Original Article

Cobalt ameliorates renal injury in an obese, hypertensive type 2 diabetes rat model

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Abstract

Background. Chronic renal hypoxia is suspected to play a pathogenic role in the genesis of diabetic nephropathy (DN). Cobalt enhances the activity of the hypoxia-inducible factor (HIF), a key factor in the defence against hypoxia. Its long-term effect on DN is evaluated.

Methods. Cobalt chloride was given to hypertensive, type 2 diabetic rats with nephropathy (SHR/NDmcr-cp). Treatment was initiated at the age of 13 weeks and continued for 26 weeks.

Results. Cobalt did not correct hypertension and metabolic abnormalities (obesity, hyperglycaemia and hyperlipidaemia) but reduced proteinuria as well as histological kidney injury. Cobalt upregulated renal HIF-1alpha and HIF-2alpha expression and increased the expression of HIF-regulated genes, including erythropoietin, vascular endothelial growth factor and heme oxygenase-1. The renal expression of transforming growth factor (TGF)-beta and connective tissue growth factor (CTGF) was significantly reduced by cobalt. The renal expression of NADPH oxidase, a marker of oxidative stress, and the renal content of pentosidine, a marker of advanced glycation, were also significantly reduced by cobalt.

Conclusions. Cobalt achieved renal protection independently of metabolic status and blood pressure. Its effect was attributed to the upregulation of HIF and HIF-regulated genes and to a mitigated advanced glycation and oxidative stress.

Keywords: chronic hypoxia; cobalt chloride; diabetic nephropathy; hypoxia inducible factor; oxidative stress

Introduction

Prevention or retardation of diabetic nephropathy (DN), now the most common cause of end-stage renal failure in developed countries, is an intensively investigated topic. The close correlation observed between tubulointerstitial damage and renal function in DN [1] has focused attention on the determinants of tubulointerstitial damage. The sensitivity of the kidney to changes in oxygen delivery suggests that hypoxia might be such a determinant. Recent studies have indeed incriminated chronic hypoxia as a possible final common pathway in end-stage kidney injury [2].

Several lines of evidence point to an early hypoxia in diabetic kidneys. Studies utilizing blood oxygen level dependent (BOLD)-MRI revealed that kidneys of streptozotocin-induced diabetic rats are hypoxic even at an early stage [3]. Sophisticated electromicrode studies by Palm et al. also demonstrated hypoxia in diabetic kidneys [4]. In a hypertensive, type 2 DN rat model (SHR/NDmcr-cp), we documented renal hypoxia by the accumulation of pimonidazole, a compound incorporated into hypoxic cells [5]. In human type 2 diabetic patients, a study of intrarenal haemodynamics showed a correlation between a decreased peritubular capillary flow and tubular dysfunction, supporting a pathogenic role of chronic hypoxia of the diabetic kidney [6].

The hypoxia-inducible factor (HIF), a heterodimeric nuclear factor, is a crucial intermediate in the defence mechanisms against hypoxia. Its activation might offer a promising approach to the protection of hypoxic tissues, as it induces a broad and coordinated downstream reaction. HIF is composed of two subunits, an oxygen-sensitive HIF-alpha subunit and a constitutively expressed HIF-beta subunit (also known as ARNT, the aryl hydrocarbon receptor nuclear translocator). Its stability is drastically reduced by the oxygen-dependent hydroxylation of proline residues within the HIF proteins by prolyl hydroxylases (PHDs). Hydroxylated HIF recruits the von Hippel Lindau tumour suppressor protein (pVHL), which in turn tags HIF with ubiquitin groups and targets it for degradation.
within the proteasome. Under hypoxic conditions, HIF is not hydroxylated but transactivates in the nucleus a host of genes involved in the adaptation to hypoxia [7]. Interestingly, cobalt inhibits HIF degradation by PHDs, thus enhancing HIF activity [8].

In the present study, we investigated whether long-term stimulation of HIF with cobalt improves DN in a rat model of type 2 diabetes and nephropathy [5,9].

