Renal protection in chronic kidney disease: hypoxia-inducible factor activation vs. angiotensin II blockade

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Submitted 11 March 2010; accepted in final form 26 September 2010


Hypoxia poses a significant challenge to cellular and organ function and plays a critical role in the progression of CKD to end stage renal failure (22, 31). Hypoxia induces the expression of a large number of genes that ultimately mediate the cell’s adaptations and maladaptations. Indeed, several hypoxia-induced proteins, vascular endothelial growth factor (VEGF; Refs. 7, 39, 44), heme oxygenase-1 (HO-1; Refs. 7, 39, 44), erythropoietin (Epo; Refs. 28, 36), and glucose transporter 1 (GLUT1), have attracted considerable research interests for their protective effects in CKD. These results suggest a potential role for hypoxia-inducible factor (16) in halting CKD progression.

We have previously shown that early application of combined angiotensin II (ANG II) blockade with losartan and captopril improves renal hemodynamics and normalizes QO2/TNa (10). In this study, we tested the hypothesis that induction of HIF, as demonstrated in kidney tissue by Western blot and indexed by the expression of VEGF, HO-1, Epo, and GLUT1 mediates the beneficial effects of ANG II blockade in CKD. We utilized two different mechanisms of HIF induction, administration of cobalt chloride (CoCl2) and dimethyloxalylglycine (DMOG). This hypothesis seems less likely given the prior observations from this laboratory in this model that combined ANG II blockade normalized kidney oxygen consumption factored by Na reabsorption, thereby eliminating the proximate cause of hypoxia, a presumed stimulus to generation of HIF-1 and related proteins, but stimulation by growth factors have also been postulated to increase HIF (10). In this study, we investigated the effects of HIF induction on renal metabolism and hemodynamics to compare with those observed with ANG II blockade in A/I and examined the expression of HIF-induced proteins with both treatments to explain the observed effects. We utilized both cobalt and DMOG therapy to induce HIF and produced similar metabolic and hemodynamic outcomes. Our unexpected results suggest that both ANG II blockade and persistent activation of the HIF pathway are highly effective and improve renal metabolic and hemodynamic functions but by mechanisms that are similar in some respects but differ significantly in other important molecular expressions.

MATERIALS AND METHODS

Animals. Animal use and care were approved by the VASDHS Institutional Animal Care and Use Committee and conducted in accordance with National Institutes of Health guidelines. Male Wistar rats with initial body weight of 225 to 250 g (Harlan, Indianapolis, IN) were randomized into four study groups: 1) control group; 2) 1-wk A/I group; 3) 1-wk A/I + ANG II blockade group; 4) 1-wk A/I + cobalt chloride group; and 5) 1-wk A/I + DMOG group. Renal A/I was...
performed as previously described (10). Cobalt chloride (10 mg kg\(^{-1}\) day\(^{-1}\)) and DMOG (5 mg/kg, twice a day) were given by subcutaneous injection for 8 days. Dual ANG II blockade was accomplished by administration of captopril (20 mg kg\(^{-1}\) day\(^{-1}\)) and losartan (20 mg kg\(^{-1}\) day\(^{-1}\)) by daily gavage for 8 days. Both cobalt chloride and dimethylxalglycine (DMOG) were used as HIF-1α stabilizers (30, 34), captopril is an angiotensin converting enzyme inhibitor (ACEI), and losartan acts as a ANG II type 1 receptor (AT\(_R\)1) blocker.

Renal function measurement and oxygen consumption calculation. In vivo renal function and renal oxygen consumption were measured as previously described (8–10). Briefly, rats were anesthetized with Inactin (100 mg/kg ip) and placed on a temperature-controlled table at 37°C. After cannulation of trachea, left jugular vein, left femoral artery, and urinary bladder, the left renal blood flow (RBF, ml/min) was monitored with a perivascular ultrasonic transit time flow probe (Transomics T420; Lithaca, NY). Systemic blood pressure and RBF were recorded after the animals were allowed 60 min for stabilization with the flow probe in place.

