemia/reperfusion (I/R) injury, in association with the up-regulation of hypoxia-inducible factor (HIF)–regulated genes. Here, we tested the hypothesis that cobalt administration not only attenuates acute ischemic insult, but also ameliorates tubulointerstitial injury secondary to chronic hypoxia.

Methods. We applied sustained cobalt treatment to the uninephrectomized Thy1 nephritis model at 3 to 5 weeks, when tubular hypoxia appeared. Histologic evaluation, including glomerular and peritubular capillary networks, was made at 8 weeks. HIF activation was confirmed by real-time polymerase chain reaction (PCR) analyses for HIF-regulated genes, such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), and heme oxygenase 1 (HO-1). Up-regulation of HIF-1α and HIF-regulated genes was also verified by Western blotting analysis. To elucidate responsible mechanisms of cobalt in the amelioration of tubuloniterstitial injury, terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) staining was conducted at 5 weeks. A combination therapy with angiotensin receptor blocker (ARB), olmesartan, was also challenged.

Results. Although the intervention did not change glomerular structural damage or urinary protein excretion rate, tubulointerstitial injury was improved in cobalt-treated animals when compared with the vehicle-treated group. The amelioration was associated with the parallel up-regulation of renoprotective, HIF-regulated gene expression. TUNEL staining revealed that the number of apoptotic cells was reduced in the cortex by cobalt administration, suggesting that renoprotection was achieved partly through its antiapoptotic properties. Furthermore, it was demonstrated that cobalt treatment exerts additional renoprotective effects with the ARB treatment in this model.

Conclusion. Maneuvers to activate HIF in the ischemic tubulointerstitium will be a new direction to future therapeutic strategies.

Key words: hypoxia, cobalt, hypoxia-inducible factor (HIF), apoptosis.

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Recent reports emphasize chronic hypoxia in the tubulointerstitium as a mechanism of progression in a number of renal diseases [1–6]. Histologic studies of human kidneys and animal models have shown that extensive tubulointerstitial injury is associated with loss of peritubular capillaries, implying that chronic hypoxia due to loss of micovasculature plays pivotal roles in the progression of tubulointerstitial injury [7–10].

In addition to peritubular capillary loss, chronic hypoxia in the tubulointerstitium can occur via several mechanisms. Previous studies from our group demonstrated a decrease in blood flow of peritubular capillaries, and hypoxia thereof, prior to the development of structural tubular and capillary damage in two distinct glomerulopathies. In the remnant kidney, hypoxia was associated with the narrowing of peritubular capillaries and treatment of angiotensin receptor blocker (ARB) restored blood flow with improvement of subsequent oxygenation in the kidney [11]. These findings suggested that changes in peritubular capillaries may be secondary to outflow constriction of the glomerular capillary bed by angiotensin II (Ang II). We also observed tubulointerstitial hypoxia alongside with decreased capillary blood flow in a model of accelerated glomerulosclerosis induced by repeated injections of anti-Thy1 antibody in uninephrectomized rats [12]. These findings indicated that blood flow of peritubular capillaries can be affected by the glomerular capillary networks and efferent arterioles, and the stagnant peritubular perfusion in turn leads to tubulointerstitial hypoxia in the early-phase of glomerulopathies, thus establishing a more solid basis on the role of hypoxia in the progression of renal diseases.

Given that renal hypoxia has pivotal roles not only on the progression to renal failure but on the early development of tubulointerstitial injury, chronic hypoxia could be a valid therapeutic target for renal diseases. To this end,

an endogenous mechanism to cope with hypoxia was investigated. Hypoxia-inducible factor (HIF), composed of α and β subunits, is a master regulator of genes activated by low oxygen tension. In normoxia, the HIF-α subunit is ubiquitinated and degraded by proteasomes. The oxygen-sensing pathway involves the oxygen-dependent prolyl hydroxylation of HIF-1α by prolyl-4-hydroxylase [13, 14], which serves as a signal for polyubiquitination and proteasomal degradation [15, 16]. And cobalt is known to cause accumulation of HIF-α protein within the cells and induce HIF-mediated transcription by inhibiting prolyl hydroxylation of the α subunit [17].

We recently demonstrated renoprotective effects of cobalt in the ischemic acute renal failure [18]. Administration of cobalt led to the induction of HIF-regulated genes, such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO), and improved tubulointerstitial damage in this model. Substantial in the acute ischemic injury, however, it remained to be elucidated whether it could also be applied to chronic, progressive disease models in which hypoxia takes on certain roles. Here, we tested whether cobalt attenuates tubulointerstitial injury secondary to chronic hypoxia, using the uninephrectomized Thy1 nephritis model. Our findings indicated that activation of HIF by cobalt exerts renoprotective roles in the ischemic tubulointerstitium, partly via suppression of apoptosis, while the density of peritubular capillaries was unaffected. Furthermore, this method had additive, positive effects to the standard antiproteinuric therapy, namely, the blockade of the renin-angiotensin system (RAS) in the hypoxic tubulointerstitium. Combination therapy suggested a potential synergistic renoprotection in this disease model.

METHODS

Experimental protocol

All experiments were conducted in accordance with the Guide for Animal Experimentation, at the Faculty of Medicine, University of Tokyo, Japan. Six-week-old male Srague-Dawley rats (Nippon Seibutsu Zairyo Center Co., Ltd., Saitama, Japan) weighing 160 to 200 g received repeated intravenous injections of IgG (OX-7) mouse monoclonal anti-Thy1.1 antibody (1.2 mg/kg body weight) ($N = 32$) or vehicle (control) ($N = 8$), at 1 and 2 weeks (week −1 and week 0) after right nephrectomy (week −2). Rats were housed in metabolic cages for overnight collection of urine, and blood samples were obtained via tail vein for the evaluation of renal functions. After collection of urine at 1 week, nephrectomized, Thy1-injected rats were administrated with cobalt chloride (Thy1 cobalt group 2.7 mg/kg, subcutaneously, once every 3 days) ($N = 17$) or vehicle (Thy1 group) ($N = 15$) for 3 weeks. The starting time point of cobalt treatment was determined based on our previous findings that tubulointerstitial hypoxia was evident at this time point [12]. The dose and period of cobalt treatment were determined to minimize the loss of body weight, according to preliminary studies. At the end of cobalt administration, 5 weeks, kidneys were obtained for reverse transcription-polymerase chain reaction (RT-PCR) analysis and terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) staining (control) ($N = 2$), Thy1 ($N = 7$), and Thy1 cobalt ($N = 9$). Remaining animals were sacrificed at 8 weeks for tissue analysis.

