TRANSLATIONAL PHYSIOLOGY

BOLD-MRI assessment of intrarenal oxygenation and oxidative stress in patients with chronic kidney allograft dysfunction

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Submitted 16 June 2006; accepted in final form 16 October 2006

Djamali A, Sadowski EA, Muehrer RJ, Reese S, Smavatkul C, Vidyasagar A, Fain SB, Lipscomb RC, Hullett DH, Samaniego-Picota M, Grist TM, Becker BN. BOLD-MRI assessment of intrarenal oxygenation and oxidative stress in patients with chronic kidney allograft dysfunction. Am J Physiol Renal Physiol 292: F513–F522, 2007. First published October 24, 2006; doi:10.1152/ajprenal.00222.2006.—Blood oxygen level-dependent (BOLD) magnetic resonance imaging (MRI) uses deoxyhemoglobin as an endogenous contrast agent for the noninvasive assessment of tissue oxygen bioavailability. We hypothesized that intrarenal oxygenation was impaired in patients with chronic allograft nephropathy (CAN). Ten kidney-transplant recipients with CAN and nine healthy volunteers underwent BOLD-MRI. Medullary R2* (MR2*) and cortical R2* (CR2*) levels (measures directly proportional to tissue deoxyhemoglobin levels) were determined alongside urine and serum markers of oxidative stress (OS): hydrogen peroxide (H2O2), F2-isoprostanes, total nitric oxide (NO), heat shock protein 27 (HSP27), and total antioxidant property (TAOP). Mean MR2* and CR2* levels were significantly decreased in CAN (increased local oxyhemoglobin concentration) compared with healthy volunteers (20.7 ± 1.6 vs. 23.1 ± 1.8/s, P = 0.03 and 15.9 ± 1.9 vs. 13.6 ± 2.3/s, P = 0.05, respectively). There was a significant increase in serum and urine levels of H2O2 and serum HSP27 levels in patients with CAN. Conversely, urine NO levels and TAOP were significantly increased in healthy volunteers. Multiple linear regression analyses showed a significant association between MR2* and CR2* levels and serum/urine biomarkers of OS. BOLD-MRI demonstrated significant changes in medullary and cortical oxygen bioavailability in allografts with CAN. These correlated with serum/urine biomarkers of OS, suggesting an association between intrarenal oxygenation and OS.

Blood oxygen level-dependent (BOLD) magnetic resonance imaging (MRI) is a noninvasive method to assess tissue oxygen bioavailability, using deoxyhemoglobin as an endogenous contrast agent (20, 58–60). Oxyhemoglobin is a diamagnetic molecule that creates no magnetic moment as oxygen molecules are bound to iron, while deoxyhemoglobin is a paramagnetic molecule that generates magnetic moments by its unpaired iron electrons (20, 40, 56, 71). Higher levels of deoxyhemoglobin result in increased magnetic spin dephasing of blood water protons and decreased signal intensity on T2* (apparent spin-spin relaxation time)-weighted MR imaging sequences (40, 56, 58–60, 71). The apparent relaxation rate (1/T2*) may be calculated as the slope of log( intensity) vs. echo time and is directly proportional to the tissue content of deoxyhemoglobin (58–60). Increased R2* levels imply increased deoxyhemoglobin (decreased oxyhemoglobin) and decreased partial pressures of oxygen (PaO2) in tissues (58–60). BOLD-MRI can therefore be used to determine intrarenal oxygen bioavailability (40). This technique has been used to investigate human and experimental models of kidney disease including aging (23, 60), diabetes (24), acute kidney transplant dysfunction (20, 65), acute ischemic kidney injury, and unilateral ureteral obstruction (5, 35, 57).

We recently demonstrated changes in medullary R2* (MR2*) levels in association with acute transplant rejection (20, 65). This led us to assess changes in intrarenal oxygenation in chronic transplant injury. We sought to determine to the development of strategies to prevent/delay its development/progression. There is evidence that both immune and nonimmune insults, including oxidative stress (OS), contribute to CAN (4, 18, 19, 43, 54, 64). OS may lead to kidney tissue injury through inflammatory, apoptotic, and fibrotic processes (2, 4, 18, 19, 29, 41, 43, 72, 73). Evidence shows that the burden of OS is increased in experimental and human CAN (4, 10, 18, 19, 68). However, it remains unknown whether intrarenal oxygenation affects OS in CAN. Insufficient oxygen supply, increased oxygen requirements, and/or inappropriate oxygen extraction could all affect OS. To date there has been no safe, noninvasive technique available to assess intrarenal oxygenation.

