Oxidative Stress Is Progressively Enhanced With Advancing Stages of CKD

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● Background: Oxidative stress appears to have a central role in the pathophysiological process of uremia and its complications, including cardiovascular disease. However, there is little evidence to suggest how early oxidative stress starts developing during the progression of chronic kidney disease (CKD). The aim of this study is to assess oxidative stress activity in a cross-sectional study of patients with CKD stages 1 to 4.

Methods: Eighty-seven steady patients (47 men, 40 women) with a median age of 62 years (range, 28 to 84 years) and mean estimated glomerular filtration rate (eGFR) of 57 mL/min (0.95 mL/s) were studied. Levels of plasma 8-isoprostanes (8-epiPGF2a) and serum total antioxidant status (TAS) were used as markers of oxidative stress. 8-epiPGF2a levels were determined by using an enzyme-linked immunosorbent assay method, whereas a chromatometric method was used to determine TAS. Results: Plasma 8-epiPGF2a levels increased significantly as CKD stages advanced (P < 0.001). There was a highly significant inverse correlation between 8-epiPGF2a level and GFR (P < 0.01). Serum TAS levels also increased in a similar fashion (P < 0.009) and showed a significant inverse correlation with GFR (P < 0.01). 8-epiPGF2a and TAS levels showed a positive correlation (P < 0.05). Multiple regression analysis showed that the most significant predictor variable for 8-epiPGF2a level was eGFR, whereas the association between eGFR and TAS was affected strongly by confounding variables, mainly uric acid level. Conclusion: Oxidative stress appears to increase as CKD progresses and correlates significantly with level of renal function. Increased TAS seems to be dependent on several confounding variables, including increased uric acid levels, and therefore does not seem to be a reliable method for assessing the antioxidant capacity of patients with CKD. These results suggest that larger studies using the correct markers to assess the timing and complex interplay of oxidative stress and other risk factors during the progression of CKD should be carried out. Am J Kidney Dis 48:752-760.

CARDIOVASCULAR DISEASE (CVD) remains the leading cause of morbidity and mortality in patients with end-stage renal disease undergoing maintenance dialysis therapy.1,2 Moreover, most patients with stages 3 to 5 chronic kidney disease (CKD) will die of a major cardiovascular event before developing end-stage renal disease.2 Traditional risk factors, such as old age, smoking, hypertension, diabetes mellitus, dyslipidemia, and left ventricular hypertrophy, associated with CVD in the general population were described primarily in the Framingham study and are fairly common among patients with CKD. However, the presence of these factors does not seem to give a satisfactory explanation for the huge cardiovascular burden in the CKD population.3-5 Recently, it was suggested that patients with CKD also are exposed to other nontraditional uremia-related risk factors, such as anemia, altered calcium-phosphorus metabolism, inflammation, malnutrition, and oxidative stress (OS).3,4 These nontraditional risk factors seem to appear early during the progression of kidney damage and probably are responsible for the large difference in cardiovascular mortality between patients with CKD and the general population.6 OS takes place when oxidant production exceeds local antioxidant capacity, resulting in increased oxidation of important macromolecules, including proteins, lipids, carbohydrates, and damage of DNA structure. OS was proposed to have a pivotal role in the pathophysiological

INDEX WORDS: Oxidative stress; isoprostanes; total antioxidant status; uric acid; chronic kidney disease (CKD) stages; progression of CKD.

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process of uremia and its complications, including CVD. There is increasing evidence associating the role of free radicals with ischemia-reperfusion injury in the heart and in the pathogenesis of atherosclerosis, hypertension, and heart failure. In a recent study, OS and, more specifically, plasma 8-isoprostane (8-epiPGF2a) level and reduced antioxidant capacity were associated with the extent and severity of coronary artery disease and the occurrence and number of different atherogenic risk factors.

There is good evidence indicating that uremia and hemodialysis treatment are associated with enhanced OS. However, the number of studies assessing OS in patients in the early stages of CKD is limited, and results are contradictory. Moreover, the critical timing that the balance between pro-oxidant and antioxidant mechanisms is disturbed and becomes harmful remains obscure.