A second set of experiments were conducted to see if cobalt treatment exhibits additive effects to the ARB treatment. Additional rats were assigned to either (*1*) Thy1 ($N = 4$), (*2*) Thy1 + ARB ($N = 7$), or (*3*) Thy1 + ARB + cobalt ($N = 7$) groups. For the ARB treatment groups, 10 mg/kg body weight of olmesartan (Pharmacology and Molecular Biology Research Laboratories, Sankyo Pharmaceuticals, Tokyo, Japan) was administered via gastric gavage (1 mg/mL in 0.5% sodium carboxymethylcellulose) at 2 to 5 weeks, 5 times a week.

In a third set of experiments, the relative contributory role of VEGF on cobalt-mediated renoprotection was investigated using anti-VEGF neutralizing antibody, as previously reported [19]. Rats were administered with anti-VEGF neutralizing antibody (4 μg/head intraperitoneally, 12 hours prior to injection of cobalt) (R&D Systems, Minneapolis, MN, USA) or control IgG, during weeks 2 through 5 ($N = 6$ in each group). These rats were euthanized for evaluation at weeks 5 and 8. Inhibition of circulating VEGF in control rats led to minimal pathologic effects [20] (not shown).

Renal histologic analyses

Tissues were fixed in methyl-Carnoy's solution and paraffin-embedded. Three micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. An indirect immunoperoxidase method was used to identify the following antigens; monocytes/macrophages with murine monoclonal IgG1 antibody endothelin-1 (ED-1) (Chemicon, Temecula, CA, USA); vimentin with murine monoclonal IgG antibody V9 (Dako, Carpinteria, CA, USA); and aminopeptidase P of microvascular endothelial cells with murine monoclonal IgG1 antibody JG-12 [21] (Bender MedSystems, San Bruno, CA, USA). Formalin-fixed and paraffin-embedded tissue was sectioned at 6 μm, for the assessment of TUNEL staining, using a commercial apoptosis detection kit (TACS2 TdT-Blue Label In Situ Apoptosis Detection Kit) (Trevigen, Gaithersburg, MD, USA).

Semiquantitative analyses of renal histologic feature

Quantification was performed in a blinded manner using 30 randomly selected glomeruli or more than 15 randomly selected fields of cortex per cross-section.

Glomerulosclerosis, defined as synechiae formation by PAS staining with focal or global obliteration of capillary loops, was graded as follows: 0, normal; 1, 0% to 25% of glomerular area was affected; 2, 25% to 50% affected; 3, 50% to 75% affected; and 4, 75% to 100% affected [22]. Tubulointerstitial injury was graded (0 to 5+) on the basis of the percentage of tubular cellularity, basement membrane thickening, cell infiltration, dilatation, atrophy, sloughing, or interstitial widening as follows: 0, no change; 1, <10% tubulointerstitial injury; 2, 10% to 25%; 3, 25% to 50%; 4, 50% to 75%; and 5, 75% to 100% [23, 24]. Tubules that were vimentin-positive or were surrounded by vimentin-positive cells, ED-1–positive cells and apoptotic (TUNEL-positive) cells were counted in 20 randomly selected cortical fields with ×20 objective.

Semiquantitative analyses of glomerular or peritubular capillary loss

Glomerular or peritubular capillary loss was assessed by immunostaining for renal microvascular endothelium with JG-12 antibody. Loss of glomerular capillary loops was graded as follows: 0, no negative glomerular tuft staining for endothelium; 1, 1% to 25% of glomerular tuft negative for endothelium; 2, 25% to 50% negative; 3, 50% to 75% negative; and 4, 75% to 100% negative [25]. Peritubular capillary loss was analyzed using rarefaction index, which is percentage area with no capillaries identified with JG-12 antibody and represents the sparseness of peritubular capillaries, as reported previously [7]. 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 10 nonoverlapping sequential fields, at ×200 magnification. The minimal possible capillary rarefaction index is 0 (i.e., every square in the grid contains a JG-12–positive peritubular capillary), whereas the maximal score is 100 (i.e., JG-12–positive peritubular capillaries are absent from every square in the grid).

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, USA). Renal mRNA levels were assessed by real-time quantitative PCR using SYBR Green PCR reagent (Qiagen, Hilden, Germany) and iCycler PCR system (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. Briefly, amplification reactions consisted of 1 μL of cDNA, 12.5 μL of the Universal 2× PCR MasterMix (Qiagen), and 5 μL each of the specific primers. Primer concentrations in the final volume of 25 μL were 500 nmol/L. In control experiments with triplicates, no false positives were detected and the variance between each of the replicates was within 5%. All PCR reactions were performed in triplicate. C_t, or threshold cycle, was used for relative quantification of the input target number. The amount of C_t for control samples was considered 1 (i.e., 2°). The number of C_ts for other samples were subtracted by cycles of control samples and recorded as ΔC_t. The relative amount of amplified genes is given by $2^{-\Delta Ct}$. The mRNA levels of target genes were normalized to levels of β-actin. PCR primers for heme oxygenase (HO-1), VEGF, and EPO have been described previously [18].

Immunoblotting

The induction of the α subunit of HIF-1 and its regulated genes were verified by Western blotting. Proteins were extracted from renal cortex of either vehicle- or cobalt-treated rats. Aliquots were separated by 7.5% (HIF-1α), 10% [glucose transporter 1 (GLUT1)] or 15% VEGF sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred onto polyvinylidine difluoride (PVDF) membranes (Millipore, Tokyo, Japan). Specific bands were detected with anti-HIF-1α (Novus Biologicals, Littleton, CO, USA), anti-GLUT1 (Chemicon) and anti-VEGF (Santa-Cruz Biochemistry, Santa Cruz, CA, USA) antibodies followed by incubation with alkaline phosphatase (AP)–conjugated secondary antibodies. Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Sigma Chemical Co., St. Louis, MO, USA) was used as substrate.

Statistical analysis

Data were reported as mean ± SE. Statistical analyses were performed using the *t* test. Nonparametric data were analyzed with the Mann-Whitney test when appropriate. Differences with P values of < 0.05 were considered significant.

RESULTS

Global features

First, the feasibility of cobalt for long-term use was estimated by measuring physical and biochemical parameters. After 3 weeks of cobalt injection to control rats, there were no significant changes in terms of body weight (vehicle, 447 ± 18 g; cobalt, 434 ± 39 g), systolic blood pressure (vehicle, 116 ± 9 mm Hg; cobalt, 114 ± 20 mm Hg), urinary protein excretion (vehicle, 13.1 ± 3.1 mg/day/kg; cobalt, 12.1 ± 2.2 mg/day/kg), and blood urea nitrogen (BUN) levels (vehicle, 17.2 ± 4.9 mg/dL; cobalt, 18.6 ± 0.9 mg/dL).

