Proinflammatory effects of Oxidative Stress in Chronic Kidney Disease: Role of Additional Angiotensin II Blockade

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Abstract

Oxidative stress plays an important role in causing progressive kidney disease. However, it is not known if the oxidative stress and the proinflammatory state of the kidney can be favorably influenced without the reduction in blood pressure or improvement in proteinuria. We examined the influence of add-on angiotensin II receptor blockade administered as losartan 50 mg per day for one month on the oxidative stress and proinflammatory state of the kidney in patients with chronic kidney disease (CKD). All subjects were taking an angiotensin converting-enzyme inhibitor, lisinopril 40 mg per day in addition to other antihypertensive agents. Oxidative stress to lipids and proteins was measured by a HPLC assay for malondialdehyde and carbonyl concentration respectively. In addition, oxidative stress to proteins was measured by western blotting of urinary and plasma proteins after derivatizing with dinitrophenylhydrazine. Urinary inflammation was measured by MCP-1 excretion rate. Sixteen patients (10 Blacks, 6 Whites; 14 males), of average age 53 ± 9 years completed the trial. The etiology of CKD was Type 2 diabetes mellitus in 12 and glomerulonephritis in the remaining. Seated BP at baseline was 156 ± 18/88 ± 12 mm Hg requiring 3.13 ± 1.2 antihypertensive drugs, creatinine was 2.0 ± 0.8 mg/dL and proteinuria 3.6 ± 0.71 g/g creatinine/24 hours. There was no change in proteinuria or 24 hour ambulatory blood pressure with add-on angiotensin II receptor blockade with losartan therapy. Prior to losartan, urinary protein oxidation was 99% higher compared to plasma (p=0.008). Urinary albumin oxidation was 71% higher than plasma albumin (p=0.045). There was a 35% reduction in urinary albumin oxidative state with add-on losartan therapy (p=0.036). Urine malondialdehyde and plasma MDA were elevated compared to age matched controls. Urinary MDA was significantly reduced from 4.75 ± 3.23 µmol/g creatinine to 3.39 ± 2.17 µmol/g creatinine with add-on losartan therapy. However, plasma malondialdehyde or plasma oxidized proteins did not change in response to additional angiotensin II blockade. A good correlation was seen between change in urinary oxidized albumin and urinary MCP-1 level (r=0.61, p=0.012). These data demonstrate that oxidant damage to urinary protein and lipids can be reduced with
additional angiotensin II receptor blockade, independent of reductions in proteinuria or blood pressure. Urinary measurements of markers of oxidative damage, both protein carbonyls and lipid hydroperoxides are more sensitive than plasma measurements in patients with CKD. The significant association of the change in MCP1 in the urine with reduction in oxidative stress supports experimental data in animals that demonstrate the important role of the redox state in the kidney with renal fibrosis and progressive kidney damage.
Introduction

Elevated blood pressure and severe proteinuria are important predictors of progressive renal injury (14). How proteinuria results in tubulointerstitial injury, the single strongest determinant of the long-term loss of glomerular filtration rate leading to end-stage renal disease, is incompletely understood (15). The current paradigm of proteinuria as a mediator of tubulointerstitial damage is based on the observations that albumin can stimulate the production of proinflammatory cytokines in proximal tubular cells via activation of the redox sensitive gene, nuclear factor kappa B (26,27). Furthermore, chemokine expression in the kidney is modulated by the redox state, which in turn is modulated by angiotensin II. It is not known if the oxidative stress and the proinflammatory state in the kidney can be favorably influenced without the reduction in blood pressure or improvement in proteinuria. Such demonstration can be of practical importance, as the current therapies of chronic kidney disease address the question of blood pressure and reduction in proteinuria, but not the direct treatment of renal inflammation.