Blood oxygen level-dependent (BOLD) magnetic resonance imaging (MRI) is a noninvasive method to assess tissue oxygen bioavailability, using deoxyhemoglobin as an endogenous contrast agent (20, 58–60). Oxyhemoglobin is a diamagnetic molecule that creates no magnetic moment as oxygen molecules are bound to iron, while deoxyhemoglobin is a paramagnetic molecule that generates magnetic moments by its unpaired iron electrons (20, 40, 56, 71). Higher levels of deoxyhemoglobin result in increased magnetic spin dephasing of blood water protons and decreased signal intensity on T2* (apparent spin-spin relaxation time)-weighted MR imaging sequences (40, 56, 58–60, 71). The apparent relaxation rate (1/T2*) may be calculated as the slope of log( intensity) vs. echo time and is directly proportional to the tissue content of deoxyhemoglobin (58–60). Increased R2* levels imply increased deoxyhemoglobin (decreased oxyhemoglobin) and decreased partial pressures of oxygen (PaO2) in tissues (58–60). BOLD-MRI can therefore be used to determine intrarenal oxygen bioavailability (40). This technique has been used to investigate human and experimental models of kidney disease including aging (23, 60), diabetes (24), acute kidney transplant dysfunction (20, 65), acute ischemic kidney injury, and unilateral ureteral obstruction (5, 35, 57).

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whether there was a relationship between impaired intrarenal oxygenation and OS in CAN. We hypothesized that CAN, as a chronic inflammatory condition, was associated with abnormal intrarenal oxygen bioavailability and increased OS. To validate these hypotheses and to assess the effect of a possible intervention, we prospectively studied 19 subjects (10 kidney transplant recipients with CAN and 9 healthy volunteers) in a pilot analysis of BOLD-MRI, OS biomarkers, and acute angiotensin receptor blockade (ARB).

MATERIALS AND METHODS

Patients

Subjects in this study included a group of healthy volunteers (n = 9) and a group of transplant recipients with CAN (n = 10) recruited between late 2003 and mid-2005. Healthy volunteers were selected from the nephrology section and through collegial relationships at the University. Healthy volunteers had to be free from chronic illness and not taking any medications with the exception of allergy medications, birth control, antidepressants, and antianxiety medications. Kidney transplant recipients were selected from the transplant clinic at the University of Wisconsin-Madison Hospital and Clinics. They were at least 12 mo posttransplant and needed to be in stage 3 chronic kidney disease [CKD; estimated glomerular filtration rate (eGFR) between 30 and 59 ml/min (42)] based on the National Kidney Foundation classification of CKD (8). The diagnosis of CAN was clinical and defined by the presence of proteinuria (>200 mg/g creatinine), a 0.3 mg/dl rise in serum creatinine compared with baseline, and hypertension. However, in most cases, the clinical diagnosis was confirmed by pathology. To participate in the study, patients also had to be off angiotensin receptor blockers (ARBs), angiotensin-converting enzyme inhibitors [ACE (→)], statins, or diuretics.

BOLD-MRI. All participants had to be off vitamin supplements for 24 h before the BOLD-MRI and free of any acute illness at the time of the study visit.

Exclusion criteria. Potential participants with hazardous metallic implants and cardiac pacemakers, hypersensitivity to losartan or any component of the formulation, hypersensitivity to other ARBs, known transplant artery stenosis, orthostatic hypotension, or who were pregnant were excluded.

Procedure. After human subjects’ committee approval was obtained, potential participants were approached by the principal investigator or study coordinator during their regular clinic visit. Individuals were given a synopsis of the study, including risks and benefits of participation. After informed consent was obtained, participants presented to the General Clinical Research Center (GCRC) for one-half day. Participants were asked to verify that they had not been recently acutely ill and then were asked to provide a brief medical history including comorbid conditions and current medications. Baseline vital signs were then recorded, and blood and urine samples were collected before the first BOLD-MRI. Blood and urine samples were assayed for standard blood chemistries, blood urea nitrogen (BUN), creatinine, albumin, C-reactive protein, and urine protein, creatinine, and Na⁺. A urine pregnancy test was performed on all female participants of childbearing age. In addition, aliquots of serum and urine samples were assayed for biomarkers of OS as described below. Following the first BOLD-MRI, patients returned to the GCRC and received a 50-mg oral dose of losartan. They then rested for about 2 h while having their vital signs monitored and had another set of blood and urine samples collected just before the second BOLD-MRI study (Fig. 1).

BOLD-MRI Technique

MR imaging was performed with a 1.5-T system (Sigma, GE Healthcare, Waukesha, WI) and a four-element torso phased-array surface coil. BOLD-MRI was performed using a multigradient-recalled-echo sequence with 16 echoes at a gap of 1 mm and a 5-mm section thickness that was prescribed in the coronal plane as described previously (20, 65). The scanning parameters were repetition/echo time, 87 ms/8–41.8 ms; flip angle, 40°; bandwidth, ±62.5 kHz; field of view, 32–34 cm; matrix, 256 × 128; and number of signals acquired, one. Each set of 16 T2*-weighted images was acquired during an 11-s breathhold. Three sections were obtained in the coronal plane for each transplanted kidney as the coronal planes have shown to present less variability (5, 65). Mean R²* values were recorded in units of 1/s. Color R²* maps were generated and regions of interest (ROIs) were placed in the medulla and cortex using Funtool on the Advantage workstation (GE Healthcare). The color R²* map was windowed to provide a visual range of R²* values from low to high, with blue representing the lowest R²* value (area of lowest deoxyhemoglobin concentration) and red representing the highest R²* value (area of highest deoxyhemoglobin concentration). Six to ten ROIs were placed in the medulla; and 6–10 ROIs were placed in the cortex, resulting in 12–20 ROIs per kidney per subject.