Materials and methods

Animals

Animal experiments were performed in accordance with the guidelines of the Committee on Ethical Animal Care and Use of Tokai University. Male spontaneously hypertensive/NIH-corpulent rats (SHR/NDmcr-cp) were purchased from SLC (Shizuoka, Japan). All rats were housed in individual cages in a temperature- and light-controlled environment with an accredited animal care. SHR/NDmcr-cp rats, aged 13 weeks, were randomly divided into two groups: rats on tap water (DM, n = 10) and rats on water containing 2 mM cobalt chloride (DM + cobalt, n = 10). Drug treatment lasted for 26 weeks.

Systolic blood pressure was determined in conscious rats by the tail-cuff method at the beginning of the study and every 8 weeks subsequently until euthanasia. Rats were housed in metabolic cages for overnight collection of urine, every 8 weeks subsequently until euthanasia. Rats were also housed in individual cages in a temperature- and light-controlled environment with an accredited animal care. SHR/NDmcr-cp rats, aged 13 weeks, were randomly divided into two groups: rats on tap water (DM, n = 10) and rats on water containing 2 mM cobalt chloride (DM + cobalt, n = 10). Drug treatment lasted for 26 weeks.

Blood pressure and urine and blood biochemistry

Systolic blood pressure was determined in conscious rats by the tail-cuff method at the beginning of the study and every 8 weeks subsequently until euthanasia. Rats were housed in metabolic cages for overnight collection of urine, and blood samples were obtained at the beginning of the study and every 8 weeks thereafter. Triglyceride and glucose concentrations in plasma and protein concentrations in urine were determined with an automatic analyser (Hitachi Automatic Clinical Analyzer 7170, Hitachi Science in Tokyo, Japan), and the percentage of total was calculated.

Semiquantitative analysis of HIF-1 expression

Kidney tissues (4-µm thickness) fixed in 10% neutral buffered formaldehyde were stained with monoclonal IgG hypoxia inducible factor (HIF)-1alpha antibody 67 (Novus Biologicals) or anti-HIF-2alpha antibody (1:1000, Novus Biologicals), followed by incubation with alkaline phosphatase (AP)-conjugated secondary antibody. Ni-trochrome tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Sigma-Aldrich, Inc., St. Lois, MO) was used as substrate.

Semiquantititative analysis of peritubular capillary loss

Peritubular capillary loss was assessed by immunostaining for renal microvascular endothelium with JG12 antibody (Bender MedSystems, Vienna, Austria). Peritubular capillary loss was analysed using rarefaction index [10]. Each square within the grid that did not contain JG12-positive cells was scored at 400x. The minimal possible capillary rarefaction index is 0 (i.e. every square in the grid contains a JG12-positive peritubular capillary), whereas the maximal score is 100 (i.e. JG12-positive peritubular capillaries are absent from every square in the grid).

Gene expression analysis

Real-time PCR was performed to evaluate the mRNA expression of transforming growth factor (TGF)-beta, connective tissue growth factor (CTGF), erythropoietin (EPO), vascular endothelial growth factor (VEGF), heme oxygenase-1 (HO-1) and NOS2 in the kidney of each group of rats. Total RNA was extracted from kidney homogenates using RNeasy mini kit (Qiagen GmbH, Hilden, Germany). Real-time PCR was performed with One Step RT-PCR Kit (Takara Bio Inc., Shiga, Japan), SYBR Green I reagent (Cambrex Bio Science, Rockland, Maine) and iCycler PCR system (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Primer sequences are listed as follows: EPO (forward 5'-TACCGTCCAGATACCAA-A-GTCAC-3', reverse 5'-GACACAGGCTTGCAAAC-TTC-3'), VEGF...
Fig. 1. Metabolic abnormalities. Body weight at the end of the study (A), and changes of blood glucose (B), triglycerides (C) and systolic blood pressure (D). SHR/NDmcr-cp rats on tap water (DM: closed square) or on water containing 0.2 mM cobalt chloride (DM + cobalt: open square). Cobalt treatment did not affect these parameters.