We have previously reported that add-on angiotensin II receptor antagonism with losartan, in a background of chronic angiotensin-converting enzyme inhibition, did not reduce proteinuria or blood pressure, (1) but caused a 38% reduction in urinary excretion of the fibrogenic cytokine, transforming growth factor β. (3) In this group of patients, who had no improvement in proteinuria or blood pressure, we found a unique opportunity to examine the role of specific angiotensin II type 1 receptor antagonism on oxidative stress and the proinflammatory effects of this oxidative stress that are precursors of renal fibrosis. We hypothesized that add-on losartan therapy will reduce oxidative lipid and protein damage in proteinuric patients with chronic kidney disease (CKD) and result in reduced urinary production of proinflammatory cytokines.

Methods

The protocol design and main results of the study have been reported in detail previously (1). Briefly, patients between the ages of 18 to 80 years with proteinuria of 1 g/d, hypertension...
defined as mean arterial pressure 97 mm Hg, serum potassium of 5.5 mEq/L and on lisinopril therapy of 40 mg/d for >3 months were eligible for the study. Patients who had previously received angiotensin receptor blockers or with estimated Ccr of < 30 mL/min were excluded. A separate group of age matched 10 normotensive volunteers with no history of kidney disease or diabetes served as the control group for plasma and urinary malodialdehyde levels and estimation of plasma protein carbonylation.

**Protocol**

The study was a two period, cross-over, randomized controlled trial and reported in detail elsewhere (1). Patients received either a sequence of losartan 50 mg/d x 4 weeks, two week washout, and placebo x 4 weeks or placebo x 4 weeks, two week washout, and losartan 50 mg/d x 4 weeks. Lisinopril 40 mg/d along with other antihypertensive therapy was continued throughout the trial. 24-hour urine was collected for protein, sodium, urea and creatinine and urinary carbonylated protein, malondialdehyde and MCP1 measurement. As the standard therapy for patients with proteinuria and renal failure includes ACE-inhibitors, we did not remove ACE-inhibitors from any patient enrolled in the study. The study was approved by the Institutional Review Board and all patients gave written informed consent.

Serum chemistries, complete blood counts, urine protein, electrolytes, urea and creatinine were measured by our hospital laboratory using routine methods. Specifically, creatinine was measured on a Hitachi 911 analyzer (Boehringer Mannheim) using the alkaline picrate method and urine protein was measured using a turbidometric method using benzethonium chloride read at 550 nm (Roche Diagnostics Corporation, Indianapolis, IN).

Ambulatory blood pressure monitoring was performed with SpaceLabs 90207 monitors and GFR measurements with continuous infusion of iothalamate as previously reported (1).

**Plasma and Urinary Malondialdehyde assay**

Malondialdehyde (MDA), a lipid hydroperoxide is formed by β-scission of peroxidized polyunsaturated fatty acids and is commonly measured by derivatization with thiobarbituric acid
(TBA) to yield a red compound (5) A rapid and sensitive fluorometric HPLC method was developed for the measurement of malondialdehyde (MDA) in plasma and urine as a biomarker of oxidative damage to lipids (2). Briefly, the mobile phase consisted of 40:60 ratio (v/v) of methanol to 50 mM potassium monobasic phosphate at pH 6.8, pumped at a rate of 1.0 mL/min on a Hewlett-Packard Hypersil 5 µ ODS 100 x 4.6 mm placed in a column warmer set to 37°C. Samples of serum and urine were treated with the antioxidant, butylated hydroxytoluene and heat derivatized at 100°C for 1 hour with thiobarbituric acid at an acid pH. Samples were extracted with n-butanol and 10 µL of the extract was injected at 1-minute intervals using an autosampler. The Hewlett-Packard model 1046A programmable fluorescence detector was set at excitation of 515 nm and emission of 553 nm. Retention time was 1.87 minutes, however absence of interfering peaks, allowed analysis to be carried out in increments of 1 minute per sample. Within day variability in estimation was between 8.6% and 10.3%. Between days variability was 3.6%-7.9%. Recovery was between 88-101%.