In the Thy1 and Thy1 cobalt groups, urinary protein excretion at 1 week, that is, before cobalt treatment, was similar (120.1 ± 26.2 mg/day/kg body weight vs. 131.3 ±

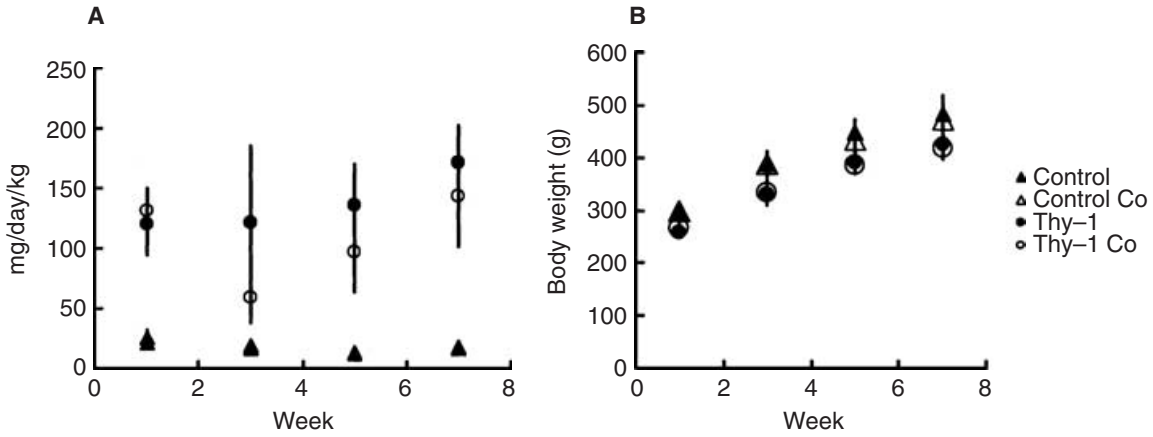

Fig. 1. Temporal profile of renal function and body weight. (*A*) Urinary protein excretion levels in the Thy1 group increased significantly as compared with controls, which were not altered by cobalt administration. (*B*) Cobalt injection did not affect the body weight in both control and Thy1 rats.

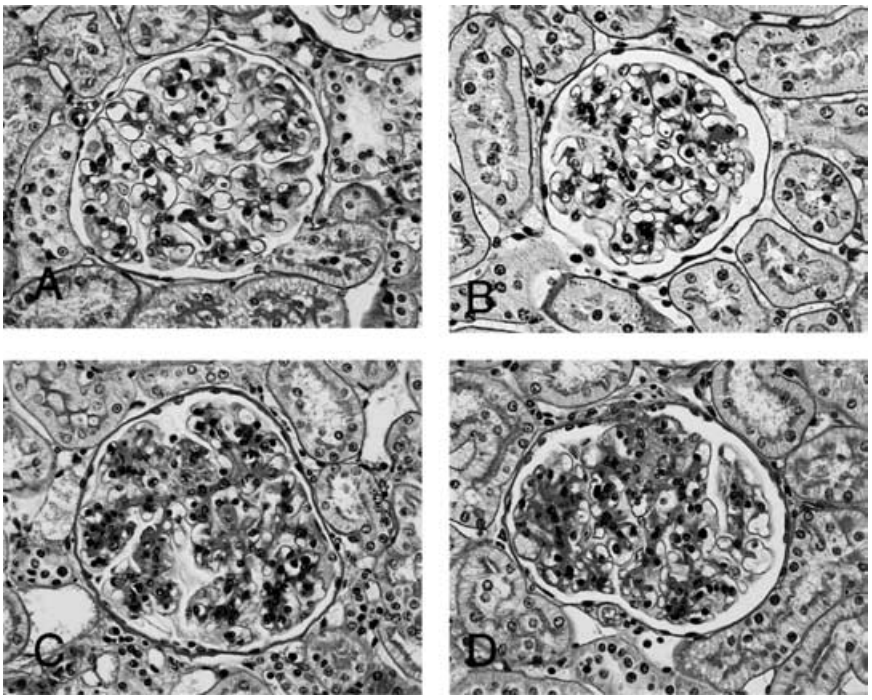

Fig. 2. Glomerular injury was not improved by cobalt administration. In the control and control cobalt groups, glomerular structure remained intact (*A* and *B*). Periodic acid-Schiff (PAS) staining of the Thy1 (*C*) and Thy1 cobalt (*D*) groups showed that glomerular sclerosis, defined by synechiae formation, developed to a similar degree (magnification, ×400).

18.9 mg/day/kg body weight) (P = NS). After the cobalt administration period, urinary protein of both groups increased in a similar manner. Cobalt injection did not affect increases in body weight (Fig. 1).

Cobalt administration did not prevent progression to the irreversible glomerulosclerosis

Glomerular morphology was evaluated to see the potential effect of cobalt on glomerular injury. Glomerular injury, which was defined according to synechiae formation by PAS staining with focal or global obliteration of capillary loops, developed to a similar degree in both Thy1 and Thy1 cobalt groups, at 8weeks (glomerular injury score control, 0.26 ± 0.08; Thy1, 2.69 ± 0.21; and Thy1 cobalt, 2.23 ± 0.22) (Fig. 2). Glomerular capillary loss, defined as the decreased staining with JG-12 antibody, was similarly observed in both groups (control, 0.06 ± 0.03; Thy1, 1.87 ± 0.30; and Thy1 cobalt, 1.92 ± 0.15), suggesting no protective effect of cobalt against glomerular injury in this model.

Cobalt administration improved tubulointerstitial injury in the uninephrectomized Thy1 nephritis model

Previously we demonstrated that chronic hypoxia underlies the progression of tubulointerstitial injury in the

Table 1. Glomerular and tubulointerstitial (TI) injury among study groups

	Glomerular injury		Tubulointerstitial injury	
	Glomerular injury (PAS)	Capillary loss score (JG = 12)	TI injury score (PAS)	Rare faction index (JG = 12)
Control	0.26 ± 0.08	0.06 ± 0.03	0.27 ± 0.08	4.54 ± 0.22
Thy1	2.69 ± 0.21[a]	1.87 ± 0.30[a]	2.28 ± 0.32[a]	11.15 ± 0.55[a]
Thy1 cobalt	2.23 ± 0.22[a]	1.92 ± 0.15[a]	1.33 ± 0.16[a,b]	11.74 ± 0.63[a]

PAS is periodic acid-Schiff.
[a] $P < 0.01$ vs. control.
[b] $P < 0.05$ vs. Thy1 group.