Biomarkers of OS

To avoid significant bias from assay variability, each sample was measured in triplicate and the experiment was repeated three times for each of the biomarkers assessed.
Heat shock protein 27. Serum and urine heat shock protein 27 (HSP27) levels were measured using an ELISA Kit from Stressgen Biotechnologies (EKS-500, Victoria, BC) according to the manufacturer’s recommendations. Briefly, recombinant HSP27 was serially diluted from 25–0.39 ng/ml with the sample diluent to generate a standard curve. The anti-HSP27 rabbit polyclonal antibody (pAb), the hors eradish peroxidase-conjugated anti-rabbit IgG antibody (cAb), and wash buffer were diluted based on kit specifications. One hundred microliters of each dilution of standard were added in duplicate to the HSP27-specific mouse monoclonal antibody-coated plate. One hundred microliters of sample diluent were added to serve as the blank. Fifty microliters of each diluted pAb were added to all wells except the blank. The plate was incubated for 1 h at room temperature (RT). After incubation, plates were washed six times with 1× wash buffer. One hundred microliters of the diluted cAb were added to all wells except the blank. The plate was incubated for 30 min at RT and washed again. The plate was developed for 15 min at RT in the dark with 100 μl/well of TMB solution and terminated with 100 μl/well of acid stop solution. The plate was read at 450 nm using a Synergy HT Multi-Detection Microplate Reader and analyzed using KC4 software (Bio-Tek). HSP27 levels were reported in picograms per milliliter.

Nitric oxide (total). Serum and urine total nitric oxide (NO) levels (nitrate/nitrite) were measured using a NO (total) Detection Kit from Stressgen Biotechnologies (EKS-310, Victoria, BC) according to the manufacturer’s recommendations. Briefly, the reaction buffer, NADH reagent, and nitrate reductase enzyme were diluted based on kit specifications. The nitrate standard solution was serially diluted from 100 to 3.125 μM. The reaction buffer to generate a standard curve. Serum and urine samples were diluted 1:10 and 1:50, respectively, in reaction buffer before addition to the plate. Fifty microliters of each diluted sample and standard were added in duplicate to the plate, including 50 μl of reaction buffer to serve as the zero standard. Twenty microliters of reaction buffer were added in duplicate to serve as the blank. Twenty-five microliters of NADH and 25 μl of nitrate reductase enzyme were subsequently added to all wells except the blanks. The plate was covered, the contents were mixed gently, and the plate was incubated at 37°C for 30 min. Fifty microliters of Griess reagent I and 50 μl of Griess reagent II were subsequently added to all wells except the blanks. The plate was covered, the contents were mixed gently, and the plate was incubated at RT for 10 min. The plate was read at 550 nm using a Synergy HT Multi-Detection Microplate Reader and analyzed using KC4 software (Bio-Tek). Total NO concentrations were reported in nanomolar (nM).

Hydrogen peroxide. Serum and urine hydrogen peroxide (H2O2) levels were measured using an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit from Invitrogen (A-22188, Carlsbad, CA) according to the manufacturer’s recommendations. Briefly, serial 50-μl dilutions of H2O2 (standard curve) and urine supernatants were placed into individual wells of a 96-well microplate. A 50-μl volume of the Amplex Red reagent/HRP solution was then added to each microplate well containing the standards and samples. The plate was incubated at RT for 30 min, protected from light. The plate was read at 590 nm using a Synergy HT Multi-Detection Microplate Reader and analyzed using KC4 software (Bio-Tek). H2O2 concentrations were reported in micromolar (μM).

8-iso PGF2α. Serum and urine 8-iso PGF2α (F2-isoprostanes) levels were measured using an ELISA Kit from Stressgen Biotechnologies (EKS-200) according to the manufacturer’s recommendations. Briefly, assay buffer and wash buffer were diluted based on kit specifications. The 8-iso PGF2α standard solution was serially diluted from 100,000 to 6.1 pg/ml with assay buffer to generate a standard curve. One hundred microliters of each dilution of standard were added in duplicate to the goat anti-rabbit IgG polyclonal antibody-coated plate. Fifty microliters of assay buffer were added to all empty wells followed by the addition of 50 μl of samples (serum and urine) in duplicate (1:4). Fifty microliters of alkaline phosphate-conjugated PGF and 50 μl of rabbit polyclonal PGF were added to all wells except the blanks. The plate was covered and shaken for 2 h at RT. The plate was then washed three times with wash buffer. Two hundred microliters of p-Npp substrate solution were added to each well and incubated for 45 min at RT. The reaction was terminated with 50 μl/well of Stop solution. The plate was read at 405 nm using a Synergy HT Multi-Detection Microplate Reader and analyzed using KC4 software (Bio-Tek). F2 isoprostane concentrations were reported in picograms per milliliter.