**Total Plasma Protein Carbonyl Measurement**

The carbonyl groups, due to oxidative damage of proteins can be detected by derivatizing with dinitrophenyl hydrazine (DNP), separating the derivatizing agent from the proteins and measuring the absorbance at 360 nm. Although the derivatizing agent can be removed with multiple washes of the protein pellet after derivatization, this entails loss of protein during the procedure. Therefore, we developed an HPLC method for measurement of carbonylated proteins using a size exclusion column to separate the derivatized carbonyl groups from the derivatizing agent and monitoring the separation with a diode array detector. Briefly, The mobile phase consisted of 200 mMol/L sodium monophosphate, pH 6.5 containing 1% SDS pumped at a rate of 1.0 mL/min on an Alltech Macrosphere GPC 7µ 250 x 4.6 mm (Alltech Associates, Deerfield, IL, USA) placed in a column warmer set to 37°C. Samples of plasma were split in two parts, one was derivatized with 20 mMol/L DNP in 10% trifluoroacetic acid (TFA) with the other sample acting as a control treated with 10% TFA. Samples were injected using a HP1100 autosampler in a volume of 25
µL of the derivatized and underivatized sample at 8-minute intervals. The Hewlett-Packard model 1100 diode array detector was programmed to retain signals every 2 nm over the 190 nm to 550 nm spectrum and data was recorded using HP LC Chemstation software (Agilent Technologies, Palo Alto, CA). Retention time of protein was 3 minutes confirmed by a maximum absorbance of the spectra at 190 nm; the derivatizing agent had a retention time of 7 minutes. The maximum absorbance of DNP was noted at at 360 nm. The area under the curve of the 360 nm peak was integrated in the underivatized sample and subtracted from the derivatized sample. A molar extinction coefficient of 22,000 absorbance units/ mole was used to determine the concentration of carbonyl in the sample. Data are expressed as nmol carbonyl per mg protein.

Estimation of Carbonylation in Plasma and Urine Protein by Western blotting technique

Oxidation of plasma and urine proteins was measured by analysis of Western blots according to the method of Shacter et al. Total protein was determined using Vitros Dry Slide system (Ortho-Clinical Diagnostics, Rochester, NY, USA) in the clinical chemistry laboratory of the hospital. Plasma was diluted 1:25 (v:v) with phosphate-buffered saline (PBS), one aliquot of the diluted sample was derivatized and another prepared as an underivatized control using the OxyBlot protein oxidation detection kit (Intergen, Purchase, NY, USA) Urine samples were derivatized with DNP or control reagent similarly except that samples were not diluted prior. Derivatized and underivatized plasma or urine samples were loaded on electrophoresis gels in volumes calculated to give 5 µg protein per sample and electrophoresed according to the method of Laemmli on 4 to 20%gradient SDS-PAGEgels (Bio-Rad, Hercules, CA, USA) for 60 minutes at 200 V. Following electroblotting to 0.2 µ nitrocellulose for 60 volt hours, the membrane was blocked with subsequent immunoblotting using OxyBlot Kit methods and reagents. Bands were visualized with chemiluminescent chemicals and captured on film at two exposure times (30 sec and 1 min). Blots were scanned on a Hewlett-Packard ScanJet 5200C scanner (Hewlett-
Packard, Palo Alto, CA, USA) and analyzed for band area using Un-Scan-It Gel software (Silk Scientific, Orem, UT, USA).

Samples for individual patients before and after losartan therapy, including derivatized and underivatized control, were analyzed on a single Western blot. This ensured response to losartan therapy was compared under the same analytical conditions. For each plasma or urine sample, carbonyl density was determined from the 30 sec exposure, which produced clearly visible bands. Density of individual albumin bands and total protein in each sample lane was determined using the same size section of each scanned blot. The analysis box included 26 lanes for each analysis. The uniform window size and analysis box ensured that data were being analyzed consistently from band to band and from blot to blot. Additionally, any density values present in underivatized controls were subtracted from density of the DNP treated sample to increase validity of comparison among patients.

