Proinflammatory effects of oxidative stress in chronic kidney disease: role of additional angiotensin II blockade

Rajiv Agarwal
(With the Technical Assistance of Shawn D. Chase)

Indiana University School of Medicine and Richard L. Roudebush Veterans Affairs Medical Center, Indianapolis, Indiana 46202

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Agarwal, Rajiv. Proinflammatory effects of oxidative stress in chronic kidney disease: role of additional angiotensin II blockade. Am J Physiol Renal Physiol 284: F863–F869, 2003. First published December 27, 2002; 10.1152/ajprenal.00385.2002.—Oxidative stress plays an important role in causing progressive chronic kidney disease (CKD). We examined the influence of add-on ANG II receptor blockade administered as losartan (50 mg/day for 1 mo) on oxidative stress and proinflammatory state of the kidney in patients with CKD. All subjects were taking an angiotensin-converting enzyme inhibitor plus other antihypertensive agents. Oxidative stress to lipids and proteins was measured by an HPLC assay for malondialdehyde (MDA) and carbonyl concentration, respectively. Urinary inflammation was measured by monocyte chemotactic protein-1 (MCP-1) excretion rate. The etiology of CKD was type 2 diabetes mellitus in 12 and glomerulonephritis in 4 patients. There was no change in proteinuria or 24-h ambulatory blood pressure (BP) with add-on ANG II receptor blockade with losartan therapy. Before losartan therapy, urinary protein and albumin oxidation were 99 and 71% higher, respectively, compared with plasma (P < 0.05). There was a 35% reduction in urinary oxidized albumin with add-on losartan therapy (P = 0.036). Urinary and plasma MDA were elevated compared with age-matched controls. Urinary MDA was significantly reduced from 4.75 ± 3.23 to 3.39 ± 2.17 μmol/g creatinine with add-on losartan therapy. However, plasma MDA or oxidized proteins did not change in response to additional ANG II blockade. A good correlation was seen between the change in urinary oxidized albumin and MCP-1 levels (r = 0.61, P = 0.012). These data demonstrate that oxidative damage to urinary protein and lipids can be reduced with additional ANG II receptor blockade, independently of reductions in proteinuria or BP. Urinary measurements of markers of oxidative damage to lipids and proteins appear to be more sensitive than plasma measurements in patients with CKD. The significant association of the change in urinary MCP-1 with a reduction in oxidative stress supports the role of the redox state in the kidney with renal fibrosis and progressive kidney damage.

carbonyl stress; malondialdehyde; proteinuria; hypertension; diabetes mellitus

ELEVATED BLOOD PRESSURE and severe proteinuria are important predictors of progressive renal injury (13). 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 (14). The present 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-κB (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 whether oxidative stress and the proinflammatory state in the kidney can be favorably influenced without a reduction in blood pressure or improvement in proteinuria. Such a demonstration can be of practical importance, because the present therapies of chronic kidney disease (CKD) 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, on a background of chronic angiotensin-converting enzyme (ACE) 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 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 and 80 yr with proteinuria of ≥1 g/day, hypertension (defined as mean arterial pressure ≥97 mmHg), serum potassium of ≤5.5 meq/l, and on lisinopril
therapy of 40 mg/day for >3 mo were eligible for the study. Patients who had previously received angiotensin receptor blockers or with estimated creatinine clearance of <30 ml/min were excluded. A separate group of 10 age-matched normotensive volunteers with no history of kidney disease or diabetes served as the control group for plasma and urinary malondialdehyde (MDA) levels and estimation of plasma protein carbonylation.

Protocol. The study was a two-period, crossover, randomized controlled trial and has been reported in detail elsewhere (1). Patients received a sequence of losartan (50 mg/day × 4 wk, 2-wk washout) and placebo × 4 wk, or placebo × 4 wk, 2-wk washout, and losartan (50 mg/day × 4 wk). Lisinopril (40 mg/day) along with another antihypertensive therapy was continued throughout the trial. Urine was collected (24 h) for protein, sodium, urea, and creatinine and urinary carbonylated protein, MDA, and monocytic chemotactic protein-1 (MCP-1) measurement. Because the standard therapy for patients with proteinuria and renal failure includes ACE inhibitors, we did not remove them from the regimen of any patient enrolled in the study. The study was approved by the Institutional Review Board, and all patients gave informed written consent.

