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Kidney Hypoxia, Attributable to Increased Oxygen Consumption, Induces Nephropathy Independently of Hyperglycemia and Oxidative Stress

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See Editorial Commentary, pp 827–828

Abstract—Diabetic nephropathy is strongly associated with both increased oxidative stress and kidney tissue hypoxia. The increased oxidative stress causes increased kidney oxygen consumption resulting in kidney tissue hypoxia. To date, it has been difficult to determine the role of kidney hypoxia, per se, for the development of nephropathy. We tested the hypothesis that kidney hypoxia, without confounding factors such as hyperglycemia or elevated oxidative stress, results in nephropathy. To induce kidney hypoxia, dinitrophenol (30 mg per day per kg bodyweight by gavage), a mitochondrial uncoupler that increases oxygen consumption and causes kidney hypoxia, was administered for 30 consecutive days to rats. Thereafter, glomerular filtration rate, renal blood flow, kidney oxygen consumption, kidney oxygen tension, kidney concentrations of glucose and glycogen, markers of oxidative stress, urinary protein excretion, and histological findings were determined and compared with vehicle-treated controls. Dinitrophenol did not affect arterial blood pressure, renal blood flow, glomerular filtration rate, blood glucose, or markers of oxidative stress but increased kidney oxygen consumption, and reduced cortical and medullary concentrations of glucose and glycogen, and resulted in intrarenal tissue hypoxia. Furthermore, dinitrophenol treatment increased urinary protein excretion, kidney vimentin expression, and infiltration of inflammatory cells. In conclusion, increased mitochondrial oxygen consumption results in kidney hypoxia and subsequent nephropathy. Importantly, these results demonstrate that kidney tissue hypoxia, per se, without confounding hyperglycemia or oxidative stress, may be sufficient to initiate the development of nephropathy and therefore demonstrate a new interventional target for treating kidney disease. (*Hypertension*. 2013;62:914-919.) • [Online Data Supplement](#)

Key Words: cell hypoxia ■ chronic kidney disease ■ dinitrophenols ■ kidney ■ nephropathy, chronic tubulointerstitial ■ oxygen consumption ■ uncoupling agents

Progressive loss of kidney function leads to end-stage renal disease (ESRD), requiring dialysis or kidney transplantation. Diabetes mellitus, hypertension, glomerulonephritis, and cystic kidney disease are all major causes for ESRD.¹ Approximately 45% of all cases of ESRD are caused by diabetes mellitus,² and the number of affected patients will continue to increase because the prevalence of diabetes mellitus is projected to increase from 171 million in 2000 to 366 million in 2030.³ Development of diabetic nephropathy is closely associated with increased levels of oxidative stress,^{4,5} and alterations in kidney metabolism and oxygen handling.^{6–8} These functional alterations precede the structural changes of the kidney.^{10,11}

Fine et al⁹ suggested already in 1998 that kidney hypoxia is a mediator of progressive kidney disease, and chronic tubulointerstitial hypoxia is now commonly acknowledged as a pathway

to ESRD.^{10–15} Although the kidneys receive 25% of cardiac output, and the blood in the renal vein is well oxygenated,¹⁶ the kidney oxygen tension (P_{O_2}) is markedly lower than arterial P_{O_2} , and the renal medulla operates in a hypoxic milieu already during physiological conditions.^{17,18} It should be noted that the kidney does not display the same relationship between oxygen demand and supply as other organs, for example, the brain. Increasing oxygen delivery (DO_2) to the kidney by increasing renal blood flow (RBF) is likely to result in a simultaneously increased tubular electrolyte load attributable to increased glomerular filtration rate (GFR). This will increase active tubular transport that, per se, increases the energy demand resulting in increased kidney oxygen consumption (QO_2). Therefore, increased intrarenal oxygen metabolism will inevitably result in kidney tissue hypoxia and potentially contribute to the

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From the Department of Medical Cell Biology, Uppsala University, Sweden (M.F.-P., P.H., F.P.); the Wallenberg Laboratory for Cardiovascular and Metabolic Research, Gothenburg University, Sweden (E.T., M.L.); School of Medicine, Division of Nephrology and Endocrinology, University of Tokyo, Japan (M.N.); Department of Medical and Health Sciences (F.P.) and Center for Medical Image Science and Visualization (F.P.), Linköping University, Sweden.

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Correspondence to Malou Friederich-Persson, Department of Medical Cell Biology, Uppsala University, Biomedical Center, Husargatan 3, SE-751 23, Uppsala, Sweden. E-mail Malou.Friederich@mcb.uu.se

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development of nephropathy.¹⁹ Indeed, kidneys from diabetic and hypertensive animals have increased renal QO_2 .^{7,20-22} However, it is difficult to mechanistically separate intrarenal tissue hypoxia from oxidative stress because it is increased oxidative stress that causes the initial increase in kidney QO_2 , which results in the lower tissue PO_2 in these kidneys.^{7,20}

In kidneys from diabetic rats, increased mitochondrial uncoupling protein-2 activity increases mitochondrial QO_2 .²¹ By releasing protons across the mitochondrial inner membrane independently of ATP production, uncoupling protein-2 controls the mitochondrial membrane potential and therefore also superoxide formation. Mitochondrial uncoupling is therefore an antioxidant mechanism but with the side effect of significantly elevated QO_2 . The chemical mitochondrial uncoupler, 2,4-dinitrophenol (DNP), also increases mitochondrial QO_2 without increasing ATP production. DNP was therefore used as a weight loss therapy in the early 1930s but was banned from the market in 1938 because of causing cataract and overdosing fatalities.^{23,24}

In this study, we tested the hypothesis that intrarenal hypoxia, per se, without confounding factors such as hyperglycemia and oxidative stress, can induce nephropathy.

Material and Methods

All animal procedures were approved by the Uppsala animal ethics committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats were treated with DNP (30 mg per day per kg bodyweight,²⁵ 1 mL dissolved in 1.5% methyl cellulose) or vehicle by gavage for 30 days. During the treatment period, the body weight, weight gain, and food and water intake were monitored and, after the treatment period, the in vivo kidney function was evaluated in terms of GFR, RBF, kidney QO_2 , kidney PO_2 , DO_2 , kidney concentrations of glucose and glycogen (analyzed using bioluminescence imaging),²⁶ and tubular reabsorption of Na^+ and proteinuria. In each group, randomly selected animals ($n=6-8$) were analyzed for urinary excretion of thio-barbituric acid reactive substances, kidney cortex content of protein carbonyls, tubular damage indicated by vimentin staining, and infiltration of immune cells by mouse monoclonal macrophage CD68 antibody (ED-1) staining. Expressions of oxygen-regulated genes were analyzed by reverse transcription polymerase chain reaction.²⁷ Nephropathy was defined as the development of proteinuria and histological alterations. See the online-only Data Supplement for a detailed Methods section.