The aim of this study is to assess OS activity in patients with CKD stages 1 to 4 by estimating 8-epiPGF2a levels, which were proposed as a reliable marker of OS and the endogenous peroxidation of lipids.

METHODS

Patient Characteristics

Eighty-seven consecutive incident adult patients from the outpatient CKD clinics of 2 hospitals were studied prospectively. These patients were followed up for 3 months before entering the study to confirm the presence of CKD. For patients with a calculated estimated glomerular filtration rate (eGFR) greater than 60 mL/min (>1.0 mL/s), the definition of CKD was based on the Clinical Practice Guidelines for Chronic Kidney Disease (Kidney Disease Outcomes Quality Initiative). There were 47 men (54%) and 40 women with a median age of 62 years (range, 28 to 84 years). Primary renal diseases were glomerulonephritis in 14 patients (16%), interstitial nephritis in 9 patients (10%), hypertensive nephrosclerosis in 9 patients (10%), diabetic nephropathy in 8 patients (8%), polycystic kidney disease in 4 patients (5%), vasculitis in 3 patients (3%), miscellaneous in 10 patients (12%), and unknown in 31 patients (36%).

For calculation of eGFR, the Cockcroft-Gault formula was used, and overall mean GFR was 57 mL/min (0.95 mL/s). The distribution of patients in CKD stages was as follows: CKD stage 1, n = 17 (20%), with a mean GFR of 107 ± 18 mL/min (1.78 mL/L); CKD stage 2, n = 26 (30%), with a mean GFR of 74 ± 8 mL/min (1.23 mL/L); CKD stage 3, n = 22 (25%), with a mean GFR of 40 ± 7 mL/min (0.67 mL/L); and CKD stage 4, n = 22 (25%), with a mean GFR of 23 ± 4 mL/min (0.38 mL/L; Fig 1). Exclusion criteria were known uncured malignancy, active inflammation at the time of enrollment, and major cardiovascular event, defined as stroke, myocardial infarction, and acute ischemic heart disease, in the last 2 months. The study was approved by local ethical committees, and patients participated after being informed and granting a formal consent.

Methods

All patients underwent a detailed review of their medical history and a careful clinical examination. The protocol of the study included recording demographic characteristics, smoking habits and alcohol consumption, history of comorbidity and medication, anthropometric measurements (height, weight, and body mass index), and measurement of blood pressure and heart rate. A full hematologic and biochemical screening was performed. For estimation of OS activity at the time of enrollment, plasma 8-epiPGF2a level and total antioxidant status (TAS) were used. 8-epiPGF2a was mea-
ured after 3 mL of venous blood was collected in standard sterile vacuum tubes containing EDTA. After immediate centrifugation at 3,000g for 10 minutes, supernatants were separated and stored at –80°C until analysis. 8-epiPGF2α concentrations in plasma samples were determined by means of a competitive enzyme-linked immunosorbent assay commercial kit for 8-epiPGF2α (Cayman Chemicals, Ann Arbor, MI). Serum TAS levels were estimated by means of a quantitative chromatometric method using commercial kits (Randox Laboratories Ltd, Admore, UK). The method is based on the production of a stable cationic radical after incubation of its unstable precursor with hyperoxidase and hydrogen peroxide. From this reaction, a stable blue-green color is produced, which is reduced depending on the concentration of antioxidants.

Statistical Analysis

Statistical analysis was performed using the statistical package SPSS, version 11 (SPSS Inc, Chicago, IL). Data for continuous variables are expressed as mean ± SD. Chi-square or Fisher exact test was used for categorical variables, whereas comparisons of means among the 4 CKD stages were analyzed by using 1-way analysis of variance (ANOVA). The relationship between 2 variables was tested by using Spearman correlation. Multiple regression analysis also was applied to identify confounding variables in the association between eGFR and estimated 8-epiPGF2α and TAS levels. Correlation was considered statistically significant for P less than 0.05.

