Angiotensin II Type 1 Receptor Blockers Reduce Urinary Oxidative Stress Markers in Hypertensive Diabetic Nephropathy

Susumu Ogawa, Takefumi Mori, Kazuhiro Nako, Taro Kato, Kazuhisa Takeuchi, Sadayoshi Ito

Abstract—We tested the hypothesis that blockade of angiotensin II type 1 receptors reduces oxidative stress markers in parallel with urinary albumin and type IV collagen excretions. Sixty-six diabetic patients with nephropathy were randomly assigned to either the angiotensin II receptor blocker (ARB; n=33) or trichlormethiazide (n=33) group. The majority of patients had been treated with angiotensin-converting enzyme inhibitors or calcium channel blockers for ≥1 year before the present study. Reduction of blood pressure was not different between the 2 groups, and HbA1c levels did not change over the study period (8 weeks). Treatment with ARB (candesartan 8 mg/day, n=11 or valsartan 80 mg/day, n=22) for 8 weeks reduced the levels of plasma monocyte chemoattractant protein 1, interleukin 6, urinary 8-epi-prostaglandin F2α, 8-hydroxydeoxyguanosine, albumin, and type IV collagen, whereas the levels of these markers were not altered with trichlormethiazide (2 mg/day). Significant correlation was observed between the reduction of the urinary 8-epi-prostaglandin F2α and 8-hydroxydeoxyguanosine and those of the urinary albumin and type IV collagen. Subjects with large oxidative stress had large reduction rates because of ARB administration and showed large urinary albumin suppression. These results suggest that ARBs reduce oxidative stress and inflammation in diabetic patients independent of their effects on blood pressure. In addition, increases in oxidative stress caused by angiotensin II may play an important role in the progression of diabetic nephropathy. Our results may help to explain the clinical observation that ARB reduces urinary albumin excretion very efficiently in some patients but not in others. (Hypertension. 2006;47:699-705.)

Key Words: oxidative stress ■ albuminuria ■ angiotensin II ■ inflammation

Albuminuria in diabetic nephropathy is not only an indicator for renal injury but also a predictor for cardiovascular events.1,2 This suggests the possibility that common mechanisms exist for renal and cardiovascular injuries.3 One possible mechanism may be an increase in oxidative stress and elicitation of inflammatory reactions induced by angiotensin II (Ang II).4–7 If that is the case, blockade of Ang II should decrease oxidative stress and inflammations and suppress organ damages, such as renal injury. Indeed, inhibition of the renin–angiotensin system with angiotensin-converting enzyme inhibitors (ACEIs) or Ang II receptor blockers (ARBs) in diabetes patients has now been well documented to decrease albuminuria and improve cardiovascular remodeling, thereby reducing the risk of end-stage renal disease and cardiovascular events.8 Studies have shown that blood pressure (BP)–independent effects are involved in reducing the risk of end-stage renal disease or cardiovascular events with ACEI or ARB.9–11 Whereas renin–angiotensin system inhibitors are now widely used in the treatment of diabetic patients with hypertension, their antialbuminuric effects vary from patient to patient. The mechanism of such variations remains largely unknown, and its clarification would be important for better management of diabetic patients.

The production of reactive oxygen species (ROS) is increased in both animal models of diabetes and diabetic human subjects. The increased production of ROS may play an important role in functional and structural alterations of the kidney in diabetes.12 The activation of the redox-sensitive gene occurs as a consequence of oxidative stress.13 Many of the redox-sensitive cytokines (interleukin [IL] 6) and chemokines (monocyte chemoattractant protein 1 [MCP-1]) are proinflammatory and may play a critical role in the initiation and progression of atherosclerosis and diabetic renal injury.14 Although it has been reported that ARB decreases oxidative stress and reverses endothelial dysfunction in patients with hypertension,7,15 there is not much evidence regarding Ang II-induced oxidative stress in diabetes patients, nor are there any studies demonstrating that the decreases in oxidative stress are related to the decrease in urinary albumin or type IV collagen (collagen IV) excretion.