Statistical analysis was performed using Student *t* test. $P<0.05$ (2-sided) was considered statistically significant, and all values are presented as mean \pm SEM.

Results

Treatment with DNP did not statistically change body weight, blood glucose, kidney weight, mean arterial pressure, RBF, GFR, or tubular reabsorption of Na^+ . Renal DO_2 , hemoglobin content, and food and water intake was increased, and

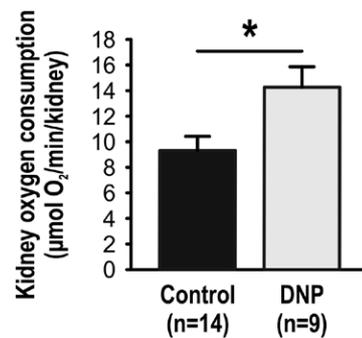


Figure 1. Total kidney oxygen consumption in rats with and without administration of 2,4-dinitrophenol (DNP). * $P<0.05$ compared with vehicle-treated controls.

transported Na^+ per consumed oxygen was reduced in rats treated with DNP (Table 1).

DNP increased QO_2 (Figure 1) and reduced kidney content of glycogen and glucose (Figure 2A and 2B). The increased metabolism induced by DNP resulted in tissue hypoxia in both kidney cortex and medulla (Figure 3). Furthermore, DNP treatment induced proteinuria and increased vimentin staining and infiltration of ED-1-positive cells (Figure 4A–4C). Importantly, chronic DNP treatment did not statistically affect markers of oxidative stress in either urine or in kidney tissue (Figure 5A and 5B).

Acute infusion of DNP to determine its acute toxicity did not statistically alter urinary protein excretion in either controls (46.9 ± 7.7 versus 50.9 ± 5.9 $\mu\text{g}/\text{min}$; ns) or in chronically DNP-treated animals (76.9 ± 7.6 versus 57.4 ± 10.2 $\mu\text{g}/\text{min}$; ns) compared with baseline. However, acute DNP administration decreased both cortical and medullary kidney PO_2 in controls (34 ± 2 versus 44 ± 1 and 26 ± 1 versus 30 ± 2 mmHg, respectively; both $P<0.05$) but did not statistically affect any of the other investigated parameters in any of the 2 groups (data not shown).

Discussion

This study demonstrates that kidney hypoxia, attributable to increased kidney QO_2 , induces nephropathy without confounding factors such as hyperglycemia or oxidative stress. The results from the present study may therefore demonstrate that kidney tissue hypoxia, per se, is sufficient to induce similar nephropathy as commonly observed in animal models of experimental diabetic and hypertensive kidney disease as well as in the corresponding patient populations.

There is growing support for kidney tissue hypoxia as a main pathogenic mechanism for development of chronic kidney disease, both in diabetes mellitus and other conditions.¹⁰⁻¹⁵ Indeed, decreased kidney PO_2 has been demonstrated in

Table. Animal Characteristics and Investigated In Vivo Parameters

Group	BW, g	BG, mmol/L	Hb, mg/dL	KW, g	Weight Gain, g/d	Food Intake, g/d	Water Intake, mL/d	MAP, mmHg	RBF, mL per min per Kidney	GFR, mL per min per Kidney	DO_2 , μmol per min per Kidney	TNa^+ , μmol per min per Kidney	TNa^+/QO_2
Control	433 \pm 21	6.1 \pm 0.4	150 \pm 4	1.3 \pm 0.0	4.4 \pm 0.2	24 \pm 1	31 \pm 1	101 \pm 3	10.3 \pm 0.4	1.5 \pm 0.1	1775 \pm 71	229.6 \pm 26.2	24.9 \pm 6.8
DNP	452 \pm 13	5.4 \pm 0.5	169 \pm 4*	1.4 \pm 0.0	3.9 \pm 0.3	29 \pm 1*	40 \pm 1*	94 \pm 9	9.1 \pm 0.7	1.3 \pm 0.1	2124 \pm 58*	174.9 \pm 17.2	12.7 \pm 1.5*

Body weight (BW), blood glucose (BG), hemoglobin (Hb), kidney weight (KW), weight gain, food intake, water intake, mean arterial pressure (MAP), renal blood flow (RBF), glomerular filtration rate (GFR), kidney delivery of oxygen (DO_2), tubular reabsorption of Na^+ (TNa^+) and transported Na^+ per consumed oxygen (TNa^+/QO_2) in rats with and without administration of dinitrophenol (DNP) for 30 days.

* $P<0.05$ compared with untreated controls, $n=8$ to 14 in each group.

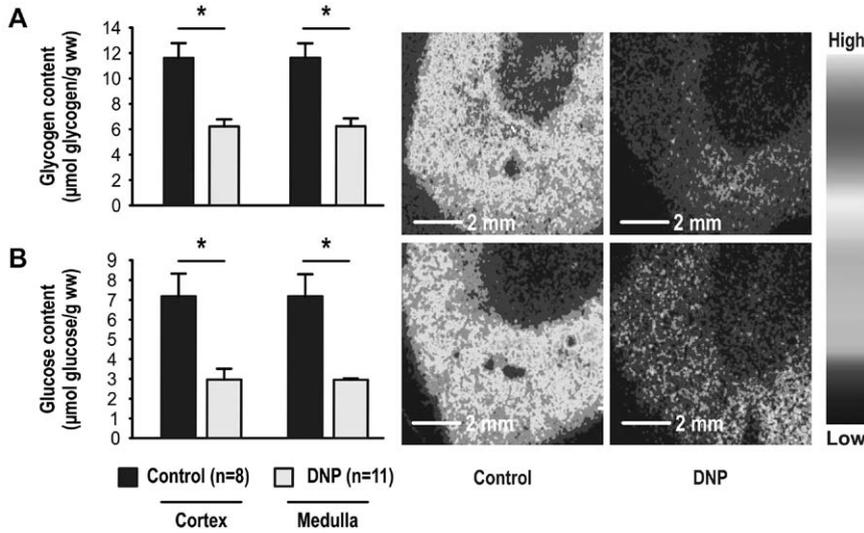


Figure 2. Glycogen (A) and glucose content (B) in kidney cortex and medulla in rats with and without administration of 2,4-dinitrophenol (DNP) for 30 days. Representative images are displayed to the right. **P*<0.05 compared with vehicle-treated controls.