RESULTS

Characteristics and clinical and laboratory features of the 87 patients and those in CKD groups 1 to 4 are listed in Table 1. There was no difference among CKD groups in age, sex, and proportions of smokers, patients with diabetes, patients with hypertension, and patients receiving therapy with angiotensin II–converting enzyme inhibitors, angiotensin T1 antagonists, and statins. In addition, serum glucose levels and lipid profiles did not differ significantly, eGFR, serum creatinine, urea, and uric acid values increased, as expected, whereas

| Table 1. Characteristics and Clinical and Laboratory Data for All 87 Patients and Those With CKD Stages 1 to 4 |
|-------------------------------------------------|---------------------------------|---------|---------|---------|---------|---------|
| CKD Stage                                      | Total                          | 1       | 2       | 3       | 4       | P       |
| No. of patients                               | 87                             | 17 (20) | 26 (30) | 22 (25) | 22 (25) | NS      |
| Sex (men)                                     | 47 (54)                        | 8 (53)  | 12 (52) | 15 (56) | 12 (55) | NS      |
| Age (y)                                       | 62 (51-71)                     | 63 (59-70) | 63 (50-69) | 65 (46-71) | 70 (53-72) | NS      |
| Body mass index (kg/m²)                       | 28.4 ± 5.6                     | 32.5 ± 5.3 | 31.1 ± 5.1 | 26.6 ± 4.9 | 24.7 ± 3.9 | <0.001  |
| Smokers                                       | 21 (24)                        | 5 (33)  | 4 (17)  | 6 (22)  | 6 (27)  | NS      |
| Diabetes                                      | 8 (9)                          | 1 (7)   | 1 (4)   | 2 (7)   | 4 (18)  | NS      |
| Hypertension                                  | 76 (87)                        | 15 (100) | 17 (74) | 23 (85) | 21 (96) | NS      |
| ACE inhibitors treatment                      | 33 (38)                        | 7 (47)  | 9 (39)  | 9 (33)  | 8 (36)  | NS      |
| AT1 treatment                                 | 20 (23)                        | 4 (27)  | 5 (22)  | 9 (33)  | 2 (9)   | NS      |
| Statin treatment                              | 32 (37)                        | 8 (53)  | 8 (35)  | 8 (30)  | 8 (36)  | NS      |
| eGFR (ml/min)                                 | 57 ± 31                        | 107 ± 18 | 74 ± 8  | 40 ± 7  | 23 ± 4  | <0.001  |
| Creatinine (mg/dL)                            | 1.7 ± 0.9                      | 0.8 ± 0.1 | 1.1 ± 0.4 | 1.8 ± 0.6 | 2.9 ± 0.7 | <0.001  |
| Urea (mg/dL)                                  | 69 ± 39                        | 34 ± 6  | 51 ± 20 | 66 ± 23 | 114 ± 43 | <0.001  |
| Uric acid (mg/dL)                             | 6.9 ± 1.6                      | 6.2 ± 1.3 | 6.3 ± 1.5 | 7.5 ± 1.9 | 7.3 ± 1.3 | <0.02    |
| Hemoglobin (g/dL)                             | 13.1 ± 1.7                     | 14.2 ± 1.1 | 13.7 ± 1.6 | 13.1 ± 1.6 | 11.9 ± 1.5 | <0.001  |
| Glucose (mg/dL)                               | 118 ± 54                       | 96 ± 19  | 121 ± 58 | 110 ± 25 | 139 ± 80 | NS      |
| Albumin (g/dL)                                | 4.2 ± 0.4                      | 4.4 ± 0.4 | 4.2 ± 0.4 | 4.2 ± 0.3 | 4.0 ± 0.5 | NS      |
| Total cholesterol (mg/dL)                     | 220 ± 44                       | 219 ± 25 | 222 ± 44 | 217 ± 45 | 220 ± 54 | NS      |
| Low-density lipoprotein cholesterol (mg/dL)   | 133 ± 33                       | 131 ± 19 | 138 ± 33 | 140 ± 35 | 132 ± 39 | NS      |
| High-density lipoprotein cholesterol (mg/dL)  | 53 ± 13                        | 56 ± 14  | 50 ± 11  | 54 ± 16  | 52 ± 12  | NS      |
| Triglycerides (mg/dL)                         | 167 ± 89                       | 162 ± 102 | 173 ± 96 | 155 ± 65 | 180 ± 101 | NS      |
| C-Reactive protein (mg/L)                     | 3.5 ± 4.9                      | 3.0 ± 3.7 | 3.5 ± 4.1 | 3.8 ± 6.8 | 3.4 ± 3.9 | NS      |
| Plasma 8-epiPGF2α (pg/mL)                    | 109 ± 31                       | 90 ± 16  | 99 ± 27  | 125 ± 36 | 118 ± 28 | <0.001  |
| Serum TAS (mmol/L)                            | 0.33 ± 0.21                    | 0.21 ± 0.10 | 0.22 ± 0.10 | 0.30 ± 0.15 | 0.33 ± 0.15 | <0.009  |