Total antioxidant property. Serum and urine total antioxidant property (TAOP) was measured using a Cayman Chemical antioxidant detection kit (70001, Ann Arbor, MI) according to the manufacturer’s recommendations. Briefly, serum (1:25) and urine (1:15) were diluted in assay buffer. Trolox was also diluted in the assay buffer to yield the standard curve from 0, 0.044, 0.088, 0.135, 0.18, 0.225, and 0.330 μM. Ten microliters of diluted standard or samples were added to each well in duplicate. Ten microliters of metmyoglobin and 150 μl of chromogen were also added to each well. Forty microliters of H2O2 solution (441 μM) were added to each well to initiate the reaction. The plate was incubated on a shaker for 5 min at RT and read at 750 nm using a Synergy HT Multi-Detection Microplate Reader and analyzed using KC4 software (Bio-Tek). TAOP concentrations were reported in millimolar.

Statistical Analyses

This was a pilot study to determine whether intrarenal oxygenation measured by BOLD-MRI was different between healthy volunteers and renal transplant recipients with CAN, whether there was a correlation between intrarenal oxygenation and biomarkers of OS in patients with CAN, and whether a single dose of losartan could alter intrarenal oxygenation. Numerical data between the groups were compared using Student’s t-test or the Wilcoxon rank sum test for parametric and nonparametric data, respectively. Paired tests were used to compare the effect of losartan. All numerical data, including R2* (1/s) values, are expressed as means ± SD unless otherwise indicated. To evaluate the frequency of nominal data between groups, χ2-analyses of contingency tables or Fisher’s exact test was performed. Multiple linear regression and Spearman rank order correlation analyses were performed to determine the strength of association between serum and urine biomarkers of OS and medullary, cortical, and medullary: cortical R2* values. These analyses were performed using SigmaStat (SPSS, Chicago, IL) and MedCalc Statistical Software (Mariakerke, Belgium). A P value ≤0.05 was considered significant.

RESULTS

Subject Characteristics

We studied 10 patients with CAN and 9 control subjects. Baseline characteristics are depicted in Table 1. The average time between the transplant and BOLD-MRI imaging was 3.9 ± 1.1 yr for the patients with CAN. All patients had clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinically indicated biopsies before the study (62, 63). Only one patient had a previous history of acute rejection with return of clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinical CAN, and 6 of 10 had biopsy-proven CAN based on clinicalCAN
imaging. Five patients received living donor transplants, and five patients had undergone deceased donor transplantation. Average donor age was 38.5 ± 3.3 yr (P = 0.3 compared with healthy volunteers). Patients were all first-time transplant recipients, except for one patient (second transplant). Induction immunosuppression included basiliximab in five patients, alemtuzumab in three patients, and OKT3 in two patients. Maintenance immunosuppression was a combination of a calcineurin inhibitor (CNI; cyclosporin A/tacrolimus in 5/5 patients), mycophenolate mofetil, and prednisone.

The CAN group was significantly older (49.3 ± 3.9 vs. 32.6 ± 4.3 yr, P = 0.01) and had significantly increased serum creatinine (1.7 ± 0.1 vs. 0.9 ± 0.1 mg/dl, P < 0.0001), proteinuria (237.35 ± 96.12 mg/dl, P = 0.005), and fractional excretion of Na⁺ levels (1.4 ± 0.3 vs. 0.7 ± 0.1%, P = 0.02) compared with the control population. The CAN group also had lower levels of serum albumin (4.2 ± 0.1 vs. 4.5 ± 0.1 g/dl), urine pH (6 ± 0.2 vs. 6.9 ± 0.3, P = 0.03), and urine specific gravity (1.010 ± 0.002 vs. 1.018 ± 0.002, P = 0.002) compared with healthy controls. Blood tacrolimus and cyclosporin A levels were 4 ± 1 and 86.8 ± 26.4 ng/ml, respectively. There were no patients or healthy controls on vitamins, over-the-counter antioxidants, or antihistamines. Two healthy volunteers were on venlafaxine (75 mg daily), and one was on a birth control pill (ethinyl estradiol/levonorgestrel 20 μg/0.1 mg). There were no significant differences in gender, ethnicity, mean arterial blood pressure, hematocrit, and C-reactive protein levels between the two groups.

**BOLD-MRI Analyses**

**Coronal color R2* maps.** Representative color maps of R2* values from a healthy volunteer and a kidney allograft with CAN in the coronal planes are presented in Fig. 2, A–D. The blue color represents the lowest R2* value (low deoxyhemoglobin concentration), and green, yellow, and red show increasing values of R2* (increased deoxyhemoglobin concentration). Usually, there are more blue areas in the cortex and more green, yellow, and red spots in the medulla, corresponding to the corticomedullary oxygen gradient. There was a loss of corticomedullary oxygen differentiation in CAN. Increasing blue areas (decreased deoxyhemoglobin) appeared in both the medulla and the cortex of kidney allografts with CAN.