**Urinary Monocyte chemotactic protein-1 assay**

MCP1 was assayed in urine using a sandwich ELISA (Quantikine® kit for Human MCP1 Immunoassay; R&D Systems, Minneapolis, MN). Corrections were made for concentration and values were expressed as ng MCP1 per gm creatinine. A standard curve was generated using a four parameter logistic curve-fit. The correlation coefficient for standards was greater than 0.99 and the lowest detectable limit was 0.7 pg/ml in 1:2 diluted urine. The intra-assay coefficient of variation was 2.5 ± 3.0% and the inter-assay coefficient of variation was 5.6 ± 4.2%.

**Statistical Analysis**

The normality assumption was tested with the Kolmogorov-Smirnov statistic. Urinary MCP1 and protein excretion were not normally distributed and were log transformed to satisfy the normality assumption. These log-transformed data were used for subsequent analysis. Data were then analyzed by paired t-test before and after losartan therapy. Results are reported as means ± SD. All tests were two sided at an \( \alpha \) level of 0.05. All statistical analysis were carried out using standard procedures on Statistica for Windows, release 5.5 (StatSoft, Inc. Tulsa, OK, USA) (4).
Results

Sixteen patients (10 Blacks, 6 Whites; 14 males), of average age 53 ± 9 years and body mass index 38 ± 5.7 kg/m², completed the trial. The etiology of CKD was Type 2 diabetes mellitus in 12 and glomerulonephritis in the remaining, Seated BP at baseline was 156 ± 18/88 ± 12 mm Hg requiring 3.13 ± 1.2 antihypertensive drugs, creatinine was 2.0 ± 0.8 mg/dL and proteinuria 3.6 ± 0.71 g/g creatinine/24 hours.

There was no change in blood pressure or proteinuria in response to add-on losartan therapy as reported earlier (1). There was an improvement in GFR noted from 63 ± 9 mL/min to 68 ± 11 mL/min (p<0.05). Prior to add-on losartan therapy, urinary protein oxidation was 99% higher than that seen in the plasma (p=0.008). Urinary albumin oxidation was 71% higher than plasma albumin (p=0.045). Oxidized urinary or plasma albumin accounted for the major fraction of total protein oxidation (Figure 2A and 2B). Although proteinuria was not reduced, losartan significantly reduced oxidative damage to urinary albumin from 102057±87149 densitometric units/µg protein to 66110±44668 densitometric units/µg protein, a reduction of 35% (Figure 2A). This effect was particularly pronounced in those patients who had a high level of oxidized albumin in the urine at baseline. After treatment with losartan urinary albumin was not more oxidized compared to plasma albumin (p=0.45), but total urinary protein remained 69% more oxidized after losartan therapy (p=0.029). There was a trend towards improvement in total urinary protein oxidation that did not reach statistical significance and plasma albumin and protein oxidation remained unchanged (Figure 2B).

Samples of urine and plasma malondialdehyde from ten normotensive volunteers, age 53 ± 14 years, were 1.94 ± 0.79 µmol/g creatinine and 0.69 ± 0.13 µmol/L respectively. In comparison, urine malondialdehyde and plasma MDA were elevated in the CKD patients. Urinary MDA was significantly reduced from 4.75 ± 3.23 µmol/g creatinine to 3.39 ± 2.17 µmol/g creatinine with add-on losartan therapy (Figure 3). However, plasma malondialdehyde (Figure 3) or plasma oxidized proteins (Figure 2B) did not change in response to additional angiotensin II blockade. Using direct measurement of protein carbonylation in plasma we found the carbonyl concentration to be
unchanged from 61.3 ± 18.9 µmol/L to 60.1 ± 19.1 µmol/L. When adjusted for plasma protein concentration the carbonyl concentration was unchanged from 0.84 ± 0.31 nmol/mg to 0.85 ± 0.44 nmol/mg confirming what was observed on Western Blot analysis. The geometric mean (SD) urinary MCP-1 fell, albeit statistically insignificantly, from 646 (3.2) pg/mg creatinine to 501 (3.0) pg/mg creatinine. Normal controls had urinary MCP-1 level of 203 (1.4) pg/mg creatinine.