Thus, the present study was designed to test the hypothesis that blockade of Ang II type 1 receptor reduces oxidative...
stress and inflammation independent of BP in human diabetic subjects and that changes in oxidative stress or inflammatory markers are closely related to changes in urinary albumin or collagen IV excretion. We, therefore, measured inflammatory and oxidative stress markers before and after the administration of ARB and compared with that of trichlormethiazide in diabetic patients.

Methods

Subjects and Protocols

This is a single-blind, randomized clinical study. The outpatient subjects with type 2 diabetes who met the following criteria were enrolled: (1) systolic arterial BP >130 mm Hg but <200 mm Hg, and (2) absence of marked hyperglycemia (HbA1c <8.0%). BP was measured at the doctor's office in the right arm after a 5-minute resting period, and the average of 3 consecutive BP measurements was used. Patients with a history of vascular events or revascularization; severe diabetic retinopathy, such as retinal bleeding, acute or chronic inflammatory disease, active liver disease, or renal dysfunction (serum creatinine >1.2 mg/dL); or current smokers were excluded. Subjects were randomly assigned to candesartan at 8 mg/day or valsartan at 80 mg/day in the ARB group or trichlormethiazide (2 mg/d, TCM) in the diuretic group.

Plasma levels of MCP-1 and IL-6 and urinary levels of 8-epi-PGF2α (8-epi-PGF2α), 8-hydroxydeoxyguanosine (8-OHdG), albumin:creatinine ratio (ACR), and type IV collagen excretion (IV collagen) were determined before and after 8 weeks of the therapy. Medication including antidiabetic, antihypertensive drugs was not changed during this period. All of the procedures were approved by the ethics committees of Tohoku University Hospital, and informed consent was obtained from all of the patients.

Measurements

Blood and urine were sampled in the morning after overnight fasting. The blood samples were drawn from the antecubital vein by venopuncture after 15 minutes of rest. Blood for the measurement of the plasma levels of MCP-1 and IL-6 was transferred to a chilled tube containing EDTA (1 mg/mL) and aprotinin (500 kallikrein inhibitor units/mL) and then centrifuged at 3000 rpm for 15 minutes at 4°C. The resulting supernatant was stored at −80°C until use. Plasma levels of MCP-1 and IL-6 were measured using commercially available immunoassay kits (Quantikine HS, R&D Systems). Intraassay coefficients of variation for all of the ELISA assays were <5%, and interassay variances were <10%.

Urine (5 mL) was transferred into a tube containing 0.25 mg of indomethacin and centrifuged at 10 000g for 10 minutes, and the resulting supernatant was stored at −80°C until assay for urinary oxidative stress markers. The urinary concentrations of 8-epi-PGF2α and 8-OHdG were determined using an enzyme immunoassay kit (8-isoprostane ELA kit; Cayman Chemical Co) and an ELISA kit (Japan Institute for Control of Aging, respectively), and they were normalized by creatinine. The intraassay and interassay coefficients of variation were 9.1% and 9.6% for 8-epi-PGF2α and 2.4% and 4.8% for 8-OHdG.

Statistical Analysis

Data were expressed as mean±SEM. Group differences in continuous variables that had a normal distribution were tested by paired or unpaired Student t test. Because the distributions of MCP-1, IL-6, ACR, IV collagen, 8-epi-PGF2α, and 8-OHdG levels were skewed rightward, the significance of any difference in these parameters was assessed by the Mann–Whitney U test or Wilcoxon signed-rank test. These parameters were also log transformed, which resulted in normal distributions, and significance was assessed by parametric analysis. Categorical data were compared by the Fisher test or χ² test. Correlations were determined by the Spearman rank correlation test. P values of <0.05 were considered as a level of significance.

Results

The baseline characteristics in each group are shown in Table 1. Patients were aged 43 to 78 years, and the duration of diabetes was 6 to 38 years. Eighteen patients in the ARB group and 20 patients in TCM group had received ACEI before the study. Calcium channel blockers had been administered in 9 and 11 patients of the ARB and TCM group, respectively. These treatments had not been changed ≥1 year before the start of this study. None of the patients have received ARB, diuretics, xanthine oxidase inhibitors, antiinflammatory drugs including aspirin, or drugs that have an antioxidant property, such as vitamin C, vitamin E, probucol, or 3-hydroxy-3-methylglutaryl coenzyme A inhibitors within 1 month before this study. Thirty-three patients received ARB (candesartan n=11, valsartan n=22), and 33 patients received TCM. No significant differences were observed between the 2 groups in the sex ratio, age, duration of diabetes, body mass index, HbA1c, serum creatinin (Cre), glomerular filtration rate, systolic BP, diastolic BP, IV collagen, or ACR.