diabetes mellitus,^{7,28–32} hypertension,^{20,33,34} polycystic kidney disease,³⁵ hypertensive nephrosclerosis, and chronic glomerulonephritis.³⁶ Fine et al⁹ proposed that the initial glomerular injury leading to decreased RBF in peritubular capillaries causes decreased tissue P_{O_2} , which promotes tubulointerstitial fibrosis. The remaining glomerular and peritubular capillaries are subjected to increased RBF and hydrostatic pressure, which in turn increases damage and promotes further development of fibrosis. Consequently, both decreased and increased RBF in the peritubular capillaries would lead to tubulointerstitial hypoxia. They proposed a critical interstitial hypoxia level that resulted in the progressive loss of kidney function.⁹ Indeed, loss of peritubular capillaries has been reported in diabetes mellitus³⁷ and ischemic injury.³⁸ Furthermore, development of hypoxia and tubulointerstitial fibrosis correlates with decreasing GFR in patients with chronic kidney disease.³⁶ Also, it has been reported that cortical interstitial fibrosis, reduced proximal tubule epithelium volume, and atrophied proximal tubules all correlate with GFR at the time of biopsy.³⁹

In the present study, we observed increased vimentin staining, a marker of tubular damage,⁴⁰ in the animals treated with DNP. Vimentin has been used to investigate the degree of tubulointerstitial damage in both patients^{41,42} and animal models.⁴³ Importantly, we also detected increased urinary protein leakage in the DNP-treated animals, which is commonly regarded as a good predictor for progressive nephropathy.⁴⁴ It

is possible that the hypoxic injury results not only in increased protein permeability across the glomerular capillary but also negatively affects protein reabsorption in the proximal tubule. Both these mechanisms promote proteinuria. It has also been demonstrated that albumin can induce elevated cytokine levels in tubular cells and thereby promote apoptosis.^{45,46} Proteinuria may therefore be a self-sustained pathway to accelerate nephropathy. Indeed, it has been demonstrated that the level of proteinuria correlated to a more rapid loss of kidney function in patients with chronic kidney disease.^{44,47}

Despite increased DO_2 in the chronically DNP-treated animals, there was still intrarenal tissue hypoxia. It may be speculated that the reason as to why kidneys with elevated QO_2 do not benefit from increased DO_2 may be attributable to arteriovenous precapillary shunting of oxygen. In the kidney, arteries run in parallel to veins, which constitutes the basis for a morphological shunt. Thus, increased kidney QO_2 results in a larger arteriovenous oxygen gradient, which facilitates shunting and prevents increased DO_2 to counteract the intrarenal tissue hypoxia. In pioneering studies, Levy et al^{48,49} injected labeled erythrocytes in supersaturated oxygen buffer and measured the transit time for the erythrocytes and the oxygen peak to travel across to the renal vein. They reported that the oxygen peak appeared on average 1.25 seconds earlier than the erythrocytes, and it was concluded that the oxygen traveled a shorter distance compared with the erythrocytes that were restricted to the vasculature.^{48,49} The only possible explanation is a functional arteriovenous shunt of oxygen, which has also been supported by later reports by others.^{17,20,50,51}

In the present study, treatment with DNP for 30 days resulted in nephropathy evident as proteinuria, increased vimentin expression, and infiltration of immune cells. However, DNP is considered an environmental toxin and may be nephrotoxic, per se. Indeed, reports of proteinuria after treatment with DNP exist. Two women treated with a low-dose DNP for 35 to 37 days and dogs treated with DNP for 6 months all developed proteinuria.^{52–54} Any influence of direct nephrotoxic effects of DNP for the observed results in the present study cannot be excluded because other mitochondrial uncouplers, such as carbonyl cyanide p-trifluoromethoxy-phenylhydrazone, have a similar chemical structure and most likely similar toxicity. Therefore,

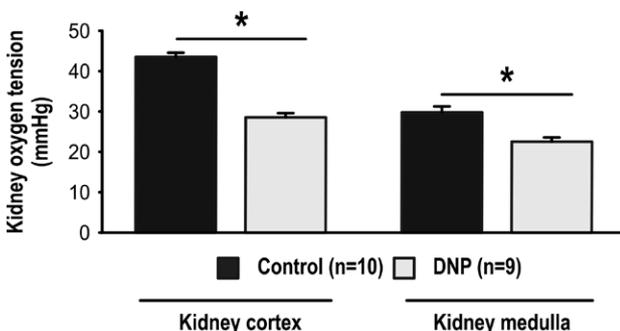


Figure 3. Oxygen tensions in kidney cortex and medulla in rats with and without administration of 2,4-dinitrophenol (DNP) for 30 days. **P*<0.05 compared with vehicle-treated controls.

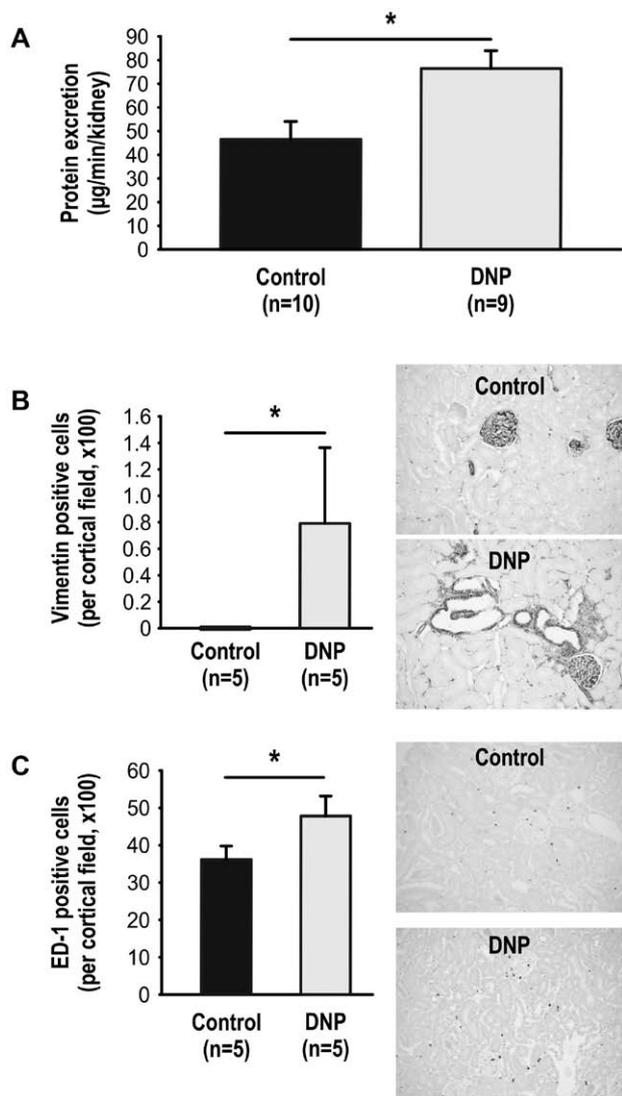


Figure 4. Urinary protein excretion (A), number of vimentin-positive cells (B), and number of mouse monoclonal macrophage CD68 antibody (ED-1)-positive cells (C) in rats with and without administration of 2,4-dinitrophenol (DNP) for 30 days. **P*<0.05 compared with vehicle-treated controls. Representative images of kidney cortex of vimentin and ED-1 staining from rats with and without administration of DNP are displayed to the right. Vimentin staining of the glomeruli in control animals is normal and does not indicate damage.