Glomerular filtration rate (GFR) was measured by clearance of \[^{1}H\]inulin in Ringer solution (111.23 mM NaCl, 4.69 mM KCl, and 29.76 mM NaHCO\(_3\)) at 12 μCi/5 ml/h.

Blood samples were taken from the femoral artery and renal vein for measurements of total arterial blood hemoglobin (tHb), O\(_2\)Hb, PO\(_2\), PCO\(_2\), PH, [Na\(^+\)], [K\(^+\)], and [HCO\(_3\);HCO\(_3\);] with a color spectrophotometer, 682 CO-Oximeter (Instrumentation Laboratory, Lexington, MA). O\(_2\) content (O\(_2c\)) was calculated by the formula:

\[
O_2c (ml/ml blood) = (1.39 \times tHb \times O_2Hb\% + PO_2 \times 0.003)/100
\]

The total left kidney oxygen consumption (Q\(_O2\)) was calculated as RBF times arterial minus renal venous O\(_2\)ct. Sodium transported (TNa) (16) equals total sodium filtered (GFR \times PNa) subtraction of excreted sodium from urine (GFR \times PNa – UNaV).

Murine cortical tubular cell culture. Murine cortical tubular cells (19) were grown in DMEM with low glucose (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% heat-inactivated FCS (GIBCO) at 37°C in 5% CO\(_2\). GLUT1 were as previously reported (20).

Quantitative RT-PCR. Total RNA was extracted from rat renal cortex using RNA STAT-60 (Tel-Test, Friendswood, TX), treated with RNaše-free DNsase to eliminate genomic DNA contamination, and purified with RNAeasy Mini kit (Qiagen, Valencia, CA). cDNA was synthesized from 2.5 μg total RNA by reverse transcription reaction using SuperScript VILO cDNA synthesis kit (Invitrogen). The primer pairs used for quantitative RT-PCR analysis of Epo and GLUT1 were as previously reported (20). Quantitative real-time PCR was conducted on a Mx3000P QPCR system (Stratagene, La Jolla, CA) using iQ SYBR Green supermix (Bio-Rad) under the following conditions: 5 min at 98°C, 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. RNA equivalents were normalized to simultaneously determined GAPDH mRNA levels in each sample. Relative RNA in renal cortex from treated rats was compared with that from the controls. Specificity of each RT-PCR reaction was checked by its dissociation curve. Single product amplification and correct product size were confirmed by agarose gel electrophoresis. The purpose of these studies was limited to examination of mRNAs for two HIF-induced molecules, GLUT1 and Epo, as evidence for active stimulation of hypoxia response element proteins by HIF at the time of evaluation. 7 days after creation of the A/I model.

Cell proliferation by 5-bromodeoxyuridine incorporation. Rats were given 5-bromodeoxyuridine (BrDU) daily (50 mg/kg ip; Sigma, St. Louis, MO) for the last 5 days. Kidneys were perfused with cold PBS, fixed with 4% paraformaldehyde in situ by cannulation of the body, and related reagents were provided from a commercial kit (Zymed Laboratories, cat. no. 93–3944; Invitrogen) according to the manufacturer’s instructions. BrDU-positive cells were counted in whole cortex regions of each section. Twenty x10 fields that show higher BrDU-positive cell numbers in each slide were used to calculate means for comparison.

Statistical analysis. Data are expressed as means ± SE. Software SPSS was used for statistical analysis. Comparisons were done by ANOVA. P < 0.05 was considered statistically significant.

RESULTS

Improvement of renal hemodynamics by cobalt treatment and ANG II blockade. The untreated A/I kidneys exhibited significant decreases in both RBF and GFR (Fig. 1). This reduction in GFR and RBF was not simply due to the reduction in renal mass in A/I kidney, since both GFR and RBF were increased significantly by ANG II blockade as well as by cobalt.