The baseline parameters were not different between patients with and without ACEI or calcium channel blockers at enrollment (data not shown). The number of patients that received antidiabetic drugs among the ARB and TCM group was 10 and 11 with sulfonylurea, 9 and 8 with α-glucosidase inhibitor, 10 and 9 with biguanide, and 9 and 10 with insulin, respectively. Diabetic retinopathy was seen in 11 and 9 patients of the ARB and TCM groups, respectively. The levels of HbA1c were unchanged in either group (from 6.7±0.5 to 6.6±0.6 in the ARB group and from 6.7±0.7 to 6.6±0.6 in the TCM group). No other clinical data, such as serum transaminase, creatinin, and electrolyte levels, were altered after 8 weeks of the treatment in either group. In all of the subjects, the urinary protein level was above the criteria of microalbuminuria (ACR ≥30 mg/g Cre).

No differences were seen in BP levels between the ARB and the TCM group before or after drug administration (ARB: from 154.1±2.4/80.3±1.3 mm Hg to 141.2±2.1/80.0±1.4 mm Hg; TCM: from 153.5±2.2/81.2±1.5 mm Hg to 142.2±1.2/80.6±1.7 mm Hg; mean±SEM was not significant). A significant decrease was seen only in SBP. The percentage changes in

<table>
<thead>
<tr>
<th>Table 1. Baseline Characteristics of Subjects</th>
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<tr>
<td>Group</td>
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<td>------------------------------------------------</td>
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<tr>
<td>Antihypertensive drugs</td>
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<tr>
<td>No.</td>
</tr>
<tr>
<td>Men/women</td>
</tr>
<tr>
<td>Age, y</td>
</tr>
<tr>
<td>Duration, y</td>
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<tr>
<td>BMI, kg/m²</td>
</tr>
<tr>
<td>HbA1C, %</td>
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<tr>
<td>SBP, mm Hg</td>
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<tr>
<td>DBP, mm Hg</td>
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<tr>
<td>S-Cre, mg/dL</td>
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<td>GFR, mL/min</td>
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ACR indicates body mass index; SBP, systolic BP; DBP, diastolic BP; S-Cre, serum creatinin; GFR, glomerular filtration rate. Mean±SEM.
SBP were not different between ARB (−8.1±0.2%) and TCM (−7.2±0.2%). Moreover, both groups showed no significant changes in BMI, HbA1c, Cre, glomerular filtration rate, uric acid, triglyceride, total cholesterol, and high-density lipoprotein cholesterol values. In the ARB group, there were significant reductions in plasma levels of MCP-1 and IL-6 and urinary excretions of ACR, IV collagen, 8-epi-PGF2α, and 8-OHdG, whereas no changes were seen in the TCM group (Figure 1). The percentage changes in urinary levels of ACR, IV collagen, 8-epi-PGF2α, and 8-OHdG and plasma levels of MCP-1 and IL-6 in the ARB group were −17.4±8.0, −7.4±6.3, −22.3±10.1, −17.5±6.9, −25.7±3.6, and −29.4±6.3%, respectively, whereas those in the TCM group were 32.5±13.3, 9.3±4.9, 17.5±7.7, 21.7±5.9, −2.1±3.0, and −10.6±3.8%, respectively. In all of these, the decreases were significantly greater in the ARB than in the TCM group (P<0.05). To identify whether these effects of ARBs are the class effect or specific to valsartan or candesartan, the changes in these markers were compared between the 2 groups. The percentage changes of urinary levels of ACR, IV collagen, 8-epi-PGF2α, and 8-OHdG and plasma levels of MCP-1 and IL-6 in the candesartan group were −15.2±12.9, −4.1±11.2, −26.0±17.9, −16.5±9.4, −24.2±6.3, and −26.1±12.0%, respectively, whereas those in the valsartan group were −19.1±10.2, −10.2±7.8, −21.3±12.3, −18.5±9.2, −26.4±4.4, and −31.0±7.5%, respectively. No significant differences were seen in any of these parameters between the candesartan and the valsartan group.