Serum chemistries, complete blood counts, urine protein, electrolytes, and 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 urinary protein was measured using a turbidometric method, with benzethonium chloride read at 550 nm (Roche Diagnostics, Indianapolis, IN).

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

Plasma and urinary MDA assay. 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 (4). A rapid and sensitive fluorometric HPLC method was developed for the measurement of MDA in plasma and urine as a biomarker of oxidative damage to lipids (2). Briefly, the mobile phase consisted of a 40:60 ratio (vol/vol) 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-ODS (5 μm, 100 × 4.6 mm) column at a 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 h with thiobarbituric acid at an acid pH. Samples were extracted with n-butanol, and 10 μl of the extract were injected at 1-min intervals using an autosampler. A Hewlett-Packard model 1046A programmable fluorescence detector was set at an excitation of 515 nm and emission of 553 nm. Retention time was 1.87 min; however, the absence of interfering peaks allowed analysis to be carried out in increments of 1 min/sample. Within-day variability in estimation was between 8.6 and 10.3%. Between-day variability was 3.6–7.9%. Recovery was between 88 and 101%.

Total plasma protein carbonyl measurement. The carbonyl groups, due to oxidative damage to proteins, can be detected by derivatizing with dinitrophenyl hydrazine (DNPH), 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 measuring carbonylated proteins using a size-exclusion col-

umn 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 mM/l sodium monophosphate, pH 6.5, containing 1% SDS pumped at a rate of 1.0 ml/min on an Alltech Macro sphere GPC (7 μm, 250 × 4.6 mm, Alltech Associates, Deerfield, IL) placed in a column warmer set to 37°C. Samples of plasma were split into two parts, one derivatized with 20 mM/l TFA in 10% trifluoroacetic acid (TFA) and the other 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 samples at 8-min intervals. The Hewlett-Packard model 1100 diode array detector was programmed to retain signals every 2 nm over the 190- to 550-nm spectrum, and data were recorded using HPLC Chemstation software (Agilent Technologies, Palo Alto, CA). The retention time of protein was 3 min as confirmed by a maximum absorbance of the spectra at 190 nm; the derivatizing agent had a retention time of 7 min. The maximum absorbance of TFA was noted at 360 nm. The area under the curve of the 360-nm peak was integrated in the derivatized and underivatized samples, and data were analyzed. A molar extinction coefficient of 22,000 absorbance units/mmol was used to determine the concentration of carbonyl in the sample. Data are expressed as nanomoles carbonyl per milligram protein.

Estimation of carbonylation in plasma and urinary protein by Western blotting. Oxidation of plasma and urinary proteins was measured by analysis of Western blots according to the method of Shacter. Total protein was determined using the Vitros Dry Slide system (Ortho-Clinical Diagnostics, Rochester, NY) in the clinical chemistry laboratory of the hospital. Plasma was diluted 1:25 (vol/vol) with PBS, one aliquot of the diluted sample was derivatized, and another was prepared as an underivatized control using an OxyBlot protein oxidation detection kit (Intergen, Purchase, NY). Urine samples were similarly derivatized with TFA or control reagent except that samples were not diluted beforehand. Derivatized and underivatized plasma or urine samples were loaded on electrophoresis gels in volumes calculated to give 5 μg protein/sample and electrophoresed according to the method of Laemmli on 4–20% gradient SDS-PAGE gels (Bio-Rad, Hercules, CA) for 60 min at 200 V. After being electroblotted to 0.2-μm 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 s and 1 min). Blots were scanned on a Hewlett-Packard ScanJet 5200C scanner (Hewlett-Packard, Palo Alto, CA) and analyzed for band area using Un-Scan-It Gel software (Silk Scientific, Orem, UT).