uninephrectomized Thy1 nephritis model, providing the rationale for testing the hypothesis that sustained low-dose cobalt treatment could remit chronic, progressive renal diseases through activation of HIF. For this purpose, we administered cobalt from the end of 1 week up until 5 weeks, when chronic hypoxia was observed in this disease model [12]. Tubulointerstitial injury developed in a diffuse manner in the Thy1 group, which was improved in the Thy1 cobalt group (tubulointerstitial injury score control, 0.27 ± 0.08; Thy1, 2.28 ± 0.32; and Thy1 cobalt, 1.33 ± 0.16; and Thy1 versus Thy1 cobalt $P < 0.05$, data summarized in Table 1). Moreover, expression of vimentin in tubular cells or in cells surrounding tubules was obviously increased in the Thy1 group, which was decreased by cobalt administration (vimentin-positive tubules per field control, 1.81 ± 0.78; Thy1, 22.0 ± 2.53; Thy1 cobalt, 7.73 ± 2.93; and Thy1 versus Thy1 cobalt $P < 0.01$) (Fig. 3). Macrophage infiltration in the tubulointerstitium, as determined by the number of ED-1–positive cells, was also reduced in rats treated with cobalt (ED-1–positive cells per field control, 14.7 ± 2.63; Thy1, 25.6 ± 2.87; Thy1 cobalt, 17.3 ± 2.21; and Thy1 versus Thy1 cobalt $P < 0.05$).

Cobalt chloride–induced expression of renoprotective, HIF-regulated genes

To investigate the possible contribution of renoprotective genes induced by cobalt, we performed real-time quantitative PCR analysis using kidneys obtained after repeated cobalt injections. Relative quantification was based on the difference in C_t of target and control samples ($= \Delta C_t$) in cobalt-treated and control groups, respectively. Although mild increases in HO-1, VEGF, and EPO mRNA were observed in the Thy1 group, cobalt administration induced much higher expression. Fold increases of each mRNA expression in this group were 11.2-, 10.2-, and 11.0-fold, for HO-1, VEGF, and EPO, respectively (Fig. 4A).

The up-regulation of HIF-1α and HIF-regulated genes were also verified at the protein level (Fig. 4B and C). The specific band corresponding to HIF-1α appeared in the cobalt group, while absent in the vehicle-treated group. Similarly, GLUT1 and VEGF, two of the representative target genes of HIF-1, were significantly up-regulated by cobalt, making it more likely that the dosage of cobalt we employed here successfully activated HIF and HIF-regulated genes.

Cobalt administration did not prevent peritubular capillary loss

Influence of cobalt administration on peritubular capillary density was of particular interest, because a considerable increase of VEGF was observed in cobalt-treated kidneys. In the Thy1 group, peritubular capillary density was decreased at 8 weeks as compared with the control group. Cobalt treatment, however, failed to prevent the decline of peritubular capillary density (rarefaction index control, 4.54 ± 0.22; Thy1, 11.15 ± 0.55; and Thy1 cobalt, 11.74 ± 0.63%).

Cobalt reduced the number of TUNEL-positive, apoptotic cells in the tubulointerstitium

To further elucidate mechanisms by which cobalt administration ameliorated tubulointerstitial injury, we conducted TUNEL staining to count the number of apoptotic cells. In the Thy1 group, TUNEL-positive, apoptotic cells increased in tubular epithelium and interstitial compartments at 5 weeks, while significantly decreased by the administration of cobalt (TUNEL-positive cell number per field control, 0.41 ± 0.03; Thy1, 4.78 ± 0.97; and Thy1 cobalt, 2.52 ± 0.58) ($P < 0.01$), implying antiapoptotic properties of this regimen (Fig. 5).

Activation of HIF by cobalt affords synergistic protection with the RAS blockade in the ischemic tubulointerstitium

Looking back on the aforementioned results that cobalt treatment conferred renoprotection in terms of tubulointerstitial injury, we plotted the correlation between proteinuria and tubulointerstitial injury in each individual rat of Thy1 and Thy1 cobalt groups (Fig. 6). In the cobalt-treated group, the y axis of the regression line shifted downward as compared with that of the Thy1 group, indicating the partial remission of tubulointerstitial injury by this method, although the damage was still remaining depending on the degree of proteinuria. In this regard, we conducted a second set of experiments to address whether cobalt works synergistically with the standard antiproteinuric therapy, the blockade of RAS.