NOTE. Values expressed as number (percent), median (percentiles 25 to 75), and mean ± SD. To convert eGFR in ml/min to ml/s, multiply by 0.01667; serum creatinine in mg/dL to μmol/L, multiply by 88.4; urea in mg/dL to mmol/L, multiply by 0.357; uric acid in mg/dL to μmol/L, multiply by 59.48; hemoglobin and albumin in g/dL to g/L, multiply by 10; glucose in mg/dL to mmol/L, multiply by 0.05551; total, low-density lipoprotein, and high-density lipoprotein cholesterol in mg/dL to mmol/L, multiply by 0.02586; triglycerides in mg/dL to mmol/L, multiply by 0.001129.

Abbreviations: NS, not significant; ACE, angiotensin II–converting enzyme; AT1, angiotensin T1 antagonist.
body mass index and hemoglobin levels decreased significantly as CKD advanced.

Plasma 8-epiPGF2a levels increased significantly as CKD progressed; namely, CKD stage 1, 90 ± 16 pg/mL (mean value in healthy subjects, 70 ± 30 pg/mL); CKD stage 2, 99 ± 27 pg/mL; CKD stage 3, 125 ± 36 pg/mL; and CKD stage 4, 118 ± 28 pg/mL (Fig 2; ANOVA $P < 0.001$). Furthermore, eGFR showed a significant inverse correlation with 8-epiPGF2a level (Fig 3; $P < 0.01$). Serum TAS level also increased in a similar mode (CKD stage 1, 0.21 ± 0.06 mmol/L [mean value in healthy subjects, 1.30 to 1.77 mmol/L]; CKD stage 2, 0.22 ± 0.10 mmol/L; CKD stage 3, 0.30 ± 0.15 mmol/L; and CKD stage 4, 0.33 ± 0.15 mmol/L [Fig 4; ANOVA $P < 0.009$]), whereas eGFR showed a significant inverse correlation with TAS (Fig 5; $P < 0.01$).

8-epiPGF2a and TAS levels showed a weak positive correlation ($P < 0.05$), whereas serum TAS level was found to have a strong correlation with serum uric acid level (Fig 6; $P < 0.01$).
We used multiple regression analysis to examine effects of confounding variables in the association of eGFR with 8-epiPGF2a and TAS levels. Statistics are listed in Table 2. eGFR showed a strong inverse association with plasma 8-epiPGF2a level ($\beta = -0.399; 95\%$ confidence interval, $-0.601$ to $-0.197; P = 0.000$), which remained highly significant after adjusting for any clinical or laboratory confounding variable. Conversely, the association between eGFR and serum TAS level ($\beta = -0.359; 95\%$ confidence interval, $-0.541$ to $-0.177; P = 0.009$) was affected by both sets of confounding variables and proved to be not a reliable marker for OS activity.