future experiments in which the possible toxicity of DNP can be excluded, possibly by reversing the hypoxia after DNP treatment, are needed to conclusively solidify the role of hypoxia, per se, as the main mechanism for development of nephropathy. Importantly, acute infusion of DNP resulted in decreased cortical and medullary P_{O_2} but did not affect the urinary protein excretion in the present study. This indicates that DNP exerts an effect on renal metabolism but without any acute nephrotoxicity. It is also unlikely that the nephropathy attributable to chronic DNP treatment is the result of ATP depletion because both groups had similar GFR and Na^+ excretion, which is in good agreement with previous reports.^{55,56} As active tubular reabsorption of Na^+ accounts for $\approx 85\%$ of total kidney QO_2 ,⁵⁷ an ATP deficiency would likely be manifested as increased urinary Na^+ excretion and deranged whole-body Na^+ homeostasis. In accordance, the

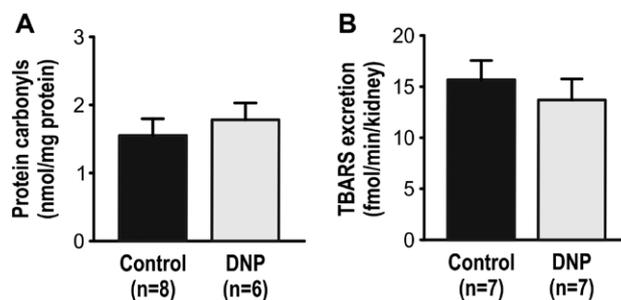


Figure 5. A and B, Urinary excretion of thiobarbituric acid reactive substances (TBARS) and kidney cortex content of protein carbonyls in rats with and without administration of 2,4-dinitrophenol (DNP) for 30 days.

kidney content of glycogen and glucose was decreased, but not depleted, by the chronic DNP treatment. These results collectively confirm that DNP elevates mitochondrial usage of these energy substrates, but that the kidney still has the necessary substrate supply to sustain sufficient ATP production to uphold tubular function. We therefore propose that DNP is indeed nephrotoxic, but the mechanism consists of kidney hypoxia resulting in tubulointerstitial damage and proteinuria.

It has been reported that DNP protects against ischemia-reperfusion injury in the heart⁵⁸ and cortical neurons,⁵⁹ which is likely because of reduced oxidative stress originating from mitochondrial sources. However, peripheral neuropathy has been reported in patients after long-term DNP treatment.⁶⁰ In the present study, we did not observe any obvious signs of neuropathy or altered behavior from the DNP treatment. Furthermore, blood flow increases in response to increased QO_2 in most organs and tissues. Thus, the DNP-induced increased QO_2 is likely to have most profound effects on the intrarenal oxygen availability because the blood flow does not increase in response to increased QO_2 in the kidney.

The present study is in accordance with numerous studies indicating chronic tubulointerstitial hypoxia as the important mediator of nephropathy.¹⁰⁻¹⁵ Interestingly, Navajo Indians living at high altitude had 1.8-fold higher prevalence of age-adjusted nondiabetes-related ESRD compared with both the general population and Native Americans in the United States.⁶¹ Furthermore, patients with type 2 diabetes mellitus living at 1700 m above sea level present with elevated baseline urinary protein excretion and increased prevalence of proteinuria compared with corresponding patients living at sea level.⁶² It should be noted that these differences are independent of glycemic control, lipidemia status, mean arterial pressure, or incidence of retinopathy. Thus, the available literature suggests that kidney tissue hypoxia is a key player for the development of chronic kidney disease in numerous pathological conditions.^{7,20,28-36}

In conclusion, the present study provides additional support that kidney tissue hypoxia, resulting from increased QO_2 induced by mitochondria uncoupling, can result in tubulointerstitial damage and proteinuria. Kidney hypoxia may therefore be an important pathogenic mechanism for the development of nephropathy.

Perspectives

Metabolic changes are likely to occur early and precede later irreversible histological alterations. Thus, being able to detect and target these early changes in the intrarenal oxygen metabolism

may provide new therapeutic targets in the fight against the growing burden of chronic kidney disease and later ESRD. The results from the present study demonstrate that increased mitochondrial QO_2 , without confounding factors such as hyperglycemia or oxidative stress, may be sufficient to induce kidney tissue hypoxia and initiate the development of kidney disease. Thus, interventions to prevent excessive mitochondrial QO_2 or to restore kidney tissue oxygen availability may prove beneficial to prevent nephropathy in diabetes mellitus and hypertension.

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Disclosures

None.