Correction of renal metabolic inefficiency by cobalt, DMOG, and ANG II blockade. When compared with the normal kidney and despite a reduction of GFR (decreased filtered load and T\(_{\text{Na}}\)), total renal oxygen consumption (Q\(_O2\)) was unchanged in the untreated A/I kidney. The resultant
Hydroxylation of proline residues on HIF-α by prolyl hydroxylases initiates degradation of HIF-α, and hydroxylation of an asparagine residue in the C-terminal transactivation domain of HIF-α by asparaginyl hydroxylase directly prevents its interaction with its coactivator p300, leading to the HIF transactivation. Both HIF-α prolyl and asparaginyl hydroxylases are dioxygenases that are iron dependent and require O₂ and 2-oxoglutarate (α-ketoglutarate, the tricarboxylic-acid-cy-

Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>GFR, ml/min</th>
<th>QO₂, ml/min</th>
<th>QO₂/TNa, ml/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 6)</td>
<td>1.05 ± 0.11</td>
<td>6.72 ± 0.47</td>
<td>6.72 ± 0.47</td>
</tr>
<tr>
<td>A/I (n = 9)</td>
<td>0.53 ± 0.04</td>
<td>0.33 ± 0.04</td>
<td>0.50 ± 0.04</td>
</tr>
<tr>
<td>A/I + C&amp;L (n = 12)</td>
<td>0.11 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>A/I + Co (n = 6)</td>
<td>0.10 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>A/I + DMOG (n = 6)</td>
<td>0.03 ± 0.03</td>
<td>0.19 ± 0.03</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>A/I + DMOG + C&amp;L (n = 6)</td>
<td>0.05 ± 0.03</td>
<td>0.19 ± 0.03</td>
<td>0.19 ± 0.03</td>
</tr>
</tbody>
</table>

Data are means ± SE. GFR, glomerular filtration rate; QO₂, renal oxygen consumption; QO₂/TNa, sodium transport efficiency; BP, mean systemic blood pressure; HCT, hematocrit. 

Elevation of QO₂/TNa designates decreased renal metabolic efficiency (Table 1 and Fig. 2). This renal metabolic inefficiency was caused by an increased demand for oxygen in the untreated A/I kidney, as dual ANG II blockade and cobalt treatment significantly increased GFR to normal levels while QO₂ remained unchanged (Table 1 and Fig. 2), correcting QO₂/TNa to normal values. We conclude that all these renal effects produced by cobalt treatment are due to the activation of the HIF pathway, since cobalt has been widely used as an inducer of HIF-1α both in vivo and in vitro studies (3, 12, 13, 34, 46). To confirm this conclusion, another HIF-1α inducer, DMOG was used in A/I rats. DMOG treatment produced similar renal protective effects, as indexed by improvement of renal function and decreased renal oxygen consumption (Table 1). 

Fig. 1. Changes in renal hemodynamics in rats one week after renal ablation and infarction (A/I) under different treatments. A: 1-wk after renal A/I the untreated kidney demonstrated a markedly decreased renal blood flow (RBF). This reduction of RBF was significantly prevented by both dual ANG II blockade with captopril and losartan (C&L) and cobalt chloride (Co). B: untreated 1-wk A/I kidney incurred a significant reduction in glomerular filtration rate (GFR). This decrease in GFR was significantly corrected only by dual ANG II inhibition (C&L) but also by cobalt chloride. Data are means ± SE. *P < 0.05 vs. normal; **P < 0.05 vs. A/I.
cle intermediate) as substrates for the hydroxylation reaction (21, 29) in which one oxygen atom is inserted into a proline residue and the other is used to convert 2-oxoglutarate into succinate (35). It has been proposed that cobalt inactivates the hydroxylases by occupying the iron-binding center of the enzyme (12). DMOG, an analog of 2-oxoglutarate, inhibits the hydroxylases via binding to the 2-oxoglutarate binding site of the enzymes.