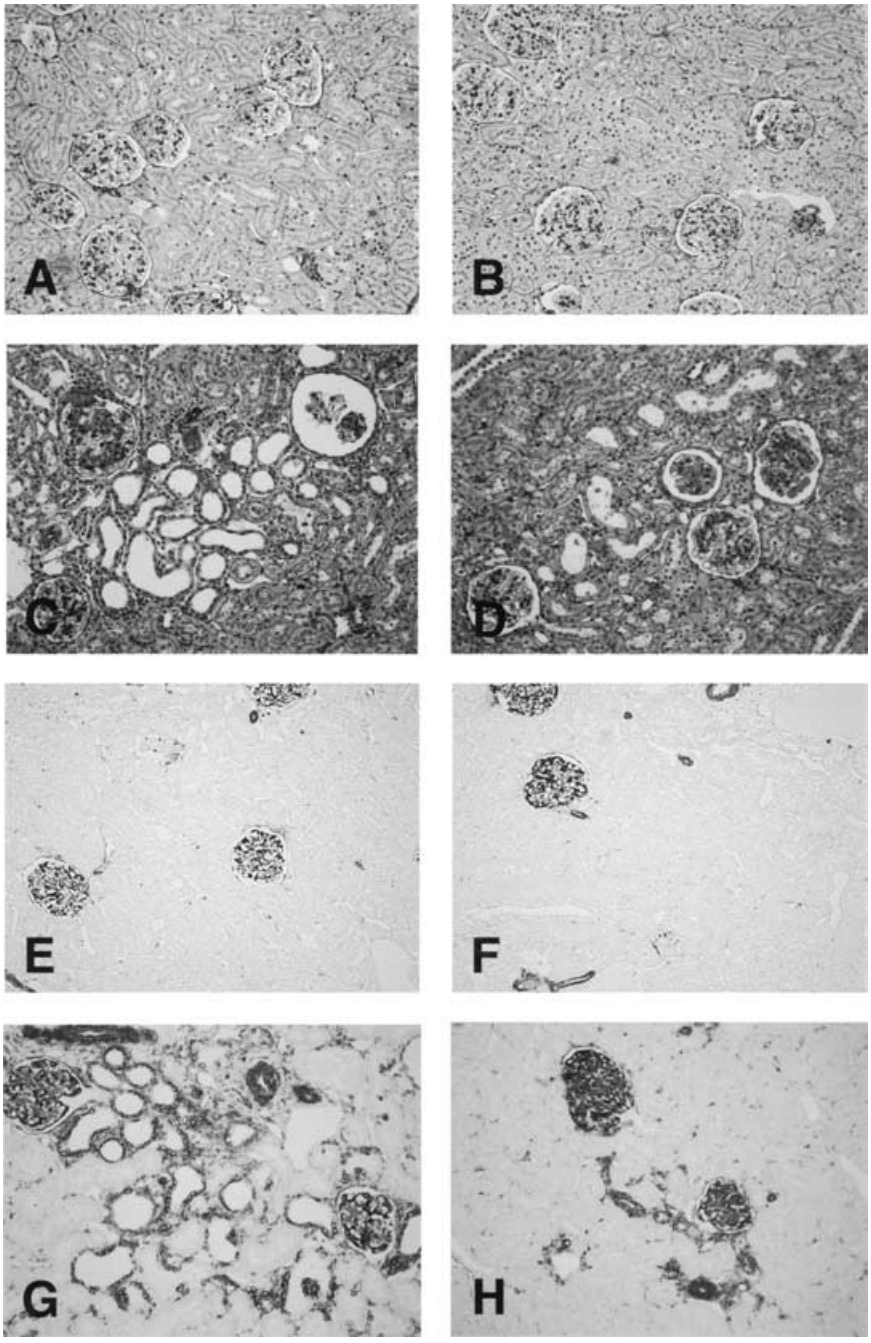

Fig. 3. Effect of cobalt administration on tubulointerstitial injury. Periodic acid-Schiff (PAS) staining revealed significant tubulointerstitial injury at 8 weeks in the uninephrectomized Thy1 nephritis (*C*), which was improved in the Thy1 cobalt group (*D*). Vimentin-positive tubules in the Thy1 group (*G*) were reduced in number in the Thy1 cobalt kidneys (*H*). Cobalt treatment alone did not induce any recognizable morphological changes in control kidneys (*A*) vs. (*B*) and (*E*) vs. (*F*) (magnification, ×200).

Proteinuria was reduced from 175.2 ± 56.8 mg/day/kg to 73.5 ± 27.1 mg/day/kg (Thy1 ARB) and 85.4 ± 37.3 mg/day/kg (Thy1 ARB + cobalt) at the end of administration period of ARB. As summarized in Table 2, a consistent, synergistic protection was observed in the tubulointerstitium, albeit seemingly independent of alterations in microvasculature (tubulointerstitial injury score Thy1, 2.91 ± 0.37; Thy1 ARB, 1.71 ± 0.19; Thy1 ARB + cobalt, 1.38 ± 0.21; and Thy1 ARB versus Thy1 ARB + cobalt $P = 0.02$) (rarefaction index Thy1, 14.16 ± 0.41; Thy1 ARB, 11.91 ± 0.60; and Thy1 ARB + cobalt, 11.98 ± 1.21). Glomerular injuries were again unaffected by cobalt treatment, both in terms of glomerular sclerosis score and of capillary loss score (glomerular injury score Thy1, 2.45 ± 0.11; Thy1 ARB, 1.99 ± 0.21; and Thy1 ARB + cobalt, 1.90 ± 0.23) (glomerular capillary loss score Thy1, 1.90 ± 0.22; Thy1 ARB, 1.39 ± 0.23; and Thy1 ARB + cobalt, 1.52 ± 0.25). Responsible mechanisms underlying synergistic renoportection were investigated by vimentin and TUNEL staining (Fig. 7). Additive, positive effects of cobalt were ascribed to its properties to suppress dedifferentiation and apoptosis of affected

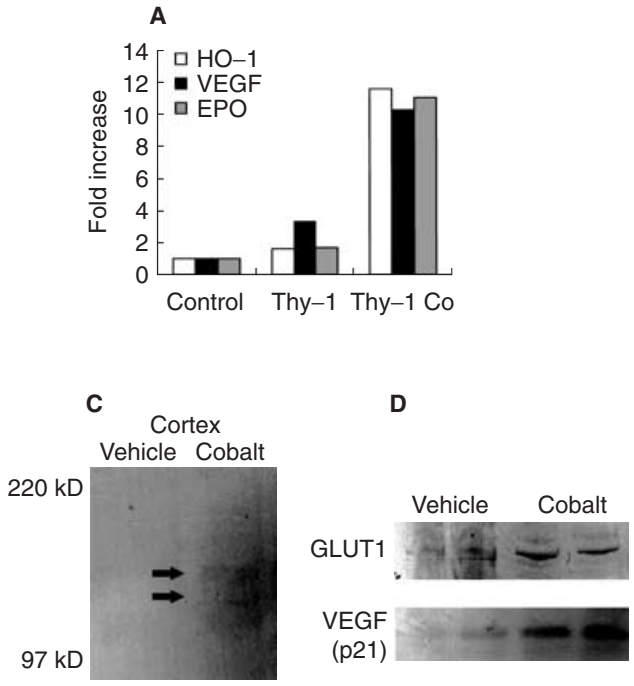

Fig. 4. Cobalt chloride induced the expression of hypoxia-inducible factor (HIF)-associated, renoprotective genes. Expression of mRNA for each gene in the whole kidney of control, Thy1, and Thy1 cobalt groups ($N = 2$, 3, and 3, respectively) at 5weeks (*A*). mRNA expression was determined by real-time quantitative reverse transcription-polymerase chain reaction (RT-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 RT were negative for each gene. Western blotting analysis shows successful up-regulation of HIF-1α (*B*), glucose transporter 1 (GLUT1) and a 21 kD fragment (p21) of vascular endothelial growth factor (VEGF) (*C*), at the protein level. HO-1 is heme oxygenase 1; EPO is erythropoietin.

renal tubules, although the latter did not reach statistical significance (vimentin-positive tubules per field Thy1, 23.0 ± 1.6; Thy1 ARB, 16.4 ± 3.0; and Thy1 ARB + cobalt, 10.4 ± 2.2; and Thy1 ARB versus Thy1 ARB + cobalt, $P < 0.01$) (TUNEL-positive cell number per field Thy1, 4.33 ± 0.71; Thy1 ARB, 2.71 ± 0.62; and Thy1 ARB + cobalt, 2.16 ± 0.51; and Thy1 ARB versus Thy1 ARB + cobalt, $P = 0.09$).

Blockade of circulating VEGF partially offsets the beneficial effect of cobalt

Finally, we attempted to estimate the relative contribution of VEGF in cobalt-mediated renoprotection. Cobalt-treated, uninephrectomized Thy1 rats were administered with either control IgG (Thy1 cobalt + IgG) or anti-VEGF neutralizing antibody (Thy1 cobalt + VEGF neutralizing antibody). The numbers of vimentin-positive tubules and TUNEL-positive cells were moderately increased in the latter group. Similarly, PAS examination at 8 weeks revealed partial, but significant, reversal of cobalt-mediated histologic improvement by this method (Table 3).