References

1. United States Renal Database; Annual Report. 2011.
2. Harvey JN. Trends in the prevalence of diabetic nephropathy in type 1 and type 2 diabetes. *Curr Opin Nephrol Hypertens*. 2003;12:317–322.
3. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care*. 2004;27:1047–1053.
4. Nishikawa T, Edelstein D, Brownlee M. The missing link: a single unifying mechanism for diabetic complications. *Kidney Int Suppl*. 2000;77:S26–S30.
5. Baynes JW. Role of oxidative stress in development of complications in diabetes. *Diabetes*. 1991;40:405–412.
6. Palm F. Intrarenal oxygen in diabetes and a possible link to diabetic nephropathy. *Clin Exp Pharmacol Physiol*. 2006;33:997–1001.
7. Palm F, Cederberg J, Hansell P, Liss P, Carlsson PO. Reactive oxygen species cause diabetes-induced decrease in renal oxygen tension. *Diabetologia*. 2003;46:1153–1160.
8. Nordquist L, Palm F. Diabetes-induced alterations in renal medullary microcirculation and metabolism. *Curr Diabetes Rev*. 2007;3:53–65.
9. Fine LG, Orphanides C, Norman JT. Progressive renal disease: the chronic hypoxia hypothesis. *Kidney Int Suppl*. 1998;65:S74–S78.
10. Mimura I, Nangaku M. The suffocating kidney: tubulointerstitial hypoxia in end-stage renal disease. *Nat Rev Nephrol*. 2010;6:667–678.
11. Nangaku M. Hypoxia and tubulointerstitial injury: a final common pathway to end-stage renal failure. *Nephron Exp Nephrol*. 2004;98:e8–e12.
12. Nangaku M. Chronic hypoxia and tubulointerstitial injury: a final common pathway to end-stage renal failure. *J Am Soc Nephrol*. 2006;17:17–25.
13. Singh DK, Winocour P, Farrington K. Mechanisms of disease: the hypoxic tubular hypothesis of diabetic nephropathy. *Nat Clin Pract Nephrol*. 2008;4:216–226.
14. Palm F, Nordquist L. Renal tubulointerstitial hypoxia: cause and consequence of kidney dysfunction. *Clin Exp Pharmacol Physiol*. 2011;38:474–480.
15. Eckardt KU, Bernhardt WM, Weidemann A, Warnecke C, Rosenberger C, Wiesener MS, Willam C. Role of hypoxia in the pathogenesis of renal disease. *Kidney Int Suppl*. 2005;S46–S51.
16. Deng A, Miracle CM, Suarez JM, Lortie M, Satriano J, Thomson SC, Munger KA, Blantz RC. Oxygen consumption in the kidney: effects of nitric oxide synthase isoforms and angiotensin II. *Kidney Int*. 2005;68:723–730.
17. Leichtweiss HP, Lübbers DW, Weiss C, Baumgärtl H, Reschke W. The oxygen supply of the rat kidney: measurements of intrarenal pO_2 . *Pflügers Arch*. 1969;309:328–349.
18. Aukland K, Krogh J. Renal oxygen tension. *Nature*. 1960;188:671.
19. Hansell P, Welch WJ, Blantz RC, Palm F. Determinants of kidney oxygen consumption and their relationship to tissue oxygen tension in diabetes and hypertension. *Clin Exp Pharmacol Physiol*. 2013;40:123–137.
20. Welch WJ, Baumgärtl H, Lübbers D, Wilcox CS. Nephron pO_2 and renal oxygen usage in the hypertensive rat kidney. *Kidney Int*. 2001;59:230–237.
21. Friederich M, Fasching A, Hansell P, Nordquist L, Palm F. Diabetes-induced up-regulation of uncoupling protein-2 results in increased mitochondrial uncoupling in kidney proximal tubular cells. *Biochim Biophys Acta*. 2008;1777:935–940.
22. Körner A, Eklöf AC, Celsi G, Aperia A. Increased renal metabolism in diabetes. Mechanism and functional implications. *Diabetes*. 1994;43:629–633.
23. Parascandola J. Dinitrophenol and bioenergetics: an historical perspective. *Mol Cell Biochem*. 1974;5:69–77.
24. Horner WD. A study of dinitrophenol and its relation to cataract formation. *Trans Am Ophthalmol Soc*. 1941;39:405–437.
25. Caldeira da Silva CC, Cerqueira FM, Barbosa LF, Medeiros MH, Kowaltowski AJ. Mild mitochondrial uncoupling in mice affects energy metabolism, redox balance and longevity. *Aging Cell*. 2008;7:552–560.
26. Levin M, Leppänen O, Evaldsson M, Wiklund O, Bondjers G, Björnheden T. Mapping of ATP, glucose, glycogen, and lactate concentrations within the arterial wall. *Arterioscler Thromb Vasc Biol*. 2003;23:1801–1807.
27. Matsumoto M, Makino Y, Tanaka T, Tanaka H, Ishizaka N, Noiri E, Fujita T, Nangaku M. Induction of renoprotective gene expression by cobalt ameliorates ischemic injury of the kidney in rats. *J Am Soc Nephrol*. 2003;14:1825–1832.
28. Edlund J, Hansell P, Fasching A, Liss P, Weis J, Glickson JD, Palm F. Reduced oxygenation in diabetic rat kidneys measured by T2* weighted magnetic resonance micro-imaging. *Adv Exp Med Biol*. 2009;645:199–204.
29. Haidara MA, Mikhailidis DP, Rateb MA, Ahmed ZA, Yassin HZ, Ibrahim IM, Rashed LA. Evaluation of the effect of oxidative stress and vitamin E supplementation on renal function in rats with streptozotocin-induced Type 1 diabetes. *J Diabetes Complications*. 2009;23:130–136.
30. Rosenberger C, Khamaisi M, Abassi Z, Shilo V, Weksler-Zangen S, Goldfarb M, Shina A, Zibretrest F, Eckardt KU, Rosen S, Heyman SN. Adaptation to hypoxia in the diabetic rat kidney. *Kidney Int*. 2008;73:34–42.
31. dos Santos EA, Li LP, Ji L, Prasad PV. Early changes with diabetes in renal medullary hemodynamics as evaluated by fiberoptic probes and BOLD magnetic resonance imaging. *Invest Radiol*. 2007;42:157–162.
32. Ries M, Basseau F, Tyndal B, Jones R, Deminière C, Catargi B, Combe C, Moonen CW, Grenier N. Renal diffusion and BOLD MRI in experimental diabetic nephropathy. Blood oxygen level-dependent. *J Magn Reson Imaging*. 2003;17:104–113.
33. Palm F, Onozato M, Welch WJ, Wilcox CS. Blood pressure, blood flow, and oxygenation in the clipped kidney of chronic 2-kidney, 1-clip rats: effects of tempol and Angiotensin blockade. *Hypertension*. 2010;55:298–304.
34. Welch WJ. Intrarenal oxygen and hypertension. *Clin Exp Pharmacol Physiol*. 2006;33:1002–1005.
35. Bernhardt WM, Wiesener MS, Weidemann A, Schmitt R, Weichert W, Lechler P, Campean V, Ong AC, Willam C, Gretz N, Eckardt KU. Involvement of hypoxia-inducible transcription factors in polycystic kidney disease. *Am J Pathol*. 2007;170:830–842.
36. Inoue T, Kozawa E, Okada H, Inukai K, Watanabe S, Kikuta T, Watanabe Y, Takenaka T, Katayama S, Tanaka J, Suzuki H. Noninvasive evaluation of kidney hypoxia and fibrosis using magnetic resonance imaging. *J Am Soc Nephrol*. 2011;22:1429–1434.
37. Ohtomo S, Nangaku M, Izuhara Y, Takizawa S, Strihou Cv, Miyata T. Cobalt ameliorates renal injury in an obese, hypertensive type 2 diabetes rat model. *Nephrol Dial Transplant*. 2008;23:1166–1172.
38. Basile DP, Donohoe D, Roethe K, Osborn JL. Renal ischemic injury results in permanent damage to peritubular capillaries and influences long-term function. *Am J Physiol Renal Physiol*. 2001;281:F887–F899.
39. Mackensen-Haen S, Bader R, Grund KE, Bohle A. Correlations between renal cortical interstitial fibrosis, atrophy of the proximal tubules and impairment of the glomerular filtration rate. *Clin Nephrol*. 1981;15:167–171.
40. Muramatsu M, Miyagi M, Ishikawa Y, Aikawa A, Mizuiri S, Ohara T, Ishii T, Hasegawa A. Estimation of damaged tubular epithelium in renal allografts by determination of vimentin expression. *Int J Urol*. 2004;11:954–962.
41. de Matos AC, Câmara NO, Tonato EJ, Durão Júnior Mde S, Franco MF, Moura LA, Pacheco-Silva A. Vimentin expression and myofibroblast infiltration are early markers of renal dysfunction in kidney transplantation: an early stage of chronic allograft dysfunction? *Transplant Proc*. 2010;42:3482–3488.
42. Rastaldi MP, Ferrario F, Giardino L, Dell'Antonio G, Grillo C, Grillo P, Strutz F, Müller GA, Colasanti G, D'Amico G. Epithelial-mesenchymal transition of tubular epithelial cells in human renal biopsies. *Kidney Int*. 2002;62:137–146.