Table 2 shows the percentage changes of various parameters in the ARB and TCM groups according to the use or nonuse of ACEI. In the ARB group, no differences were seen in the percentage changes of the parameters between the use and nonuse of ACEI. In the TCM group, however, all of the parameters except MCP-1 and SBP increased in the ACEI(−) group. Moreover, when the effects of ARB and TMC were compared separately in the subjects with ACEI(+) or in the subjects with ACEI(−), the ARB-induced decreases were significantly greater than those induced by TMC in most of the parameters regardless of the use of ACEI.

Treatment with ARB reduced urinary ACR and IV collagen (Figure 1). Despite a similar reduction in BP, TCM had no significant effect on urinary ACR and IV collagen. Changes in BP did not correlate with changes in any of the parameters examined. However, there were significant correlations between the percentage changes in the urinary markers of oxidative stress (urinary 8-epi-PGF2α and 8-OHdG) and those in the markers of nephropathy (ACR and IV collagen; Figure 2). In contrast, the changes in the plasma

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Figure 1. Changes in levels of urinary ACR, type IV collagen excretion, 8-epi-PGF2α, and 8-OHdG in each patient by 8 weeks of treatment with ARB (candesartan or valsartan) or TCM (top). The significance of difference between the levels of these markers at before (B) and after 8-week treatment of ARB or TCM was assessed by the Wilcoxon signed rank test (*P<0.001). Changes in log-transformed values of ACR, IV collagen, 8-epi-PGF2α, and 8-OHdG in patients by 8 weeks of treatment with ARB or TCM (bottom). After log transformation of the concentrations of the markers, the significance of any difference between mean values obtained before (B) and after (A) the ARB or TCM administration was assessed by paired Student t test (**P<0.001).
levels of the inflammation markers (MCP-1 and IL-6) did not correlate with changes in urinary ACR or IV collagen excretion.

There was no relationship between the basal levels of urinary markers of oxidative stress and the basal levels of ACR. Neither was there any relationship between the baseline ACR and ARB-induced changes in ACR. However, there were significant relationships between the basal levels of urinary oxidative stress markers and ARB-induced changes in these markers or ACR. Figure 3 shows plots of the baseline values of either urinary 8-epi-PGF2α (Figure 3, top) or 8-OHdG (Figure 3, bottom) on the horizontal axis against the percentage changes in either these markers (Figure 3, left) or ACR (Figure 3, right) on the vertical axis in the ARB group. Subjects with high baseline values showed large reduction of the oxidative stress markers and ACR.

### Discussion

The present study demonstrates that in diabetic patients with nephropathy, administration of ARB suppresses both oxidative stress and inflammation. It is the suppression of renal oxidative stress markers that is strongly correlated with the renoprotective effect of ARB. The higher the renal oxidative stress marker value, the greater the ARB-induced reductions of both renal oxidative stress markers and urinary albumin excretion rate. These actions can be similarly expected in patients who are already treated with ACEI. Thus, ARB is expected to exert renoprotective actions in patients whose oxidative stress is increased, regardless of the presence or absence of past treatment with ACEI, or how large or small the baseline ACR is. Our results would help to explain the clinical fact that some patients respond very well to ARB and some do not.