A proposed diagram of our current study is summarized in Figure 8. Induction of HIF-regulated genes by cobalt protects ischemic tubulointerstitium in this model, partly by inhibiting tubular cell apoptosis. And, when combined with the antiproteinuric therapy, it more strongly attenuates tubulointerstitial injury.

DISCUSSION

We previously demonstrated that cobalt pretreatment protects kidneys against acute ischemic insult, suggesting beneficial roles of cobalt, and activation of HIF, in protecting tissues from hypoxia [18]. This study was undertaken to address whether this strategy also applies to chronic renal diseases in which hypoxia exists and plays certain roles in their progression. Cobalt administration was started at the end of 1 week, when decline of oxygen tension in cortical tubules occurred in the uninephrectomized Thy1 nephritis model [12].

In vivo activation of HIF was accomplished by administering cobalt to rats. In normoxia, HIF-α protein is quickly degraded through ubiquitin-proteasomal pathways, which is triggered by prolyl hydroxylation, and the enzymatic activity of prolyl hydroxylases depends on iron as the activating metal, 2-oxoglutarate as a cosubstrate and ascorbic acid as a cofactor. Cobalt has been suggested to induce the "hypoxia-mimetic" effect via its ability to substitute for iron [17], and by depleting intracellular ascorbate [26]. Although the mechanisms appear to be multifaceted, cobalt administration to rats led to successful activation of HIF and induction of its associated genes, which we confirmed by real-time PCR and immunoblotting of HIF target genes.

Repeated injections of anti-Thy1 antibody after uninephrectomy directly injured mesangial cells and led to irreversible structural damage in glomeruli [12]. The glomerular lesion and glomerular capillary loss were not improved by cobalt treatment. This was not surprising because primary mediators of glomerular injury in this model are complement-mediated mesangial injury and subsequent release of cytokines and growth factors [27, 28]. Structural glomerular damage may have reached the threshold not to be overcome by the up-regulation of HIF-regulated, renoprotective genes. This would also explain similar degrees of proteinuria observed in the Thy1 and Thy1 cobalt groups.

On the other hand, tubulointerstitial injury was clearly improved by cobalt administration, in association with the induction of HIF-regulated, renoprotective genes such as EPO, HO-1, and VEGF. The first place we looked for was the vascular network of glomerular and peritubular capillaries, because HIF stimulates the expression of a number of target genes essential for angiogenesis. In

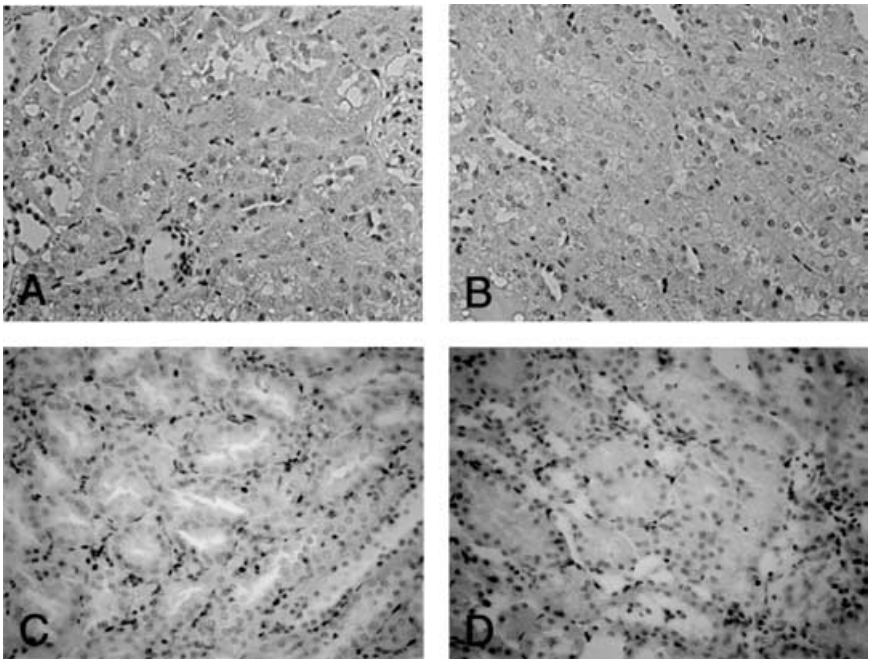

Fig. 5. Detection of apoptosis by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) staining. In the Thy1 group, TUNEL-positive, apoptotic cells increased in tubular epithelium and the interstitial compartment at 5 weeks (*C*), which was decreased in the Thy1 cobalt group (*D*). Minimal positive cells were observed in the control (*A*) and control cobalt (*B*) groups (magnification, ×200).

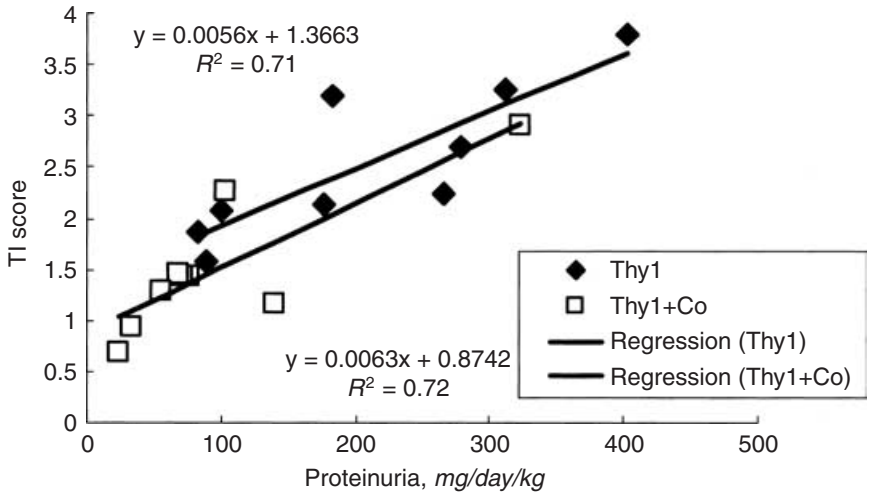

Fig. 6. Correlation between urinary protein excretion and tubulointerstitial injury. Correlations between proteinuria and tubulointerstitial injury were plotted with each individual rat in both Thy1 and Thy1 cobalt groups. The decrease in the y axis in the Thy1 cobalt group indicates that cobalt administration attenuated tubulointerstitial injury, although the injury was remaining substantially, depending on the degree of proteinuria developed in each individual rat. There was a positive correlation between proteinuria and tubulointerstitial injury in both groups (Thy1, $y = 0.0056x + 1.3663$, $R^2 = 0.71$; Thy1 cobalt, $y = 0.0063x + 0.8742$, $R^2 = 0.72$).