**Medullary, cortical, and medullary: cortical R2* values.** Bar graphs of medullary (MR2*), cortical (CR2*), and medullary: cortical R2* ratios (MCR2*) are presented in Fig. 2E. Mean MR2* levels were significantly (~10%) decreased in CAN (increased local oxygen bioavailability) compared with healthy volunteers (20.7 ± 6.1 vs. 23.1 ± 8.5, P = 0.03). Similarly, mean CR2* levels were decreased (by 14%) in CAN (15.9 ± 3.1 vs. 13.6 ± 2.3, P = 0.05). Intrinsic regulatory mechanisms maintain intrarenal oxygen bioavailability and perfusion gradients between the medullary and cortical regions of the kidney (9, 21, 25, 26). We have previously defined the MCR2* ratio as a surrogate marker of normalized intrarenal oxygen bioavailability (20). The MCR2* ratios were nearly unchanged between the two groups (1.5 ± 0.2 and 1.53 ± 0.2, P = 0.67).

**Biomarkers of OS**

We simultaneously assayed serum and urine levels of H2O2, total NO, F₂-isoprostanes, HSP27, and TAOP. Each of these molecules may represent some component of OS from both pro- and antioxidant states, thus providing a panel of kidney-related ROS.

Surrogate markers of OS were measured in the serum and urine of all subjects at baseline and following losartan. H₂O₂ levels were significantly higher in the serum (24.8 ± 9.3 vs. 5.9 ± 1.6 μM, P = 0.04) and urine (8.3 ± 1.6 vs. 6.2 ± 2.3 μM, P = 0.05) of patients with CAN (Fig. 3A). Similarly, serum HSP27 levels were increased in patients with CAN (8.6 ± 2.9 vs. 2.3 ± 1.1 pg/ml, P = 0.02, Fig. 3C). Conversely, urine NO levels and TAOP were significantly increased in healthy volunteers (945 ± 149 vs. 443 ± 134 nM, P = 0.009 and 67.6 ± 12.8 vs. 29.4 ± 5.5 mM, P = 0.03, Fig. 3, B and E, respec-

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**Table 1. Baseline characteristics**

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<th></th>
<th>Normal</th>
<th>CAN</th>
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<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>10</td>
<td>N/A</td>
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<tr>
<td>Gender, male/female</td>
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<td>7/3</td>
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<td>Caucasian ethnicity</td>
<td>9/9</td>
<td>7/10</td>
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<td>Age, yr</td>
<td>32.6 ± 4.3</td>
<td>49.3 ± 3.9</td>
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<td>BMI, kg/m²</td>
<td>27 ± 1.7</td>
<td>28.3 ± 1.2</td>
<td>0.51</td>
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<td>MAP, mmHg</td>
<td>87.7 ± 2.5</td>
<td>95.5 ± 3.5</td>
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<td>Serum creatinine, mg/dl</td>
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<tr>
<td>Estimated GFR (MDRD), ml/min</td>
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<td>Hematocrit, %</td>
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<td>Albumin, g/dl</td>
<td>4.5 ± 0.1</td>
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Calcineurin inhibitor levels, ng/ml

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<th>CAN</th>
<th>P Value</th>
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<tr>
<td>TAC (n = 5)</td>
<td>4 ± 1</td>
<td></td>
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<tr>
<td>CsA (n = 5)</td>
<td>86.8 ± 26.4</td>
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<tr>
<td>CRP, mg/dl</td>
<td>0.9 ± 0.8</td>
<td>0.2 ± 0.1</td>
<td>0.4</td>
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<td>Urine protein:creatinine, mg/g</td>
<td>96 ± 12.1</td>
<td>237 ± 35.3</td>
<td>0.005</td>
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<tr>
<td>Urine pH</td>
<td>6.9 ± 0.3</td>
<td>6.5 ± 0.2</td>
<td>0.03</td>
</tr>
<tr>
<td>Urine specific gravity</td>
<td>1.018 ± 0.002</td>
<td>1.010 ± 0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>Urine Na, meq/l</td>
<td>109.5 ± 17.9</td>
<td>83.9 ± 15.7</td>
<td>0.31</td>
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<tr>
<td>FeNa, %</td>
<td>0.7 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>0.02</td>
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</table>

Values are means ± SD. CAN, chronic allograft nephropathy; BMI, body mass index; CRP, C-reactive protein; CsA, cyclosporin A; FeNa, fraction of excretion of Na⁺; MAP, mean arterial pressure; GFR, glomerular filtration rate; MDRD, Modification of Diet in Renal Disease formula; TAC, tacrolimus; N/A, not applicable.
tively). Serum and urine F2-isoprostane levels were not statistically different between the two groups (Fig. 3D). Inter- and intra-assay variability (coefficient of variation) was <15% for all assays. However, when considered as a whole, these experiments demonstrated a prooxidant profile in patients with CAN.