### Table 2. Percentage Changes of Each Parameter

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ARB</th>
<th>TCM</th>
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<tr>
<td>n</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>ACR</td>
<td>-23.4±9.6*</td>
<td>-23.7±7.1</td>
</tr>
<tr>
<td>11.0±13.3†</td>
<td>74.5±25.3</td>
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</tr>
<tr>
<td>IV collagen</td>
<td>-11.2±7.8*</td>
<td>-5.5±5.6</td>
</tr>
<tr>
<td>4.6±10.4†</td>
<td>24.7±7.0</td>
<td></td>
</tr>
<tr>
<td>8-Epi-PGF2α</td>
<td>-26.7±14.5*</td>
<td>-5.5±8.8</td>
</tr>
<tr>
<td>18.2±13.7†</td>
<td>37.3±12.2</td>
<td></td>
</tr>
<tr>
<td>8-OHdG</td>
<td>-16.8±11.2*</td>
<td>2.2±4.7</td>
</tr>
<tr>
<td>-16.9±7.1†</td>
<td>43.1±9.3</td>
<td></td>
</tr>
<tr>
<td>MCP</td>
<td>-21.7±5.4*</td>
<td>-2.8±3.4</td>
</tr>
<tr>
<td>-28.3±5.1†</td>
<td>1.8±5.1</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>-33.1±8.5*</td>
<td>-19.1±4.7</td>
</tr>
<tr>
<td>-21.8±9.1†</td>
<td>1.6±5.1</td>
<td></td>
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<tr>
<td>SBP</td>
<td>-6.4±2.0</td>
<td>-8.7±1.5</td>
</tr>
<tr>
<td>-10.1±1.4†</td>
<td>-4.8±1.9</td>
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NS indicates not significant; SBP, systolic BP. Mean±SEM.

*P<0.05 vs TCM ACEI(+) P1: ARB ACEI(+) vs ARB ACE(−).
†P<0.05 vs TCM ACEI(−) P2: TCM ACEI(+) vs TCM ACE(−).
In the present study, suppression of plasma inflammation markers and urinary oxidative stress markers was observed only in the candesartan- or valsartan-treated group but not in the TCM group. Because the 2 ARBs caused similar effects, obtained results were attributed to the ARB class effect rather than their unique actions. In addition, because no differences were seen in the changes in BP between the TCM and the ARB group, and because no correlation was observed between the changes in BP and changes in various markers, even within the ARB group, it may be reasonable to speculate that the changes in the markers were strongly affected by factors other than BP changes. However, because we only measured office BP, but did not evaluate ambulatory BP, we cannot entirely rule out the possible influence of BP, particularly the nighttime BP, which has been shown to correlate cardiovascular events more closely than office BP.23,24 Additional studies are needed that include ambulatory BP monitoring or the measurement of home BP.

Before our present study, some investigated the effects of ARB administration on oxidative stress and inflammations in subjects with essential hypertension or diabetes.7,15 However, our study is the first to demonstrate the relationship between changes in urinary markers of oxidative stress and changes in the marker of renal injury. Based on our observations, it may be suggested that an increase in in vivo (most probably intrarenal) oxidative stress constitutes a powerful factor for promoting renal injury and that Ang II type 1 receptor-mediated actions of Ang II play a significant role in such increase. The reason why changes in plasma inflammatory markers did not correlate to changes in renal injury markers may be because plasma inflammatory markers reflect the presence of inflammations not only in the kidneys but also in a variety of organs (such as blood vessels). It is also reported that patients with chronic kidney disease have a greater concentration of biomarkers of ROS in the urine than in the plasma and that ARB reduces urinary but not plasma concentrations of these markers.25

Figure 1 gives the impression that the higher the subject’s urinary oxidative stress marker before ARB, the greater the decline in these values after ARB. Thus, we examined the relationship between the baseline values of the oxidative stress markers and the percentage change of these markers or ACR (Figure 3). Whereas ARB demonstrated antialbuminuric actions effectively in subjects with high values of oxidative stress markers, its effects were equivocal in subjects with low values. The reason for this may be that Ang II plays a significant role in increasing intrarenal oxidative stress and that the level of urinary oxidative stress markers reflects the intrarenal level of oxidative stress.