Samples for individual patients before and after losartan therapy, including derivatized and underivatized control, were analyzed on a single Western blot (Fig. 1). This ensured that the response to losartan therapy was compared under the same analytic conditions. For each plasma or urine sample, carbonyl density was determined from the 30-s exposure, which produced clearly visible bands. Density of individual albumin bands and total protein in each sample lane were determined using a section of the same size from 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 TFA-treated sample to increase the validity of comparison among patients.
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 to 68 ± 11 ml/min (P < 0.05). Before add-on losartan therapy, urinary protein oxidation was 99% higher than that seen in 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 (Fig. 2, A and B). Although proteinuria was not reduced, losartan significantly reduced oxidative damage to urinary albumin from 102,057 ± 87,149 to 66,110 ± 44,668 densitometric units/µg protein, a reduction of 35% (Fig. 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 with plasma albumin (P = 0.45), but total urinary protein remained 69% more oxidized after losartan therapy (P = 0.029). There was a trend toward improvement in total urinary protein oxidation that did not reach statistical significance, and plasma albumin and protein oxidation remained unchanged (Fig. 2B).

Samples of urinary and plasma MDA from 10 normotensive volunteers, aged 53 ± 14 yr, were 1.94 ± 0.79 µmol/g creatinine and 0.69 ± 0.13 µmol/l, respectively. In comparison, urinary and plasma MDA were elevated in the CKD patients. Urinary MDA was significantly reduced from 4.75 ± 3.23 to 3.39 ± 2.17 µmol/g creatinine with add-on losartan therapy (Fig. 3). However, neither plasma MDA (Fig. 3) nor plasma oxidized proteins (Fig. 2B) changed in response to additional angiotensin II blockade. Using direct measurement of protein carbonylation in plasma, we found carbonyl concentration to be unchanged (61.3 ± 18.9 vs. 60.1 ± 19.1 µmol/l). When adjusted for plasma protein concentration, carbonyl concentration was unchanged (0.84 ± 0.31 vs. 0.85 ± 0.44 nmol/mg), confirming what was observed by Western blot analysis. The geometric urinary MDA fell, albeit statistically insignificantly, from 646 ± 3.2 to 501 ± 3.0 pg/mg creatinine. Normal controls had a urinary MCP-1 level of 203 ± 1.4 pg/mg creatinine. Finally, a good correlation was seen between the change in urinary oxidized albumin and urinary MCP-1 levels (Fig. 4).

DISCUSSION

The major findings of our study are that patients with CKD with proteinuria have a greater concentration of biomarkers of oxidative stress in the urine compared with in plasma. Among proteins, albumin is the major target of this oxidative damage. This oxidative stress can be reduced with angiotensin II receptor blockade independently 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 with healthy controls suggests increased sys-

**RESULTS**

Sixteen patients (10 African Americans, 6 Caucasians; 14 men), with an average age of 53 ± 9 yr and body mass index of 38 ± 5.7 kg/m², completed the trial. The etiology of CKD was type 2 diabetes mellitus in 12 patients and glomerulonephritis in the remaining 4. Seated blood pressure at baseline was 156 ± 18/88 ± 12 mmHg, requiring 3.13 ± 1.2 antihypertensive drugs; creatinine was 2.0 ± 0.8 mg/dl; and proteinuria was 3.6 ± 0.71 g·g creatinine⁻¹·24 h⁻¹.

**Urinary MCP-1 assay.** MCP-1 was assayed in urine using a sandwich ELISA (Quantikine kit for human MCP-1 immunoassay; R&D Systems, Minneapolis, MN). Corrections were made for concentration, and values were expressed as nanograms MCP-1 per gram creatinine. A standard curve was generated using a four-parameter logistic curve fit. The correlation coefficient for standards was >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 interassay coefficient of variation was 5.6 ± 4.2%.