HIF activation by HIF-1α inducers in vitro and in vivo. With the use of murine cortical proximal tubular cells in culture, administration of CoCl2 at 200 μM for 12 h induced not only HIF-1α protein expression but also carbonic anhydrase 9 (CN9), a prototype HIF-1 target protein, as demonstrated by Western blot (Fig. 3), indicating HIF pathway activation by cobalt in cultured renal proximal tubular cells occurred as early as at 8 h after cobalt stimulation. HIF-1α was undetectable in renal tissue in any groups at any time windows by the Western blot applied for the detection of HIF-1α in cell culture. Induction of HIF-1α in renal tissue by both cobalt and DMOG at day 7 after A/I was visualized by Western blot only after immunoprecipitation of 500 μg of cortical proteins with anti-HIF-1α (Fig. 4). Induction of CN9 in renal cortex was also detectable in A/I kidney treated with cobalt as early as at 18 h after cobalt treatment, suggesting an early HIF activation (Fig. 5).

The expression of other HIF-induced targets was also investigated at either protein level by Western blot or mRNA level for certain molecules by real-time PCR. As shown in Fig. 6, VEGF protein was constitutively expressed in normal kidney, tended to increase in untreated A/I kidneys, further increased in ANG II-blocked kidneys and demonstrated maximal expression in A/I kidneys treated with cobalt. HO-1 protein (Fig. 7) was undetectable in normal kidneys, induced in the untreated group, suppressed in the ANG II-inhibited group, and greatly induced in the cobalt-treated group. When GLUT1 was examined, the pattern was similar to that observed with VEGF, whereby it was modestly elevated in A/I kidneys and further increased in the cobalt and ANG II blockade kidneys (Fig. 8).
Cobalt-treated kidneys were associated with increased Epo mRNA by >20 fold at day 3, while untreated kidneys demonstrated a 30% reduction of Epo, and ANG II blocked A/I kidney exhibited an 80% reduction of Epo mRNA (Fig. 9). Epo mRNA returned to lower levels of expression by day 7. Likewise, expression of GLUT1 mRNA peaked at 3 days of cobalt treatment in A/I animals (Fig. 10) but was relatively unaffected by combined ANG II blockade. Both of these HIF target genes maximally increased in expression in A/I kidneys treated with cobalt, and combined ANG II blockade either decreased mRNA expression or remained unchanged.

Renal cell proliferation. We compared renal cortical cell proliferation among four groups by quantification of BrdU incorporation. We counted BrdU-positive cells in whole cortex of each section. The estimation of the mean for BrdU-positive cells was based on 20 of 100 fields that showed higher BrdU-positive cell numbers in each group, not including scar areas. As shown in Fig. 11, in normal kidney cortex BrdU-positive cells are negligible. There was cortical cell prolifera-
tion in untreated A/I kidney cortex. Cortical cell proliferation was slightly but significantly increased by dual ANG blockade (BrdU-positive cells 26 ± 1.9 vs. 20 ± 1.9/field). Cobalt treatment markedly increased the number of cortical BrdU-positive cells by fivefold, when compared with untreated A/I kidney. Cellular proliferation occurred in glomerular, tubular, and interstitial cells. When examined by PCNA, the results differed somewhat from those by BrdU. PCNA was increased in untreated A/I kidneys and those treated with cobalt to approximately the same degree (Fig. 12). ANG II blockade resulted in normal levels of PCNA. The differences between PCNA and BrdU results may relate to the fact that PCNA was detected on day 7 and BrdU results represent cell proliferation integrated over the last 5 days.

DISCUSSION

The major finding of this study is that activation of the HIF pathway and ANG II blockade improved renal hemodynamics and corrected renal metabolic efficiency by different molecular mechanisms. HIF activation in A/I kidney treated with either cobalt or DMOG was demonstrated by dramatic increases in the expression of HIF-1α protein and increases in the expressions of the HIF targets CN9, VEGF, HO-1, GLUT1, and Epo. In contrast, the HIF pathway was in a deactivated status shown by significant decreases in the expressions of HIF-1α protein and Epo mRNA.