Correlation Between Intrarenal Oxygenation and OS

To determine whether there was a correlation between intrarenal oxygenation and OS in CAN, we performed multiple linear regression and Spearman rank order correlation analyses using MR2*, CR2*, and MCR2* as dependent variables and serum and urine biomarkers of OS as independent variables (Table 2). Multiple linear regression analyses showed that MR2* levels could be predicted by a combination of CR2*, urine F2-isoprostane, and NO levels ($R^2 = 0.97$, $P < 0.001$, Table 2), whereas CR2* levels could be predicted by a combination of urine NO and H2O2 levels ($R^2 = 0.71$, $P < 0.03$, Table 2). Conversely, urine F2-isoprostanes, NO, HSP27, and H2O2 levels could be predicted by various combinations involving MR2* and CR2* levels. Interestingly, mean arterial pressure (MAP) could be accurately predicted by a linear regression formula involving MCR2*, hematocrit, urine F2-isoprostanes, and H2O2 levels as variables ($R^2 = 0.92$, $P < 0.03$).

Spearman rank order correlation analyses demonstrated a significant correlation between MR2* levels and age, CR2*, serum HSP27, and urine NO (Table 2) as well as between CR2* and MCR2* levels and age, urine NO, and urine F2-isoprostanes. MAP correlated with eGFR, hemoglobin, serum F2-isoprostanes, and total NO levels (Table 2). There was no correlation between CNI dose/blood levels and intrarenal oxygenation or OS biomarkers, and we failed to demonstrate a statistical correlation between MR2* and CR2* levels and OS biomarkers in healthy volunteers (data not shown). Overall, these analyses showed a significant correlation between intrarenal oxygenation measured by BOLD-MRI and serum/urine biomarkers of OS.

Impact of Losartan on Intrarenal Oxygenation

ARBs, including losartan, may improve/delay disease progression in CAN (7, 11, 44). Losartan has also antioxidant properties (12, 16) that could lead to decreased cortical oxygen consumption through increased local NO availability (4). We hypothesized that losartan might improve BOLD-MRI-measured intrarenal oxygenation. In this pilot study, we decided to first examine the short-term effects of the drug. Because the terminal half-life of the drug, taken orally, is 2 h (PDR-2006), BOLD-MRI as well as blood and urine sample analyses were repeated 1.5 to 2.5 h after patients ingested losartan (50 mg).

Losartan was associated with a modest, yet significant decrease in CR2* levels in healthy volunteers (15.5 ± 1.9 prelosartan vs. 14.5 ± 1.3/s postlosartan, $P = 0.05$, Fig. 4A2). This corresponded to increased MCR2* levels in these subjects (1.5 ± 0.2 vs. 1.6 ± 0.2, $P = 0.04$, Fig. 4A3). MR2*, CR2*, and MCR2* levels did not change significantly in patients with CAN. Similarly, losartan had no significant impact on serum/urine levels of OS biomarkers (data not shown). Finally, there was no significant change in urine Na+ excretion rates (Fig. 4B).

DISCUSSION

The current study shows that oxyhemoglobin bioavailability measured by BOLD-MRI is increased in the medulla and cortex.
of allografts with CAN. Impaired intrarenal oxygenation was associated with impaired OS balance as there was a significant relationship among MR2*, MC2*, and MCR2* levels and urinary and serum biomarkers of OS. Finally, a single oral dose of losartan increased cortical oxygenation in healthy volunteers but had minimal effect in kidneys from patients with CAN.

**Table 2. Correlation between intrarenal oxygenation and oxidative stress in CAN**

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Independent variable</th>
<th>Correlation coefficient</th>
<th>P</th>
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<tbody>
<tr>
<td>MR2*</td>
<td>Age</td>
<td>0.80</td>
<td>0.002</td>
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<tr>
<td></td>
<td>CR2*</td>
<td>0.78</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Serum HSP27</td>
<td>0.73</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Urine nitric oxide</td>
<td>-0.70</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>0.65</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Urine nitric oxide</td>
<td>-0.68</td>
<td>0.03</td>
</tr>
<tr>
<td>CR2*</td>
<td>Urine F2-isoprostanes</td>
<td>0.88</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>0.65</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Urine nitric oxide</td>
<td>-0.68</td>
<td>0.03</td>
</tr>
<tr>
<td>MCR2*</td>
<td>Urine F2-isoprostanes</td>
<td>0.88</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>MAP, mmHg</td>
<td>0.67</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>eGFR</td>
<td>-0.66</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Hgb</td>
<td>-0.67</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Serum F2-isoprostanes</td>
<td>0.67</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Serum nitric oxide</td>
<td>-0.6</td>
<td>0.05</td>
</tr>
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</table>

BOLD, Blood oxygen level dependent; UNO, urinary nitric oxide; HSP27, heat shock protein 27; H2O2, hydrogen peroxide; Ht, hematocrit; OS, oxidative stress.
disease (Table 3) (5, 20, 23, 35, 57, 60, 65, 69). These studies have shown impaired regional oxygenation patterns in unilateral ureteral obstruction (57), acute ischemic kidney injury (6, 35), experimental hypertension (57), acute kidney transplant rejection (20, 65), and aging (69). While ischemic models of kidney injury (acute ischemic renal failure and aging) have understandably resulted in increased MR2* and CR2* levels (decreased regional oxyhemoglobin concentrations) (6, 35, 69), inflammatory and immune-mediated kidney injury have been associated with decreased MR2* (increased oxyhemoglobin) values, probably as a result of decreased tissue oxygen extraction (20, 57, 65) (Table 3). In addition, studies inhibiting Na+/H+ reabsorption (by furosemide, water diuresis, and tempol) have shown increased medullary oxygenation through reduced nephron workload (23, 57, 60). However, there have been no studies assessing regional oxygenation patterns in CAN.