43. Gröne HJ, Weber K, Gröne E, Helmchen U, Osborn M. Coexpression of keratin and vimentin in damaged and regenerating tubular epithelia of the kidney. *Am J Pathol.* 1987;129:1–8.
44. Ruggerenti P, Perna A, Mosconi L, Pisoni R, Remuzzi G. Urinary protein excretion rate is the best independent predictor of ESRF in non-diabetic proteinuric chronic nephropathies. “Gruppo Italiano di Studi Epidemiologici in Nefrologia” (GISEN). *Kidney Int.* 1998;53:1209–1216.
45. Tang S, Leung JC, Abe K, Chan KW, Chan LY, Chan TM, Lai KN. Albumin stimulates interleukin-8 expression in proximal tubular epithelial cells *in vitro* and *in vivo*. *J Clin Invest.* 2003;111:515–527.
46. Wang Y, Rangan GK, Tay YC, Wang Y, Harris DC. Induction of monocyte chemoattractant protein-1 by albumin is mediated by nuclear factor kappaB in proximal tubule cells. *J Am Soc Nephrol.* 1999;10:1204–1213.
47. Hunsicker LG, Adler S, Caggiula A, England BK, Greene T, Kusek JW, Rogers NL, Teschan PE. Predictors of the progression of renal disease in the Modification of Diet in Renal Disease Study. *Kidney Int.* 1997;51:1908–1919.
48. Levy MN, Imperial ES. Oxygen shunting in renal cortical and medullary capillaries. *Am J Physiol.* 1961;200:159–162.
49. Levy MN, Saucedo G. Diffusion of oxygen from arterial to venous segments of renal capillaries. *Am J Physiol.* 1959;196:1336–1339.
50. Schurek HJ, Jost U, Baumgärtl H, Bertram H, Heckmann U. Evidence for a preglomerular oxygen diffusion shunt in rat renal cortex. *Am J Physiol.* 1990;259(6 pt 2):F910–F915.
51. Schurek HJ, Kriz W. Morphologic and functional evidence for oxygen deficiency in the isolated perfused rat kidney. *Lab Invest.* 1985;53:145–155.
52. Beinhauer L. Urticaria following the use of dinitrophenol. *W V Med J.* 1934;7:466–477.
53. Imerman S, Imerman C. Dinitrophenol poisoning with thrombocytopenia, granulopenia, anemia and purpura complicated by lung abscess. *JAMA.* 1936;106:1085–1088.
54. Tainter M, Cutting W, Wood D. Dinitrophenol. Studies of blood, urine and tissues on dogs on continued medication and after acute fatal poisoning. *A.M.A Arch Pathol.* 1934;18:881.
55. Evans RG, Goddard D, Eppel GA, O’Connor PM. Stability of tissue PO₂ in the face of altered perfusion: a phenomenon specific to the renal cortex and independent of resting renal oxygen consumption. *Clin Exp Pharmacol Physiol.* 2011;38:247–254.
56. Johannesen J, Lie M, Kiil F. Renal energy metabolism and sodium reabsorption after 2,4-dinitrophenol administration. *Am J Physiol.* 1977;233:F207–F212.
57. Lassen NA, Munck O, Thaysen JH. Oxygen consumption and sodium reabsorption in the kidney. *Acta Physiol Scand.* 1961;51:371–384.
58. Minners J, van den Bos EJ, Yellon DM, Schwalb H, Opie LH, Sack MN. Dinitrophenol, cyclosporin A, and trimetazidine modulate preconditioning in the isolated rat heart: support for a mitochondrial role in cardioprotection. *Cardiovasc Res.* 2000;47:68–73.
59. Korde AS, Pettigrew LC, Craddock SD, Maragos WF. The mitochondrial uncoupler 2,4-dinitrophenol attenuates tissue damage and improves mitochondrial homeostasis following transient focal cerebral ischemia. *J Neurochem.* 2005;94:1676–1684.
60. Phillips L, Singer MA. Peripheral neuropathy due to dinitrophenol used for weight loss: something old, something new. *Neurology.* 2013;80:773–774.
61. Hochman ME, Watt JP, Reid R, O’Brien KL. The prevalence and incidence of end-stage renal disease in Native American adults on the Navajo reservation. *Kidney Int.* 2007;71:931–937.
62. Sayarlioglu H, Erkoc R, Dogan E, Topal C, Algun E, Erem C, Atmaca H, Kocak E, Yilmaz R, Erdol H, Cinal A. Nephropathy and retinopathy in type 2 diabetic patients living at moderately high altitude and sea level. *Ren Fail.* 2005;27:67–71.

Novelty and Significance

What Is New?

- This study demonstrates that increased kidney oxygen consumption and subsequent kidney tissue hypoxia are sufficient to induce nephropathy.

What Is Relevant?

- Most conditions associated with development of kidney disease also have confounding factors such as hyperglycemia or oxidative stress,

which, per se, could contribute to disease development. By using 2,4-dinitrophenol to induce kidney hypoxia, we were able to demonstrate that hypoxia alone is sufficient to initiate disease development.