(foward 5′-TGGACCCTGGCTTTACT-GCTG-3′, reverse 5′-GGCAATAGCTGCCTGTAAGA-3′), HO-1 (forward 5′-AGGTGCACATCGTGAGA-3′, reverse 5′-TCCAGGGCCGTAGATATGGTAC-3′), TGF-beta (forward 5′-TGCAGGGTCACAGTGTTGCTA-3′, reverse 5′-AGGTAAAGCCAGGATTTGGTTGCTA-3′), CTGF (forward 5′-CACCAGGGTTACCAATGACAGA-3′, reverse 5′-AGCCCGGTAGAGTCTCCACACGTAAGA-3′), Nox2 (forward 5′-CGGGACTTCGGACCCATATTC-3′, reverse 5′-ATTCCTGTGATGCCAGCCAAC-3′), and beta-actin (forward 5′-TAGCCACAAATCCTACAC-3′). Beta-actin mRNA expression was used for normalization. The mRNA expressions are presented as the relative values from the untreated SHR/NDmcr-cp rats (DM).

Results

Metabolic and physical characteristics of the animals

SHR/NDmcr-cp rats exhibited metabolic abnormalities derived from hyperphagia due to a genetic mutation in the leptin receptor gene (i.e. obesity, hyperglycaemia and hyperlipidaemia). They were also hypertensive as part of their genetic background (SHR). Cobalt chloride, given to SHR/NDmcr-cp rats for 26 weeks, failed to modify these parameters (Figure 1).

Renal involvements

SHR/NDmcr-cp rats developed progressive diabetic nephropathy with severe proteinuria (Figure 2A) and histological abnormalities (Figure 2B and C).

At the end of the study, urinary protein excretion was significantly lower in cobalt-treated than in control SHD/NDmcr-cp rats (Figure 2A). In parallel with the reduction of proteinuria, cobalt improved both glomerular sclerosis and tubulointerstitial fibrosis. The glomerular score and the percentage of fibrosis in the interstitium were significantly reduced by cobalt (Figure 2B and C, P < 0.01).

Representative PAS- or MT-stained pictures of each animal group are shown in Figure 2D–G. They illustrate the benefits of cobalt on the glomerular (focal and segmental glomerular sclerosis, mesangial expansion and thickening of basement membrane) and tubulointerstitial (inflammatory cell infiltration and tubulointerstitial fibrosis) lesions.

Pentosidine measurement

The pentosidine content of the kidney was measured. Kidney tissue (100 mg) was minced, rinsed with 10% TCA, dried under vacuum and acid hydrolyzed in 500 µl of 6 N HCl for 16 h at 110 °C under nitrogen. Pentosidine was analysed on a reverse-phase HPLC as described previously [5,9].

Statistical analysis

All data are expressed as the mean ± SE. Comparisons between two groups were performed using an unpaired t-test. P < 0.05 was considered significantly different.
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Fig. 2. Renoprotective effects of cobalt. Urinary protein excretion (A) and histological evaluation of glomerular sclerosis (B) and tubulointerstitial fibrosis (C) at the end of the study. Representative pictures of PAS-stained (D: DM, E: DM + cobalt) and MT-stained (F: DM, G: DM + cobalt) renal tissues. Cobalt significantly reduced proteinuria and improved glomerular sclerosis and tubulointerstitial fibrosis. *P < 0.05, **P < 0.01 versus DM. Original magnification ×400 (D and E) and ×200 (F and G).

Table 1. Immunohistological analyses

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<th>DM</th>
<th>DM + cobalt</th>
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<td>HIF-1alpha positive tubular cells (cell number/high power field)</td>
<td>8 ± 1</td>
<td>20 ± 2**</td>
</tr>
<tr>
<td>Rarefaction index</td>
<td>10.1 ± 0.6</td>
<td>7.0 ± 0.2**</td>
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**P < 0.01 versus DM.