Table 2. Administration of cobalt worked synorgistically with the angiotensin receptor blocker (ARB) treatment against ischemic tubulointerstitial (TI) injury

	Glomerular injury		Tubulointerstitial injury	
	Glomerular sclerosis (PAS)	Capillary loss score (JG-12)	TI injury score (PAS)	Rare faction index (JG-12)
Thy1 (vehicle)	2.45 ± 0.11	1.90 ± 0.22	2.91 ± 0.37	14.16 ± 0.41
Thy1 ARB	1.99 ± 0.21[a]	1.39 ± 0.23[a]	1.71 ± 0.19[a]	11.91 ± 0.60[a]
Thy1 ARB + cobalt	0.90 ± 0.23[a]	1.52 ± 0.25[c]	1.38 ± 0.21[a,b]	11.98 ± 1.21[a]

PAS is periodic acid-Schiff.
[a] $P < 0.01$ vs. vehicle.
[b] $P < 0.05$ vs. Thy-ARB group.
[c] $P < 0.05$ vs. vehicle.

addition, evidence has been accumulating that tubulointerstitial injury is associated with the loss of peritubular capillaries, and therapies targeting angiogenesis and the maintenance of microvasculature successfully retards the progression of renal diseases [29–31]. In this study, however, the density of peritubular capillaries was not improved in the Thy1 cobalt group, despite the marked induction of VEGF mRNA and protein by this method.

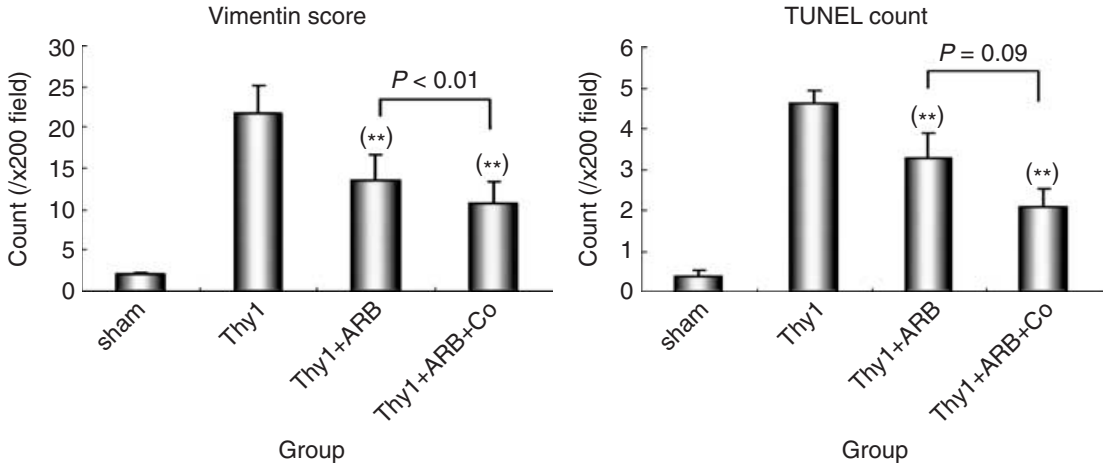

Fig. 7. Cobalt treatment protected renal tubules from dedifferentiation and apoptosis synergistically with ARB. Administration of cobalt in combination with angiotensin receptor blocker (ARB) synergistically reduced the number of vimentin-positive tubular cells (*A*) and terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL)-positive, apoptotic cells (*B*). The numbers of vimentin-/TUNEL- positive cells decreased from 23.03 ± 0.63/4.33 ± 0.71 to 16.41 ± 3.04/2.71 ± 0.61 by ARB treatment ($P < 0.01$ each), with further reduction to 10.42 ± 2.17/2.16 ± 0.51 by the additional cobalt treatment ($P < 0.01$ and $P = 0.09$, respectively) [counting per ×200 field $N = 5$ (Thy1), $N = 7$ (Thy1 ARB), $N = 7$ (Thy1 ARB + cobalt). $^{**}P < 0.01$ vs. Thy1 group].

Table 3. Effect of anti-vascular endothelial growth factor (VEGF) nutralizing antibody in the cobalt-treated rats

	Tubulointerstitial score (PAS)	Vimentin-positive tubules (per field)	TUNEL-positive cells (per field)
Thy1 (vehicle)	2.25 ± 0.15	18.6 ± 1.76	4.60 ± 0.57
Thy1 cobalt + IgG	1.13 ± 0.10[a]	6.53 ± 0.48[b]	2.87 ± 0.86[b]
Thy1 cobalt + VEGF nutralizing antibody	1.70 ± 0.13[b,c]	13.5 ± 2.83	3.76 ± 0.70
P value vs. control IgG	($P = 0.01$)	($P = 0.09$)	($P = 0.07$)

Abbreviations are: PAS, Periodic acid–Schiff; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling.
[a] $P < 0.01$ vs. Thy1.
[b] $P < 0.05$ vs. Thy1.
[c] $P < 0.05$ vs. Thy cobalt + IgG group.

In an effort to elucidate other mechanisms likely responsible for the amelioration of tubulointerstitial injury by cobalt, we carried out TUNEL staining and clarified that the number of apoptotic cells was significantly reduced by cobalt. These results confirmed and extended previous observations that apoptosis plays an important role in hypoxia-mediated kidney injury [32–35].

EPO, one of the up-regulated genes in the current study, has been emphasized to exhibit antiapoptotic effects through activation of signaling pathways downstream of the EPO receptor in various tissues, including brain, spinal cord, retina, testis, and the kidney. In vivo, therapeutically applied EPO has been shown to reduce the infarct volume in various stroke animal models, to prevent retinal degeneration, and to ameliorate spinal cord injury [36]. Parsa et al [37] reported that EPO treatment improved cardiac function following myocardial infarction, which was associated with mitigation of myocyte apoptosis. In the kidney, exogenous administration of EPO has been shown to protect tubular cells from injury in the ischemia/reperfusion (I/R) model [38, 39]. Although we have not investigated responsible mechanisms for the reduced number of apoptosis, local EPO production might have contributed to the alleviation of tubulointerstitial injury by cobalt treatment.