Finally, there was a good correlation seen between change in urinary oxidized albumin and urinary MCP-1 level (Figure 4).
Discussion

The major findings of our study are that patients with chronic kidney disease (CKD) with proteinuria have greater concentration of biomarkers of oxidative stress in the urine compared to plasma. Albumin is the major target of this oxidative damage amongst proteins. This oxidative stress can be reduced with angiotensin II receptor blockade independent of blood pressure reduction or reduction in proteinuria. This improvement in reduced oxidative stress is correlated with improvement in urinary inflammation.

Increased plasma MDA in patients with CKD compared to healthy controls suggests increased systemic oxidative stress. Although vascular superoxide production is increased via NADH/NADPH oxidase via angiotensin II may be responsible for increased systemic oxidative stress (19), angiotensin II may play a larger role in the kidney due to its effects on superoxide anion production by the mesangial cells and tubular cells (9;12). Animal models of increased oxidative stress induced by diets deficient in vitamin E and selenium show an increased ROS generation, glomerular and tubular hypertrophy and subsequent injury (18). Thus the kidney may be particularly susceptible to oxidative stress. Our data demonstrating greater urinary protein carbonylation compared to plasma are therefore consistent with the above observations made in cell cultures and animals.

The urinary environment is a prooxidant one with measured amounts of hydrogen peroxide attaining micromolar quantities in rats and humans (17;23). Plasma and the urinary excretion rates of MDA, a biomarker of lipid peroxidation, were elevated in patients with CKD, compared to normal controls. These data are consistent with observations in animals with reduced renal mass who show increased tubular oxygen consumption accompanied by increased MDA per tubule, and increased urinary and plasma levels of MDA (16). The fall in MDA excretion rate with additional angiotensin II antagonism, despite no change in plasma levels suggests that the renal generation of MDA, but not the systemic production of MDA, was reduced. The known
pro-oxidant effects of angiotensin II on the kidney lend biologic plausibility to these observations (9;12). Stimulation of lipid peroxide production involved protein kinase C (8), an enzyme whose activity is reduced by AT1 receptor antagonism may partly explain these results (6). Although we did not find a correlation in the fall in urinary MDA excretion and reduction in urinary MCP-1, oxidized lipids can increase chemokine expression in monocytes (22) and mesangial cells (13). Therefore, fall in urinary MDA excretion may be a marker of reduced renal inflammation.

Angiotensin II blockade improved biomarkers of oxidative stress in the urine but not in plasma. In a model of chronic renal failure, in which 5/6th nephrectomized rats are treated with the ACE inhibitor, enalapril, direct measurements of antioxidant enzymes in the kidney such as superoxide dismutase and glutathione peroxidase is increased (24). The recruitment of antioxidant defenses, brought about by abrogation of actions of angiotensin II, may account for improvement in oxidative state in the kidney in preference of plasma oxidative state.