The purpose of these studies was to identify proteins linked to HIF that serve as indicators as to whether the beneficial effects of combined ANG II blockade also result from expression of these proteins. It is recognized that hundreds of malarian genes are regulated by HIF, and dozens of these have been established as direct targets by identification of critical hypoxia response element binding sites (11, 40). Several of these target genes act to inhibit oxidative metabolism, e.g., effects on pyruvate dehydrogenase kinase, as well as promote glycolysis, activation of GLUT1, and effects on lactate dehydrogenase (2, 40). The patterns of protein expression between HIF activation and combined ANG II blockade are not identical, yet the final physiologic and metabolic outcomes are remarkably similar. Similar responses were noted for VEGF and GLUT1 protein expression, but the HO-1 responses differed markedly. Cobalt increased HO-1 and ANG II blockade markedly decreased HO-1. Gene expression of Epo and GLUT1 by quantitative RT-PCR differed markedly between these target genes act to inhibit oxidative metabolism, e.g., effects on pyruvate dehydrogenase kinase, as well as promote glycolysis, activation of GLUT1, and effects on lactate dehydrogenase (2, 40). The patterns of protein expression between HIF activation and combined ANG II blockade are not identical, yet the final physiologic and metabolic outcomes are remarkably similar. Similar responses were noted for VEGF and GLUT1 protein expression, but the HO-1 responses differed markedly. Cobalt increased HO-1 and ANG II blockade markedly decreased HO-1. Gene expression of Epo and GLUT1 by quantitative RT-PCR differed markedly between these two regimens at two time periods.

Our current results suggest that, at 1 wk, VEGF increases in the untreated remnant kidney. Treatments with either cobalt or captopril and losartan further magnified the expression of VEGF, the magnitude of increase being considerably greater with induction of HIF. These effects correlated with normalization of oxygen consumption and improvement in both GFR and RBF. Two different treatments produced similar effects with different mechanisms of VEGF induction. Induction of VEGF in cobalt-treated A/I kidney is HIF dependent, whereas
increased VEGF in ANG II blocked A/I kidney is the consequence of the inhibition of ANG II type I receptor. The main VEGF effects on vasculature are angiogenesis and vasodilation (38). VEGF is produced in the normal kidney at modest levels, with VEGF receptors expressed on endothelial cells of the kidney. Via interaction with its receptors, VEGF promotes endothelial cell proliferation, migration, survival, and new vessel formation. Increased VEGF levels may have contributed to the increase in cell proliferation as indexed by increased BrdU incorporation and increased PCNA after cobalt induction of HIF. A vasodilatory effect of VEGF results from upregulation of the production of nitric oxide (NO). This VEGF-induced increase in NO production also exerts an effect on the regulation of mitochondrial oxygen consumption (6, 26). A reduction in VEGF protein expression has been observed 2 wk after surgery in the remnant kidney model (25), and this observation may be the result of the progressive glomerulosclerosis and tubulointerstitial fibrosis. (38). VEGF administration results in increases in renal endothelial cell proliferation, activation of endothelial nitric oxide synthases, improvement of renal function, and reduction of renal fibrosis (24). However, we do not suggest that these physiologic and metabolic corrections have been proven solely the result of increases in VEGF, as other proteins are likely involved. HO-1, the inducible form of heme oxygenase, catalyzes heme to produce iron, carbon monoxide (CO), and bilirubin, which is further converted to bilirubin. Bilirubin is an important antioxidant. CO is thought to be essential to regulating vascular relaxation and mitochondrial oxygen consumption in a manner similar to NO. Increased HO-1 may also exert effects by limiting or decreasing the supply of porphyrins. We demonstrate herein that HO-1 was substantially increased with cobalt treatment. Increased production of CO and bilirubin due to upregulation of HO-1 could contribute several effects, including enhanced renal vasodilation, reduced oxygen consumption, and reductions in inflammation. Combined ANG II blockade completely prevented any rise in HO-1 by eliminating the normally stimulatory effects of ANG II on HO-1 (1, 18). Taken in the aggregate, it seems unlikely that the beneficial effects of cobalt treatment and HIF activation were mediated principally by induction of HO-1, since ANG II blockade also corrected the metabolic and hemodynamic effects in the absence of significant influences on HO-1. The levels of HO-1 during cobalt treatment were quite clearly increased and different from both the untreated and combined ANG II blockade animals. The changes in GLUT1 protein expression were similar with both treatments and are part of a variety of metabolic alterations that occur with HIF-induced activation of target proteins. The kidney is the primary organ for the production of Epo, and regulation of Epo expression is mainly at the transcriptional level. It is well documented that Epo mRNA is upregulated by HIF in response to hypoxia (4, 41, 42). Recent studies (23, 27) have shown that hypoxic induction of Epo is suppressed by inflammatory cytokines via activation of other inhibitory transcription factors. Therefore, in the diseased kidney, Epo gene expression is determined by the balance between positive and negative transcription factors. It is no surprise that a characteristic of cobalt-treated A/I kidney is a striking increase in Epo mRNA, while the untreated group demonstrates a significant early decrease in Epo mRNA. The latter is likely due to the inflammatory cytokines generated after A/I. The negative influence from the inflammatory cytokines might overcome the positive influence from HIF, resulting in a reduction of Epo in A/I kidney at early stages. Combined ANG II blockade was associated with a further reduction in Epo mRNA, which is an unexpected finding but is consistent with the observations that ACEI is linked to worsening anemia in patients with CKD on dialysis (33) and to lowering serum Epo levels in renal transplant recipients (15). Our finding provides a mechanism that transcriptional regulation of Epo gene is ANG II dependent and probably requires the participation of AT1R (14). In addition to erythropoiesis, Epo exerts a mitogenic effect on nonerythroid tissues via interaction with its receptors that are widely expressed in a variety of tissues, including kidneys (47).