Summary

The present study provides further support that hypoxia is a common pathway for development of chronic kidney disease.

KIDNEY HYPOXIA, DUE TO INCREASED OXYGEN CONSUMPTION, INDUCES
NEPHROPATHY INDEPENDENTLY OF HYPERGLYCEMIA AND OXIDATIVE
STRESS

Malou Friederich-Persson¹, Erik Thörn², Peter Hansell¹, Masaomi Nangaku³, Max Levin² and
Fredrik Palm^{1,4,5}

¹Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden

²The Wallenberg Laboratory for Cardiovascular and Metabolic Research, Gothenburg
University, Gothenburg, Sweden

³University of Tokyo, Tokyo, Japan

⁴Department of Medical and Health Sciences, Linköping University, Linköping, Sweden

⁵Center for Medical Image Science and Visualization, Linköping University, Linköping,
Sweden

Address correspondence to:

Malou Friederich-Persson
Department of Medical Cell Biology
Uppsala University
Biomedical Center, Husargatan 3
SE-751 23
Uppsala, Sweden
Phone: +46(0)18 471 4378
Fax: +46(0)18 471 4938
E-mail: Malou.Friederich@mcb.uu.se

Online data supplement.

Animals, treatments, surgical procedures and *in vivo* measurements.

Male Sprague-Dawley rats (Charles River Laboratories, Sulzfeld, Germany, n=32) were housed in a temperature controlled environment and had free access to standard rat chow (R3, Ewos, Södertälje, Sweden) and tap water and randomly assigned to either treatment with DNP (30 mg/kg bw/day for 30 days by oral gavage) or vehicle (1 ml 1.5% methylcellulose). Blood glucose was measured using a reagent test strip (MediSense, Bedford, MA, USA) in a blood sample obtained from the cut tip of the tail.

Animals were anaesthetized with sodium thiobutabarbital (Inactin, 120 mg/kg bw, i.p) and placed on a servo-controlled heating pad, tracheotomy performed and polyethylene catheters placed in the femoral artery and vein to allow monitoring of mean arterial pressure (MAP, Statham P23dB, Statham Laboratories, Los Angeles, CA, USA), blood sampling and infusion of saline (5 ml/kg bw/h). The left kidney was exposed by a subcostal flank incision and immobilized. Total RBF was measured by and ultrasound flow probe (Transonic Systems Inc., Ithaca, NY, USA). The left ureter and bladder was catheterized to allow for timed urine sampling and urinary drainage, respectively. After surgery, the 40-minute recovery period was followed by a 40-minute experiment period at the end of which a blood sample was carefully drawn from the renal vein and analyzed in a blood gas analyzer (IStat, Abbott Laboratories, Abbott Park, IL, USA). Thereafter, 0.1 ml DNP-saturated saline (0.6 µg/kg bw) was administered as a bolus intravenous infusion and kidney function monitored for an additional 40 minutes in order to determine any acute nephrotoxicity of DNP. Urinary Na⁺ concentration was determined by flame photometry (IL943, Instrumentation Laboratory, Milan, Italy) and urinary protein excretion by DC Protein Assay (Bio-Rad Laboratories, CA, USA). Kidney tissue pO₂ was measured using Clark-type oxygen electrodes (Unisense, Aarhus, Denmark). GFR was measured by clearance of ³H-inulin (185 kBq bolus followed by 185 kBq/kg bw/h, American Radiolabelled Chemicals, St Louis, MO, USA). Kidney QO₂ was estimated from the arterio-venous difference in oxygen content (O_{2ct}) multiplied by RBF according to the formula $O_{2ct} = ([Hb] * \text{oxygen saturation} * 1.34 + pO_2 * 0.003) * RBF$. Kidney oxygen delivery (DO₂) was calculated according to arterial O_{2ct}*RBF. Tubular Na⁺ transport (TNa⁺) was calculated from $[Na^+]_{\text{plasma}} * GFR - [Na^+]_{\text{urine}} * \text{urine flow rate}$ and electrolyte transport efficiency according to TNa^+ / QO_2 .

Kidney content of glucose and glycogen.

An enzyme solution containing luciferase was applied to a kidney cryosection in a microscope placed in a dark box, resulting in emission of photons in proportion to the concentration of the studied metabolite (either glucose or glycogen).¹ The photons was registered by a photon-counting camera (C2400-47, Hamamatsu Photonics, Japan) mounted on the microscope (Axiovert 135 M, Carl Zeiss, Germany). The light intensity (gray value) in different parts of the resulting digital bioluminescence image reflects the local metabolite concentrations. A dark field image of the same section was obtained to outline histological structures in the corresponding bioluminescence image. To calibrate the bioluminescence signal, standards were made by dissolving different concentrations of glucose or glycogen in physiological saline with 8% low-molecular-weight gelatin. The solution was frozen, and 16 µm cryosections were cut, analyzed exactly as the tissue sections and grey values in the different standards used to convert grey values in kidney sections into metabolite concentrations (µmol/gram wet weight). Photon-counting images were obtained using Wasabi software (Hamamatsu Photonics). Further analysis of images was performed using KS400 software (Carl Zeiss).

Immunohistochemistry

A separate set of animals (n=6-8 per group) was used for tissue collection and subsequent analysis of histological alterations (vimentin expression and infiltration of ED1 positive cells) and mRNA analysis of hypoxia-related genes. The animals were anesthetized with Inactin and a polyethylene catheter placed in the carotid artery followed by infusion of 20 ml ice-cold phosphate buffered saline and the renal vein cut opened in order to facilitate complete drainage of the kidneys. The kidneys were dissected on ice and placed in methyl Carnoy's fixative (methanol:chloroform:acetic acid, 6:3:1) or snap frozen using liquid nitrogen. Carnoy-fixed tissue sections were paraffin-embedded and indirect immunoperoxidase methods were used to identify vimentin (a marker of tubular injury) using mouse monoclonal antibody V9 (Dako, Carpinteria, CA, USA) and monocytes and macrophages using mouse monoclonal antibody ED-1 (Chemicon, Temecula, CA, USA) on 3 μ m thick sections. Computer-based counting of ED1 positive infiltrating cells was performed utilizing image J software (NIH, Bethesda, MD, USA) and the number of vimentin positive tubules surrounded by healthy tubules was counted as previously described.² Nuclei were not counterstained. Quantification was performed in a blinded manner using 20 randomly selected fields of cortex per cross-section (x100).