Although there are a variety of cytokines that can be measured in the urine, we elected to measure MCP-1 for were several reasons. MCP-1 has been previously measured in the urine of patients with a variety of glomerular diseases, found to be biologically active and without correlation with plasma levels (20). Urinary excretion of MCP-1 correlates with extent of renal inflammation (25) as well as MCP-1 gene expression in the tubules, parietal epithelial cells and infiltrating monocytes (7). Therefore, we reasoned that urinary MCP-1 would serve as an important measure of the inflammatory state in the kidney and its reduction would be biologically plausible based on the animal experiments. Our data show a good correlation between reduction in oxidative stress in urine and the reduction in renal inflammation as measured by MCP-1. These data can be reconciled with the observation that ROS generation is involved in MCP-1 gene transcription in response to tissue injury likely via NADPH-oxidase (21). Furthermore, in animal models of inflammatory kidney disease, administration of AT1 receptor antagonists (28) or genetic absence of the AT1a receptor (21), abrogates the early expression of MCP-1 in the glomerulus and the infiltration of monocyte/macrophages.
There are several limitations of our study. First, we did not study whether ACE inhibitors alone can lower urinary oxidative damage in patients with CKD and proteinuria. Although we show the effects of angiotensin II receptor blockade to be independent of blood pressure and proteinuria, the converse study was not performed. In other words, it is not known whether blood pressure reduction or antiproteinuria therapies will result in similar reduction in renal oxidative stress. Finally, a much larger trial would need to be conducted to show if this strategy would translate into protection from end-stage renal disease and death.

In this randomized controlled trial of additional angiotensin II blockade we have demonstrated that protein in the urine undergoes oxidative damage (urinary albumin was 71% more oxidized compared to plasma albumin). Thus, in proteinuric patients urinary albumin can serve as a decoy of oxidative injury as it passes from the glomerulus to the urine. Although such oxidative damage to plasma proteins in patients with CKD has previously been reported (10;11), we believe that this is the first demonstration of oxidative damage to urinary proteins in humans. Furthermore, our data demonstrate that oxidant damage to urinary protein and lipids can be reduced with additional angiotensin II blockade. This is particularly notable because the reduction of oxidant stress occurred independent of reduction in proteinuria or blood pressure, the key mediators of progressive renal damage. Furthermore, there was no change in the markers of protein or lipid damage in the plasma of these patients. Thus, the data are consistent with the hypothesis that the urinary measurements of markers of oxidative damage, both carbonyls and lipid hydroperoxides are more sensitive than plasma measurements in patients with CKD. These observations are further strengthened with the significant association of the change in MCP1 in the urine with oxidized albumin, which supports experimental data in animals that demonstrate the important role of the redox state in the kidney with renal fibrosis and progressive kidney damage.


Figure Legends

Figure 1: Western Blots of oxidized proteins in urine (top panel) and plasma (bottom panel). Each sample was run after derivatization with dinitrophenyl hydrazine before (pre) and after (post) angiotensin II receptor blockade administered for 1 month. Underivatized controls are negative for dinitrophenyl hydrazine antibody as expected. Pt1, Pt2 and Pt3 refer to the first, second and third patients in the study. Background chemiluminescence was subtracted from the parent blot to obtain the true carbonylated fraction of protein.

Figure 2: Panel A shows changes in urinary oxidized albumin and total protein (densitometric units/µg protein loaded on gel). Urinary oxidized albumin improved from mean of 102,057 to 66,110 DU/µg protein (p=0.036) whereas urinary oxidized total protein remained unchanged. Panel B shows unchanged plasma oxidized albumin and total protein.

Figure 3: Changes in plasma and urinary malondialdehyde (MDA), a maker of lipid peroxidation. Although mean plasma MDA levels were elevated (1.10±0.88 µmol/L) compared to healthy controls (0.69±13 µmol/L) there was no improvement with therapy (0.81±0.39 µmol/L post drug). However, angiotensin II receptor blockade caused significant improvement in urinary MDA levels from 4.75 ± 3.23 µmol/g creatinine to 3.39 ± 2.17 µmol/g creatinine.

Figure 4: Reduction in urinary monocyte chemotactic protein-1 (MCP1) was correlated with reduction in urinary oxidized albumin with angiotensin II receptor blockade (r=0.61, p=0.012).
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A: Urinary Proteins

B: Plasma Proteins

Figure 2
Figure 3

**Urine**

MDA (µmol/L) vs. Creatinine (µmol/g)

**Plasma**

MDA (µmol/L) vs. Creatinine (µmol/g)