**DISCUSSION**

In this study, we tried to estimate OS activity in patients with CKD stages 1 to 4. Plasma 8-epiPGF2a level was used as the main marker
reflecting OS. 8-isoprostanes were proposed as a reliable marker of OS in patients with uremia.\textsuperscript{18,19} They represent a family of compounds produced by polyunsaturated fatty acids through a free radical-catalyzed mechanism. F2-Isoprostanes are isomers of the F2a prostaglandin derived from nonenzymatic peroxidation of arachidonic acid, resulting in free radical production. The most abundant isomer of the F2-isoprostanes is 8-epiPGF2a, considered to be an accurate marker of endogenous lipid peroxidation. From results of the present study, it is clearly evident that OS appears early during the progression of CKD because GFR is the main parameter that determined 8-epiPGF2a level.

During the last years, several studies reported that uremia was associated with increased OS activity,\textsuperscript{3,7} and both hemodialysis and peritoneal dialysis further enhance OS and contribute to a decrease in antioxidant capacity.\textsuperscript{19} More recently, it was found that 8-epiPGF2a levels were 2 to 4 times greater in hemodialysis patients compared with healthy controls matched for age and sex and were related to serum C-reactive protein, albumin, and aptoglobulin levels.\textsuperscript{19,21,22}

![Fig 6. Correlation of serum TAS and uric acid levels in 87 patients. To convert uric acid in mg/dL to μmol/L, multiply by 59.48.](image)

![Table 2. Regression Analysis of the Association of eGFR With Plasma 8-epiPGF2a and Serum TAS Levels in 87 Patients](table)

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*Sex, age, body mass index, smoking, diabetes, hypertension, therapy with angiotensin II–converting enzyme inhibitors and angiotensin T1 antagonists, and therapy with statins.

†Hemoglobin, uric acid, albumin, total cholesterol, low-density lipoprotein, high-density lipoprotein cholesterol, triglycerides, and C-reactive protein levels.
However, the number of studies that tried to assess OS development in the early stages of CKD are few and contain conflicting results. Himmelfarb et al. in a small number of subjects (10 healthy controls, 10 patients with advanced renal failure, and 10 patients on hemodialysis therapy), used serum carbonyl radicals and thiols as markers of prooxidant and antioxidant activity, respectively. They showed a progressive increase in carbonyl radicals from healthy subjects to hemodialysis, which was accompanied by a simultaneous decrease in levels of the thiols. Annuk et al. studied 37 predialysis patients with a mean GFR of 25 mL/min (0.42 mL/s) and observed that levels of end-products of lipid peroxidation and glutathione increased and were related to endothelial dysfunction in these patients. However, these results were not in accord with those reported by Oberg et al. In a study of 60 patients with CKD stages 3 to 5, they failed to describe a correlation between levels of reduced thiols, F2-isoprostanes, and carbonyl radicals with eGFR. However, the investigators admitted that the number of patients was small and their distribution in CKD stages 3 to 5 was not representative because 60% were in stages 4 and 5.

As far as we know, there are 3 reports describing an inverse correlation between OS and GFR in patients with CKD. Witko-Sarsat et al. studied the end-products of protein and carbohydrate oxidation, malondialdehyde and glutathione peroxidase, in 162 patients with GFRs ranging from 80 to 20 mL/min (1.33 to 0.33 mL/s). They found that levels of end products of protein oxidation were higher compared with controls and correlated inversely with GFR. Terawaki et al. found that serum oxidized albumin levels increased progressively as CKD advanced in 55 predialysis patients with a mean GFR of 40 mL/min (0.67 mL/s). More recently, in 159 patients with stages 1 to 5 CKD, Yilmaz et al. showed that levels of such markers of OS as malondialdehyde and oxidized low-density lipoprotein increased, whereas the antioxidants erythrocyte superoxide dismutase, glutathione peroxidase, plasma selenium, erythrocyte zinc, and copper decreased as CKD stage advanced. In our study, it was clearly shown that there was a strong inverse correlation between eGFR and 8-epiPGF2a level, which is in accord with these 3 studies. However, distribution of our patients was more representative for CKD stages 1 to 4 compared with the studies of Witko-Sarsat et al. and Terawaki et al. and similar to the study by Yilmaz et al.