Thiobarbituric acid reactive substances (TBARS) and protein carbonyls

50 μ l cortex homogenate was added to 42 μ l 0.67% thiobarbituric acid, vortexed and heated to 97°C for 60 minutes. After cooling the samples on ice 50 μ l methanol:1 mM NaOH (91:9) was added, the samples vortexed and centrifuged at 3000 rpm for 5 minutes at roomtemperature. The supernatant was analyzed for fluorescence using excitation/emission of 532/553 nm and the concentration calculated using a standard curve of malondialdehyde ranging from 250 nM to 0.98 nM and multiplied with the urine flow to be displayed as TBARS excretion.³

Protein carbonyls was analyzed using a kit from Cayman Chemicals, Ann Arbor, Michigan, USA according to the manufacturer's instructions.

Expressions of oxygen-regulated genes

Total RNA was extracted from kidney homogenates with Isogen RNA isolation kit (Nippon Gene, Tokyo, Japan). Superscript II reverse transcriptase (Life Technologies BRL, Rockville, MD, USA) was used to synthesize cDNA from total RNA and levels were assessed by real-time quantitative PCR using SYBR green PCR reagent (Qiagen, Hilden, Germany) and the iCycler PCR system (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. Briefly, amplification reactions consisted of 1 μ l of cDNA, 12.5 μ l of the Universal 2 \times PCR mastermix (Qiagen, Hilden, Germany), and 5 μ L each of the specific primers. Primer concentrations in the final volume of 25 μ L were 500 nmol/l. In control experiments, no false positives were detected and the variance between each of the replicates was within 5% and all PCR reactions were performed in triplicate. Ct, or threshold cycle, was used for relative quantification of the input target number. The amount of Ct for control samples was considered 1. The number of Cts for other samples was subtracted from that of the control samples. The relative amount of amplified genes is given by $2^{-\Delta Ct}$ after normalization for β -actin. The PCR primers for heme oxygenase (HO-1), vascular endothelial growth factor (VEGF), and erythropoietin (EPO) were the same as previously reported.⁴

Hypoxia-inducible factor system

DNP-treated animals presented with increased mRNA levels of HIF-1 α and HO-1, whereas the levels of EPO, VEGF and GLUT-1 were not significantly changed compared to controls (Table S1). An important mechanism to counteract sustained hypoxia is via the heterodimeric

transcription factor HIF. In the presence of oxygen, the α -subunit is rapidly degraded and HIF therefore remains inactive. However, during hypoxia the α -subunit escapes degradation and forms an active heterodimer with the β -subunit, resulting in transcription of genes involved in anaerobic metabolism, angiogenesis, oxygen delivery, antioxidant defenses and iron metabolism.^{5,6} In the present study, the kidney tissue hypoxia induced by DNP resulted in up-regulation of HIF-1 α and HO-1, without affecting EPO, VEGF or GLUT-1 which is in agreement with previous results.⁷ However, the increased hemoglobin levels in the DNP-treated animals might seem contradictory, but it should be noted that circulating EPO levels are regulated also by other tissues than the kidney.⁸ Furthermore, mRNA may not reflect actual EPO protein levels and the level in the seemingly hypoxic kidneys from DNP-treated animals might therefore be a result of a negative feedback mechanism involving several organ systems. The HIF system can be artificially activated independently of hypoxia by inhibiting prolyl hydroxylases. Cobalt is commonly referred to as a hypoxia mimetic due to its ability to cause accumulation of the α -subunit. Administration of cobalt has been reported to reduce proteinuria, tubulointerstitial damage and loss of peritubular capillaries in a type 2 model of diabetes. Therefore, drugs that activate HIF may prove to be beneficial in reducing kidney hypoxia and subsequent development of nephropathy.^{5,9} However, drug-induced activation of the HIF system should be thoroughly evaluated before translated into clinical practice since there are reports that HIF also can promote fibrosis in the kidney.

References

1. Levin M, Leppanen O, Evaldsson M, Wiklund O, Bondjers G, Bjornheden T. Mapping of ATP, glucose, glycogen, and lactate concentrations within the arterial wall. *Arterioscler Thromb Vasc Biol.* 2003;23:1801-1807.
2. Palm F, Nangaku M, Fasching A, Tanaka T, Nordquist L, Hansell P, Kawakami T, Nishijima F, Fujita T. Uremia induces abnormal oxygen consumption in tubules and aggravates chronic hypoxia of the kidney via oxidative stress. *Am J Physiol Renal Physiol.* 2010;299:F380-386.
3. Palm F, Cederberg J, Hansell P, Liss P, Carlsson PO. Reactive oxygen species cause diabetes-induced decrease in renal oxygen tension. *Diabetologia.* 2003;46:1153-1160.
4. Matsumoto M, Makino Y, Tanaka T, Tanaka H, Ishizaka N, Noiri E, Fujita T, Nangaku M. Induction of renoprotective gene expression by cobalt ameliorates ischemic injury of the kidney in rats. *J Am Soc Nephrol.* 2003;14:1825-1832.
5. Haase VH. Hypoxia-inducible factors in the kidney. *Am J Physiol Renal Physiol.* 2006;291:F271-281.
6. Maxwell P. HIF-1: an oxygen response system with special relevance to the kidney. *J Am Soc Nephrol.* 2003;14:2712-2722.
7. Harvey AJ, Kind KL, Thompson JG. Effect of the oxidative phosphorylation uncoupler 2,4-dinitrophenol on hypoxia-inducible factor-regulated gene expression in bovine blastocysts. *Reprod Fertil Dev.* 2004;16:665-673.
8. Weidemann A, Johnson RS. Nonrenal regulation of EPO synthesis. *Kidney Int.* 2009;75:682-688.
9. Higgins DF, Biju MP, Akai Y, Wutz A, Johnson RS, Haase VH. Hypoxic induction of Ctgf is directly mediated by Hif-1. *Am J Physiol Renal Physiol.* 2004;287:F1223-1232.

Table S1. Gene products of the hypoxia-inducible factor (HIF)-1 and HIF-regulated genes. mRNA levels of hypoxia-inducible factor (HIF-1 α), hemeoxygenase-1 (HO1-), erythropoietin (EPO), glucose transporter-1 (GLUT-1) and vascular endothelia growth factor (VEGF) in rats with and without administration of dinitrophenol (DNP) for 30 days.

Group	HIF-1 α	HO-1	EPO	GLUT-1	VEGF
Control	1.0 \pm 0.3	1.0 \pm 0.4	1.0 \pm 0.4	1.0 \pm 0.2	1.0 \pm 0.3
DNP	3.2 \pm 0.8*	4.2 \pm 0.1*	1.1 \pm 0.4	0.7 \pm 0.3	0.7 \pm 0.3

Values are normalized to controls. * denotes P<0.05 compared to untreated controls, n=6-8 in each group.