The current study shows decreased MR2* and CR2* levels in CAN, suggesting that oxygen bioavailability (oxyhemoglobin levels) is increased in both the medulla and cortex of kidney allografts. Enhanced medullary and cortical oxyhemoglobin concentrations may result from increased oxygen delivery, decreased oxygen extraction/consumption, or a combination of these two factors. It is unlikely that allografts with CAN have greater oxygen delivery through increased perfusion (34, 67). We have recently demonstrated that both medullary and cortical blood flow rates are decreased in kidney allografts undergoing acute rejection (unpublished data). Although the current study has not examined regional blood flow rates, it is

**Table 3. BOLD-MRI, MR2*, and CR2* levels in experimental and human studies**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Potential Mechanism</th>
<th>Species</th>
<th>MR2*</th>
<th>CR2*</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Unilateral ureteral obstruction</td>
<td>Inflammation</td>
<td>Pig</td>
<td>↓</td>
<td>↑</td>
<td>57</td>
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<tr>
<td>Acute ischemic renal failure</td>
<td>Ischemia</td>
<td>Swine</td>
<td>↑</td>
<td>↑</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pig</td>
<td>↑</td>
<td></td>
<td>35</td>
</tr>
<tr>
<td>Tempol in hypertension</td>
<td>Free radical scavenger</td>
<td>Rat</td>
<td>↓</td>
<td>No change</td>
<td>57</td>
</tr>
<tr>
<td>Furosemide</td>
<td>Na⁺ reabsorption</td>
<td>Human</td>
<td>↓</td>
<td>No change</td>
<td>23</td>
</tr>
<tr>
<td>Water diuresis</td>
<td>Na⁺ reabsorption</td>
<td>Human</td>
<td>↓</td>
<td>No change</td>
<td>60</td>
</tr>
<tr>
<td>Age</td>
<td>Ischemia</td>
<td>Human</td>
<td>↑</td>
<td></td>
<td>69</td>
</tr>
<tr>
<td>Acute transplant rejection</td>
<td>Immune/inflammation</td>
<td>Human</td>
<td>↓</td>
<td>No change</td>
<td>65</td>
</tr>
<tr>
<td>CAN</td>
<td>Immune/inflammation</td>
<td>Human</td>
<td>↓</td>
<td></td>
<td>20</td>
</tr>
</tbody>
</table>

MRI, magnetic resonance imaging.
unlikely that increased intrarenal oxygen bioavailability in CAN is secondary to greater renal blood flows. We believe that the mechanisms of decreased oxygen uptake in CAN may include salt wasting and tubular damage, inflammation and OS, decreased GFR, or a combination of these factors.

Tubular dysfunction and/or dropout is a histopathological hallmark of CAN (70). Renal oxygen consumption is largely dedicated to Na\(^+\) reclamation (9, 22, 53), of which the majority occurs in the cortex with adjustment of the final urine Na\(^+\) content in the medulla (9, 22, 53). Urine concentrating ability is impaired in CKD, resulting in isosthenuria and increased Na\(^+\) excretion, a finding consistent with our observations. It is therefore likely that salt wasting (increased fractional Na\(^+\) excretion and decreased urine specific gravity) in CAN contributed to increased oxygen bioavailability. Furthermore, it has been recently shown that increases in luminal Na\(^+\) concentration and/or flow rate can increase the generation of superoxide in the medullary thick ascending limb and reduce NO bioavailability, thereby increasing local OS (1). It is therefore possible that salt wasting results in OS, with further limitation of O\(_2\) uptake. Congruent with this hypothesis, BOLD-MRI studies have shown that decreased nephron workload through inhibition of Na\(^+\) reabsorption may increase medullary oxygen bioavailability experimentally (23, 24, 60).

CAN is a state of chronic inflammation/OS. This proinflammatory/oxidant milieu results from immune and nonimmune insults, including graft-infiltrating cells, proinflammatory cytokines, glomerular hyperfiltration, and hypertension. These insults result in tubulointerstitial injury and atrophy (2, 4, 18, 19, 29, 41, 43, 72, 73) and the progressive reduction in nephron mass could lead in turn to salt wasting, OS, and decreased oxygen uptake and consumption by tubules, creating a “vicious circle” (Fig. 5). Finally, a decrement in GFR may be a protective mechanism to reduce oxygen consumption in the medulla (9). It is thus likely that multiple factors (decreased GFR, OS, inflammation, and salt wasting) contribute to increased intrarenal oxygen availability in CAN.