HO-1, an enzyme responsible for catalysis of heme to iron, carbon monoxide (CO), biliverdin, and bilirubin, has been shown to inhibit apoptosis in various cell types such as endothelial cells, fibroblasts, and neuronal cells [40–42]. In kidneys, recent studies have suggested that HO-1 confers resistance to apoptosis and exerts renoprotective roles [43, 44]. We speculate that similar mechanisms also participate in the histologic improvement observed in this study.

In the current study, contribution of VEGF to the maintenance of microvasculature was not observed. Nonetheless, it should be emphasized that the blockade of circulating VEGF in cobalt-treated rats resulted in partial reversal of histologic improvement, as determined by TUNEL, vimentin, and PAS staining. This is important for the following reasons. First, in spite of several lines of studies showing positive effects of VEGF, the role of it in nephrology in general is still under debate and needs to be verified according to each pathologic context. Second,

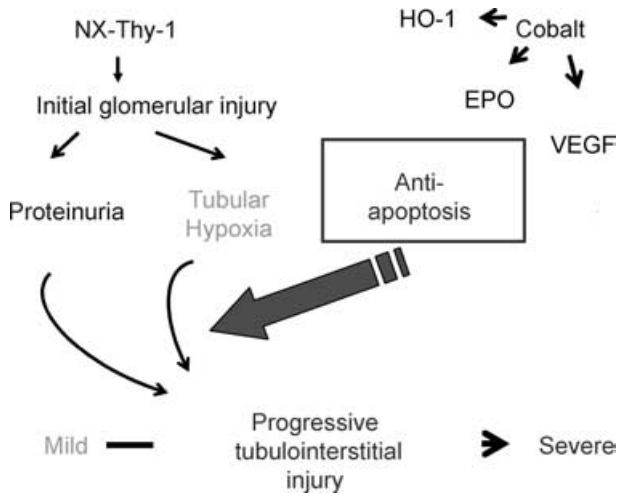

Fig. 8. Proposed diagram of our study. A summarized diagram out of this study is shown. Cobalt stimulates hypoxia-inducible factor (HIF)-regulated gene expression and protects ischemic tubulointerstitium in this model, partly via suppression of tubular cell apoptosis. By blocking proteinuria simultaneously, this strategy exerts more effective renoprotection (see text for details). Abbreviations are: NX, nephrectomy; HO-1, heme oxygenase 1; EPO, erythropoietin; VEGF, vascular endothelial growth factor.

blocking of VEGF alone does not lead to the complete reversal of HIF-mediated renoprotection. Indisputably VEGF played significant roles in this disease model, yet other factors induced by HIF must have contributed to the histologic improvement.

Regarding specific properties of VEGF, there remains a possibility that the induced VEGF prevented apoptosis of peritubular capillary endothelial cells, because TUNEL-positive, apoptotic cells were observed not only in tubular epithelium but also in the interstitial compartment. Previous studies also suggested a role of VEGF as a survival factor in tubular cells [45]. Another possibility is left open that VEGF expression by this method fails to protect renal vasculature in this model, but exerts angiogenic properties in other disease models where hypoxia takes part. Further work will be needed to completely clarify this issue.

A second set of experiments were carried out to investigate whether cobalt administration exhibits synergistic renoprotective effects with the ARB treatment in the ischemic tubulointerstitium. Despite lots of exertions to eradicate the progression of glomerular diseases, the renoprotective role of any single regimen had been partial, if any, and even the current gold standard therapy, the blockade of RAS, has failed to halt the progression of this disease model completely [46, 47]. Therefore, a new antidote had been awaited that targets any aspect modulating the disease progression. And our new method of activating HIF apparently served as an additive factor to protect the kidney from the ischemic insult. This would signify a novel approach against progressive ischemic renal diseases and provide a rationale for developing new drugs that activate HIF, such as inhibitors of prolyl hydroxylases.

The local action of Ang II can be either hemodynamic or nonhemodynamic. On the one hand, it is known to raise both systemic and glomerular/peritubular capillary pressures, through disturbances of afferent and efferent vascular tones [48, 49]. In the Thy1 nephritis model, it has been established further that local Ang II promotes proliferation of mesangial cells and stimulates the production of proinflammatory and fibrogenic growth factors [46, 50, 51] such as transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF), which increase the production of extracellular matrix (ECM) and retard the ECM degradation, thus accumulating the ECM component in the affected tubulointerstitium. Tubules surrounded by ECM proteins are exposed to further impairment of tissue oxygenation and in turn will be a secondary site of releasing inflammatory cytokines and growth factors [4]. There is no known evidence, however, that TGF-β released by hypoxic tubular cells is mediated by HIF-1. These molecular mechanisms would also justify our therapeutic strategies to combine HIF-activation with the RAS blockade. Once again, no data have been available suggesting that the administration of angiotensin-converting enzyme (ACE) inhibitors or ARB alone can halt the disease progression completely in the Thy1 model [46, 47] or in human nephropathies [52]. These current limitations clearly highlight the significance to seek for new antidotes that can be combined with the RAS blockade and act through distinct mechanisms.

The present study must be interpreted within the context of certain limitations. First, we focused on only a few of the HIF-regulated genes, so it cannot be concluded that all genes activated by HIF are renoprotective. Indeed, some are apparently positive factors, as in the case of VEGF, EPO, and HO-1, but others may be at times detrimental. For example, a possibility has been suggested that connective tissue growth factor (CTGF), a candidate mediator of tubulointerstitial fibrosis [53], is induced by hypoxia in a HIF-1–dependent manner [54]. Obviously, we need to take both sides into consideration and details downstream of HIF deserve further analysis. Second, the administration period of cobalt was only 3 weeks, with subsequent follow-up for up to 8 weeks, precluding the feasibility of HIF-activation for longer periods of time. Third, cobalt is toxic to the organic body. Although we failed to observe toxic effects in terms of body weight, urinary protein excretion, blood pressure and BUN levels, it is obvious that cobalt per se cannot be applied in treating renal disorders. A new pharmacologic approach has been awaited that successfully activates HIF with minimal toxic effects.

CONCLUSION

We demonstrated that cobalt administration activates HIF-regulated genes and protects hypoxic tubulointerstitium from injury in the uninephrectomized Thy1 nephritis model. Responsible mechanisms included inhibition of tubular cell apoptosis and this strategy exhibited synergistic, renoprotective effects with the antiproteinuric therapy. To our knowledge, this is the first study to address the therapeutic, not preventive, approach against chronic renal diseases. These findings imply that multifactorial mechanisms could be utilized as a valid target for progressive renal diseases.

NOTE ADDED IN PROOF

After the manuscript was submitted for publication, we uncovered the angiogenic properties of cobalt in the rat remnant-kidney model, which we reported separately (Tanaka T et al, *Lab Invest* 85:1292–1307, 2005).

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