In the cobalt-treated A/I kidney, we observed that there was a profound proliferation of glomerular, tubular, vascular, and interstitial cells (Deng A, Blantz R, unpublished observations), while the untreated kidney exhibited modest tubular cell proliferation, which was not influenced by combined ANG II inhibition. BrdU expression indicates cell division or proliferation. Studies examining PCNA showed similar findings except that combined ANG II blockade was associated with reductions in PCNA. PCNA does not necessarily indicate cell division. We assume that this extensive renal parenchymal cell proliferation may be associated with the mitogenic effects of Epo. Somehow these events were associated with normalization of both metabolic activity and renal hemodynamics.

The current results suggest that induction of HIF, as identified by increased HIF-1α protein expression and by downstream HIF-1 products, VEGF, HO-1, GLUT1, and Epo, acts to improve renal hemodynamics and renal function and corrects renal metabolic efficiency. Results suggest that HIF induction also promotes renal cell growth, stimulates angiogenesis, and reduces inflammation. We have shown that in early experimental CKD major activation of HIF pathway is renal protective. This is in accord with recent data from Bernhardt et al. (5), where HIF-1α induction provided protective effects and prolonged survival in a model of kidney transplantation and ischemia, and from Tanaka et al. (45) and by Song et al. (43) in this model at a later stage. Thus, in addition to ANG II blockade, HIF induction may present a major mechanism for kidney protection and to ameliorate disease progression of CKD. Since the patterns of protein expression differ between induction of HIF and combined ANG II blockade, it is intriguing to speculate that some form of combined therapy might be highly beneficial, either additive or synergistic to progression of CKD. The mechanisms of protection of combined ANG II blockade and HIF-1 appear to differ. Further studies are required to define the specific HIF-1-induced proteins and mechanisms related to ANG II blockade that produce major physiologic and metabolic benefits in this experimental model of CKD.

ACKNOWLEDGMENTS

We thank Dr. Nissi M. Variki’s Histology and Immunohistochemistry Shared Resource Group at the University of California San Diego Moores Cancer Center for excellent technical BrdU stain services.

GRANTS

This work was supported by the National Institute of Diabetes and Digestive and Kidney Disease Grants R01-DK-28602 and P30-DK-079337 and includes the Department of Veterans Affairs Research Service. Prabhleen Singh was supported by grants through the National Kidney Foundation and now is funded by a K08 award from the National Institutes of Health.
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