Other transplant-related factors may affect intrarenal oxygen bioavailability. For example, CNIs may affect intrarenal perfusion and decrease urinary NO levels (7). Similarly, sympathetic denervation in transplanted kidneys results in a significant decrease in local norepinephrine concentrations (28), with a potential effect on intrarenal oxygen bioavailability. However, experimental studies suggest that the medullary blood flow may be insensitive to sympathetic nerve activity (25) and that changes in renal vascular resistance are not explained by denervation (27). As far as CNI use and oxygen bioavailability are concerned, we have been unable to show a correlation between the dose and/or the blood level of CNIs and MR2* or CR2* values in both acute (20) and chronic allograft dysfunction (current study, data not shown). A group of kidney transplant recipients with similar GFRs (stage 3 CKD) and no CNI therapy would have been an ideal control group, but this was out of the scope of this study.

OS is increased in CAN (4, 10, 18, 19, 68), but the relationship between intrarenal oxygenation and OS remains obscure. We assayed a number of different biomarkers to complete an OS profile for this pilot study. Urinary H\(_2\)O\(_2\) levels may be a valuable biomarker of OS in certain conditions (30, 76). NO is a free radical synthesized by endothelial and inducible NO synthase enzymes (39). Detrimental effects of NO are observed when it reacts with the superoxide anion to form peroxynitrite (ONOO\(^-\)), a potent prooxidant molecule (49). In the kidney, however, NO counters the effects of the superoxide anion and H\(_2\)O\(_2\) (26), balancing out potential OS injury. Isoprostanes are biologically active products of arachidonic acid metabolism formed by nonenzymatic lipid peroxidation (49). Urinary F\(_2\)-isoprostanes provide a noninvasive index of lipid peroxidation in patients with obesity, hypertension, and cardiovascular disease (15, 26, 48, 55). Finally, HSP27 is a constitutive and inducible stress protein with antioxidant properties, including inhibition of H\(_2\)O\(_2\)-mediated cell death (18, 32, 61). Normally, high concentrations of HSP27 are present in medullary tissue (18, 38, 50). Urinary and serum H\(_2\)O\(_2\) and serum HSP27 levels were increased in CAN, whereas urinary TAOP and total NO levels were decreased, confirming an increased OS burden (Fig. 3). We believe that greater serum HSP27 levels in CAN were a result of an upregulated response to OS. There was a close correlation between biomarkers of OS and intrarenal oxygenation. Multiple linear regression and Spearman rank order correlation analyses demonstrated that MR2*, CR2*, and MCR2* could accurately be predicted by various combinations of OS markers (Table 2). It is therefore possible that intrarenal oxygenation is involved in the generation of reactive oxygen/nitrogen species (ROS/RNS) and that, conversely, increased ROS/RNS result in tubular injury and decreased oxygen extraction/consumption in CAN. Various stimuli, e.g., CNIs (7, 14), inflammation (31, 52, 74), hypoaalbuminemia (31), and acidic urine (47), could have contributed to the generation of ROS/RNS (4, 10, 18, 19, 68) (Fig. 5).

Losartan has antioxidant properties and may improve or delay progression in CAN (6, 11, 12, 16, 44). We hypothesized that losartan would improve intrarenal oxygenation by decreasing oxygen consumption through increased local NO availability (4). However, losartan did not improve intrarenal oxygenation in patients with CAN and produced only modest, yet significant decreases in CR2* levels in healthy volunteers. This may be secondary to the large burden of OS/inflammation in kidney allografts with CAN. A greater dose, multiple doses, intravenous drug delivery, or prolonged treatment may be necessary to observe differences in intrarenal oxygenation in CAN. There is also the possibility that the solitary injured

![Figure 5](http://ajprenal.physiology.org/)

In patients with CAN, intrarenal oxidative stress (OS) may be increased by calcineurin inhibitors (CNIs), decreased kidney function (↓ GFR), anemia, acidosis, greater oxyhemoglobin levels (↑ O\(_2\)), and salt wasting. In turn, a greater burden of OS may result in kidney tubular injury, preventing oxygen uptake.
kidney has changed in perfusion in response to the ARB distributed equally throughout the cortex and medulla. Unfortunately, the nature of these studies prohibited a formal assessment of perfusion by BOLD-MR techniques.

Our study was thus limited by the lack of perfusion analyses, the absence of longitudinal data on these patients, and the inability to demonstrate a causal relationship between increased OS and greater intrarenal oxyhemoglobin levels in CAN. However, despite these limitations, this pilot study is provocative in suggesting that oxygenation patterns are different in CAN and, moreover, are strongly associated with OS. Our therapeutic to date have not used oxygen delivery as an outcome of therapy, but it may well be the case that optimal tissue oxygenation, not hypoxia nor hyperoxia, is a target of therapy. The association in CAN between aberrant kidney oxygenation and OS is important and may provide leads as to how to slow loss of transplant function.

GRANTS

Parts of this work were supported by an AST/Fujisawa Award, National Institutes of Health (NIH) Grants DK-067981–02 (A. Djamali), 5K24 DK-616962–04 (B. N. Becker), R01-DK-073680 – 01 and R21/R33 DK-070243–01 (S. B. Fains, E. A. Sadowski, T. M. Grist), and MO1 RR-03186 from the General Clinical Research Centers Program of the National Center for Research Resources, NIH, and American Heart Association Grant 0235290N (M. Samaniego-Picota).

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