Molecular biochemical analyses

Despite the persistence of metabolic abnormalities and hypertension, cobalt provided renoprotection in this obese, hypertensive type 2 diabetes rat model. In order to understand molecular biological mechanisms underlying the renal benefit of cobalt, we investigated several factors implicated in the pathology of diabetic nephropathy, i.e. HIF-1alpha, HIF-2alpha, peritubular capillary loss, HIF-regulated genes, TGF-beta, CTGF, oxidative stress and advanced glycation.

HIFs

We examined whether cobalt treatment activates HIFs. The upregulations of intranuclear HIF-1alpha and HIF-2alpha proteins by long-term cobalt treatment were verified by Western blot analysis. The specific band corresponding to HIF-1alpha and HIF-2alpha appeared in the cobalt-treated rats, while being absent in the untreated rats (Figure 3A). Immunohistochemistry revealed that HIF-1alpha was expressed in the tubular cells (predominantly in the proximal tubular cells) in the outer medulla (Figure 3B and C). Cobalt treatment significantly increased HIF-1alpha positive cells (Table 1).

Peritubular capillary loss

Influence of cobalt administration on peritubular capillary density was of interest. We examined whether cobalt treatment protects against peritubular capillary loss by immunostaining for JG12 (Figure 3D and E). Cobalt treatment significantly decreased the rarefaction index, indicating protecting of the peritubular capillary (Table 1).

HIF-regulated genes

To confirm our hypothesis that cobalt treatment may protect diabetic kidneys through activation of HIF-1alpha and its downstream genes, we investigated expression of HIF-regulated protective genes, i.e. EPO, VEGF and HO-1, by real-time PCR analysis utilizing kidney tissues obtained at the end of the study (Figure 4A). Cobalt treatment increased the mRNA expression of EPO (P < 0.01), VEGF (P < 0.01) and HO-1 (P < 0.05) above the levels observed in control SHR/NDmcr-cp rats.

TGF-beta and CTGF expressions

TGF-beta and CTGF are well-known pivotal factors in the development of renal fibrosis [11–13]. As shown in Figure 4B, cobalt treatment decreased mRNA expressions of TGF-beta (P < 0.05) and CTGF (P < 0.05) below the levels observed in control SHR/NDmcr-cp rats.

Oxidative stress

Oxidative stress is a therapeutic target for diabetic renal injury [14]. NADPH oxidase is one of the major sources of cellular reactive oxygen species. Cobalt treatment
Fig. 3. Expressions of hypoxia inducible factors (HIFs) and peritubular capillary loss. The intranuclear HIF-1alpha and HIF-2alpha protein of experimental rats were verified by Western blot analysis (A). The specific band corresponding to HIF-1alpha and HIF-2alpha appeared only in the cobalt-treated DM rats. Immunostaining for HIF-1alpha (B: DM, C: DM + cobalt) or JG12 (D: DM, E: DM + cobalt) in renal tissues. Original magnification ×400 (B–E).

suppressed the mRNA expression of a NADPH subunit Nox2 (Figure 4C, \( P < 0.05 \)) below the levels observed in control SHR/Ndmcr-cp rats.

Advanced glycation end products

Advanced glycation has been incriminated in diabetic renal injury [15–18]. We thus measured, by HPLC analysis, the renal contents of pentosidine, a marker of oxidative stress and advanced glycation (Figure 4D). Cobalt treatment significantly reduced renal pentosidine contents (\( P < 0.01 \)).

Of note, the AGE lowering mechanisms of cobalt in vivo in SHR/Ndmcr-cp rats differ from those of aminoguanidine, pyridoxamine and angiotensin receptor blockers (ARBs). Half-maximal inhibition (IC_{50}) values of in vitro pentosidine formation during 1-week incubation of uraemic plasma for aminoguanidine, pyridoxamine and olmesartan (ARB) are 7.18, 4.31 and 1.78 mM, respectively [5], whereas that for cobalt is >15 mM. Despite the lack of in vitro AGE lowering ability, cobalt significantly lowered local AGE formation, probably as a result of a lowering of oxidative stress.

Discussion

The present data demonstrate for the first time that cobalt, a HIF activator through the inhibition of PHDs, mitigates the development of DN as evidenced by improved pathological changes and a decreased proteinuria. A similar effect of cobalt has been previously reported in other renal injury animal models [10,19–21].

