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

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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 thibutabarbital (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 (O₂ct) multiplied by RBF according to the formula O₂ct=([Hb]*oxygen saturation*1.34+pO₂*0.003)*RBF. Kidney oxygen delivery (DO₂) was calculated according to arterial O₂ct*RBF. Tubular Na⁺ transport (TNa⁺) was calculated from [Na⁺]plasma*GFR-[Na⁺]urine*urine flow rate and electrolyte transport efficiency according to TNa⁺/QO₂.

**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 µm 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 µl cortex homogenate was added to 42 µl 0.67% thiobarbituric acid, vortexed and heated to 97°C for 60 minutes. After cooling the samples on ice 50 µl 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 Arbour, 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). Supercript 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 µl of cDNA, 12.5 µl of the Universal 2×PCR mastermix (Qiagen, Hilden, Germany), and 5 µL each of the specific primers. Primer concentrations in the final volume of 25 µL 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^−ΔCt 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-1α 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.\textsuperscript{5,6} In the present study, the kidney tissue hypoxia induced by DNP resulted in up-regulation of HIF-1α and HO-1, without affecting EPO, VEGF or GLUT-1 which is in agreement with previous results.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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

Table S1. Gene products of the hypoxia-inducible factor (HIF)-1 and HIF-regulated genes. mRNA levels of hypoxia-inducible factor (HIF-1α), hemeoxygenase-1 (HO-1), 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.

<table>
<thead>
<tr>
<th>Group</th>
<th>HIF-1α</th>
<th>HO-1</th>
<th>EPO</th>
<th>GLUT-1</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0±0.3</td>
<td>1.0±0.4</td>
<td>1.0±0.4</td>
<td>1.0±0.2</td>
<td>1.0±0.3</td>
</tr>
<tr>
<td>DNP</td>
<td>3.2±0.8*</td>
<td>4.2±0.1*</td>
<td>1.1±0.4</td>
<td>0.7±0.3</td>
<td>0.7±0.3 *</td>
</tr>
</tbody>
</table>

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