**Statistical analysis.** The normality assumption was tested with the Kolmogorov-Smirnov statistic. Urinary MCP-1 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 α level of 0.05. All statistical analysis was carried out using standard procedures on Statistica for Windows, release 5.5 (StatSoft, Tulsa, OK, USA) (21).
temic oxidative stress. Although the increase in vascular superoxide production via NADH/NADPH oxidase via angiotensin II may be responsible for increased systemic oxidative stress (18), 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 (8, 11). Animal models of increased oxidative stress induced by diets deficient in vitamin E

Fig. 2. A: changes in urinary oxidized albumin and total protein [densitometric units (DU)/μg protein loaded on gel]. Urinary oxidized albumin improved from mean of 102,057 to 66,110 DU/μg protein (P = 0.038), whereas urinary oxidized total protein remained unchanged. B: unchanged plasma oxidized albumin and total protein.

Fig. 3. Changes in plasma (A) and urinary (B) malondialdehyde (MDA), a maker of lipid peroxidation. Although mean plasma MDA levels were elevated (1.10 ± 0.88 μmol/l) compared within healthy controls (0.69 ± 0.13 μmol/l), there was no improvement with therapy (0.81 ± 0.39 μmol/l posttherapy). However, angiotensin II receptor blockade caused significant improvement in urinary MDA levels, from 4.75 ± 3.23 to 3.39 ± 2.17 μmol/g creatinine.
and selenium show increased generation of reactive oxygen species, glomerular and tubular hypertrophy, and subsequent injury (17). Thus the kidney may be particularly susceptible to oxidative stress. Our data demonstrating greater urinary protein carbonylation compared with 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 (16, 23). Plasma and the urinary excretion rates of MDA, a biomarker of lipid peroxidation, were elevated in patients with CKD compared with 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 (15). 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 prooxidant effects of angiotensin II on the kidney lend biological plausibility to these observations (8, 11). That stimulation of lipid peroxide production involved protein kinase C (7), an enzyme whose activity is reduced by AT1 receptor antagonism, may partly explain these results (5). Although we did not find a correlation between the fall in urinary MDA excretion and reduction in urinary MCP-1, oxidized lipids can increase chemokine expression in monocytes (22) and mesangial cells (12). Therefore, a 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 5/6 nephrectomized rats are treated with the ACE inhibitor enalapril, direct measurements of antioxidant enzymes in the kidney such as superoxide dismutase and glutathione peroxidase are increased (24). The recruitment of antioxidant defenses, brought about by abrogation of actions of angiotensin II, may account for an improvement in the oxidative state in the kidney in preference to the plasma oxidative state.

Although there are a variety of cytokines that can be measured in the urine, we elected to measure MCP-1 for several reasons. MCP-1 has been previously measured in the urine of patients with a variety of glomerular diseases and was found to be biologically active and not correlated with plasma levels (19). Urinary excretion of MCP-1 correlates with the extent of renal inflammation (25) as well as MCP-1 gene expression in the tubules, parietal epithelial cells, and infiltrating monocytes (6). 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 reactive oxygen species generation is involved in MCP-1 gene transcription in response to tissue injury, probably via NADPH-oxidase (20). Furthermore, in animal models of inflammatory kidney disease, administration of AT1 receptor antagonists (28) or the genetic absence of the AT1a receptor (20) 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, a study of the converse was not performed. In other words, it is not known whether blood pressure reduction or antiproteinuria therapies will result in a similar reduction in renal oxidative stress. Finally, a much larger trial would need to be conducted to show whether 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 with plasma albumin). Thus in proteinuric patients urinary albumin can serve as a decoy of oxidative injury as it passes from the glomerulus into the urine. Although such oxidative damage to plasma proteins in patients with CKD has previously been reported (9, 10), we believe that this is the first demonstration of oxidative damage to urinary proteins in humans. Furthermore, our data demonstrate that oxidative 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 independently of a 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 through the significant association of the change in urinary MCP-1 with that in oxidized albumin, which supports experimental data in animals demonstrating the important role of the redox state in the kidney with renal fibrosis and progressive kidney damage.

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REFERENCES