It is of interest to note that cobalt improved DN without modification of metabolic abnormalities and of hypertension, characteristic of the SHR/Ndmcr-cp rat model. This finding stands in sharp contrast with our previous observations of a similar benefit obtained, in the same rat model, by the correction of both the metabolic syndrome and hypertension induced either by diet alone or by the administration of ARBs [5,9,22].

Renoprotective mechanisms of cobalt remain elusive. Cobalt inhibits HIFs degradation by PHDs and thus enhances its activity [8]. It is therefore likely to protect the kidney through the upregulation of HIF-regulated genes. Cobalt given to this model (1 mg/day/animal) indeed augmented expressions of HIFs, assessed by Western blotting and immunohistochemistry, and of HIF-regulated genes (i.e. EPO, VEGF and HO-1) evaluated by real-time PCR analysis. Unfortunately, we did not inject pimonidazole, a hypoxic probe, intravenously to detect the hypoxic regions. It would be of interest to investigate whether hypoxic regions are identical to those of HIF-stained regions.

Previously, some researchers demonstrated renoprotective effects of EPO including anti-apoptosis and recruitment of endothelial precursor cells, thus protecting the peritubular capillary plexus [23]. Indeed, our present study demonstrated that cobalt treatment protected against peritubular capillary loss, indicating the renoprotective effect against chronic hypoxia. It has recently been demonstrated that EPO directly ameliorates podocyte injury resulting in the prevention of glomerulosclerosis [24]. In our preliminary study with normal rats, cobalt did not induce significant erythrocytosis at the dose used in our present study.

The role of VEGF in DN appears to be more complex. Some researchers showed that the inhibition of VEGF or angiogenesis ameliorates glomerular injury in DN [25], whereas others reported that VEGF administration ameliorates experimental non-diabetic kidney diseases [26].

We further investigated the effects of cobalt treatment on several factors implicated in the pathology of DN. First, several lines of evidence point to the role played by advanced glycation and its attendant formation of toxic AGEs [15–18]. Cobalt significantly reduced the renal content of pentosidine. However, its mechanism of action differs from that of previous AGE inhibitors and ARBs, as cobalt is inactive on in vitro AGE formation. Second, cobalt significantly interferes with local oxidative stress, assessed by the expression of a NADPH oxidase subunit Nox2, and increases the expression of HO-1, a defensive enzyme against oxidative stress. Recently, we demonstrated a crucial role of HIF-2 in the anti-oxidative defence utilizing mouse molecular genetics [27]. We have previously suggested that the inhibition of advanced glycation and oxidative stress in the present rat model improved both glomerular and tubulointerstitial injury [5].
Cobalt treatment has more possibilities for renal protection. First, several studies showed that deficiency of renal endothelial nitric oxide synthase (eNOS) production in prolonged diabetes has been implicated in the glomerular haemodynamics changes and the progression of DN [28]. Cobalt could enhance eNOS expression via HIF-2 activation, and may potentially normalize changed glomerular haemodynamics in diabetic kidney [29]. Second, cobalt could potentially inactivate collagen prolyl 4-hydroxylases, which play a key role in collagen synthesis, and may possibly improve the accumulation of extracellular matrix [8,30]. Protective effects of cobalt on tubulointerstitial fibrosis were supported by our MT-staining and real-time PCR analysis: cobalt treatment significantly lowered expressions of TGF-beta and CTGF genes.

Whatever be the sequential mechanisms of cobalt renoprotection, our observations confirm that cobalt treatment has unique properties including the improvement of chronic hypoxia and the inhibition of advanced glycation and oxidative stress.

Unfortunately, the benefits of cobalt given in clinical practice to diabetic patients with nephropathy are sharply limited by its toxicity [31]. As yet no other compound enhancing organ resistance to hypoxia is clinically available. The future availability of non-toxic small molecular PHD inhibitors or HIF activators might open new therapeutic avenues.

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Conflict of interest statement. None declared.

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