It was established that there are other than GFR factors contributing to the increased prooxidant activity in patients with CKD, such as advanced age, diabetes, chronic inflammation, malnutrition, intravenous administration of iron, and factors associated with the hemodialysis session per se. In our study using multiple regression analysis, eGFR was found to be the main predictor determining 8-epiPGF2a levels, and this strong association was not affected by any confounding clinical or biochemical variable (Table 2). Terawaki et al. in a similar multivariate analysis, found that oxidized albumin level correlated significantly not only with GFR, but also with serum uric acid and magnesium levels.

Estimation of antioxidant reserve using TAS as a marker did not prove to be a successful choice. Our results show that serum TAS levels increased in parallel with 8-epiPGF2a levels as CKD stages advanced. This finding was not in accord with previous results, which showed that antioxidant reserve, reflected by such reliable markers as reduced thiols, erythrocyte superoxide dismutase, glutathione peroxidase, and platelet activating factor-acetylhydrolase, decreased as uremia advanced. Our multivariate analysis showed that serum TAS levels were confounded by several clinical and laboratory parameters, including serum acid, which showed a strong positive correlation with TAS level (Fig 6). Bergesio et al. also reached the same conclusion describing a strong correlation between serum TAS and uric acid levels in uremic patients, whereas a more recent study found that serum uric acid contributed importantly to the elevated serum TAS levels observed in patients with severe sepsis. Since the early 1980s, it was proposed that uric acid may have an important antioxidant role because it was shown by means of in vitro experiments to be a powerful scavenger of reactive oxygen species. More recently, in a prospective case-control study, greater serum uric levels were associated with elevated TAS levels in individuals with atherosclerosis, confirming experimental evidence that hyperuricemia may be a compensatory mechanism to counteract oxidative damage related to
ischemia reperfusion and atherosclerosis. Nevertheless, the role of uric acid as an antioxidant remains elusive. Hyperuricemia has been labeled as both a risk factor and marker for CVD. Therefore, estimation of antioxidant capacity in the context of our study is not reflected reliably by serum TAS levels, and these findings emphasize the importance of choosing reliable markers to define OS in different pathological entities.

The balance between free radical formation and antioxidant mechanisms is determined by the activity of such enzymes as catalases, nitric oxide synthetase, glutathione peroxidase, and superoxide dismutase, which appear to be disturbed in patients with CKD. OS represents the biological effect of the break of balance between these 2 components and appears to have a central role in the gradual endothelial dysfunction, constituting what was called “the elephant in uremia.” Endothelial dysfunction in turn may lead to arteriosclerotic remodeling of the vascular bed, which, to a great extent, is responsible for the increased cardiovascular morbidity and mortality in patients with CKD. Inflammation and malnutrition also constitute important features in the course of CKD and are in a close interplay with OS, which appears to be the connecting link through which both uremia-related and conventional risk factors affect endothelial dysfunction in patients with CKD. Results of our study show that OS presents early during CKD progression, well in advance of starting hemodialysis therapy. Given the complexity of redox balance and known differences between markers of this balance in different body components (eg, red blood cells versus plasma), it would have been more meaningful to use additional measures of oxidative damage and antioxidant status in our study (eg, protein and DNA oxidation and antioxidant enzyme activities, circulating dietary antioxidants, and thiols). Nevertheless, despite this limitation, our findings deserve attention because the assessment and understanding of OS during the early stages of CKD remains limited and obscure. Furthermore, the timing of its presentation during the course of CKD and the pathways through which it exerts its deleterious effects in the progression of renal and cardiac damage have not been defined as yet.

In conclusion, 8-epiPGF2a levels, as a marker of OS, increased significantly early during the progression of CKD, whereas TAS level does not represent a reliable marker for estimation of antioxidant capacity. These results suggest it is necessary to define clearly the timing of the break of the balance between pro-oxidant and antioxidant mechanisms and the interplay of OS with other risk factors during progression of cardio renal damage.

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