In the present study, we did not find any correlations between the baseline value of renal oxidative stress markers and the baseline value of ACR nor did we found any
relationship between the baseline ACR and ARB-induced reduction of ACR. However, the ARB-induced reduction of ACR was found to be closely related to baseline levels of oxidative stress, as well as ARB-induced decreases in renal oxidative stress markers. Thus, it seems that the urinary albumin excretion rate is determined not only by oxidative stress but also many other factors, and the contributions of each factor vary from patient to patient. This may be one of the reasons why the antialbuminuric effect of ARB is variable among patients. Hinokio et al\textsuperscript{22} reported that diabetic patients with high urinary excretion rates of oxidative stress markers developed nephropathy more frequently than those with lower values during the 5-year follow-up period. Our study showed a strong possibility that administration of ARB is effective in these patients with high oxidative stress marker values and that ARB can reduce renal oxidative stress and inhibit the increase in albuminuria. Therefore, in patients with high renal oxidative stress markers, it seems important to use ARB from an early stage to prevent the development of nephropathy.

Our study included subjects who had already been treated with ACEI. The ACEI is well known to reduce albuminuria in diabetic patients, and it is likely that our subjects with ACEI had a high ACR before ACEI treatment. Because the ACEI possesses actions other than suppressing Ang II production (eg, bradykinin activation and transforming growth factor \(\beta\) suppression), the combination therapy of ACEI and ARB may have an additive or synergistic effect as compared with the treatment with either drug alone. Indeed, Mogensen et al\textsuperscript{26} have shown that the combination of candesartan and lisinopril reduced urinary albumin excretion and BP to a greater extent than either drug did it alone. Our study is not aimed at comparing the combination versus monotherapy. Thus, all of the subjects had taken ACEI for an extended period (\(\geq 1\) year), so the effects of the drug may have reached a plateau. Still, our study would be deemed significant in that we were able to demonstrate the potential for obtaining additional effects with additional administration of ARB, even in subjects whose oxidative stress markers and ACR failed to return to normal levels despite using ACEI. Consistent with our study, Rossing et al\textsuperscript{27} reported that an addition of ARB reduced albuminuria in diabetic patients who had already been treated with maximal recommended doses of ACEI. Our results are also consistent with the study by Agarwal\textsuperscript{28} that the addition of ARB reduced levels of urinary markers of ROS in patients with chronic kidney disease who were already treated with ACEI.

The use or nonuse of ACEI did not affect the outcome the ARB group, but it did so in the TCM group (Table 2). In those without prior administration of ACEI, TCM aggravated most of the parameters examined, whereas they were mostly unchanged in those with prior administration of ACE-I. This may indicate that, unless used in combination with ACEI, TCM may not be expected to reduce either oxidative stress or inflammations or to prevent the progression of renal injury. However, even without ACEI, the TCM treatment resulted in a substantial reduction in BP, which would inhibit renal injuries. Thus, it may be possible that subjects not treated with ACEI may have experienced worse results over time if they had not been treated with TMC (or left untreated). However, this remains speculative, because we did not have a placebo group. Nevertheless, 1 thing that became clear was that TCM was more effective when used in combination with ACEI than when it was used alone.

In the present study, we observed a substantial reduction of albuminuria with candesartan at a dose of 8 mg and valsartan at a dose of 80 mg. These doses are rather low as compared with the doses used in various clinical trials. Recent studies have shown that the antialbuminuric effect of ARB is dose dependent and that the effect reaches a plateau at doses much higher than the maximal recommended doses for BP control.\textsuperscript{29} Thus, if we used higher doses of ARB, we might have observed the antialbuminuric effects independent of urinary markers of oxidative stress. On the other hand, there is evidence for racial differences in the renoprotective effects of ARB. Chan et al\textsuperscript{20} reported that Asian diabetic patients with nephropathy benefited from ARB treatment more than whites. Thus, it may be possible that our observation is unique to the Asian or Japanese population. However, these issues remain to be clarified by additional studies.

We conclude that ARBs reduce systemic inflammation and renal oxidative stress in diabetic patients and protect against diabetic nephropathy. There is a possibility that the greater the subject’s renal oxidative stress, the greater the renoprotective effect of ARB. This may provide useful information for determining therapeutic strategies to minimize renal injury in diabetic patients.

**Perspectives**

ARBs are now widely used to lower BP and to protect from end-organ damage in diabetic patients. It has been shown that ARBs have pressure-independent effects via inhibition of inflammatory and redox pathway and inhibit the induction of adhesion molecules. Increased urinary excretion rates of albumin and collagen IV have been used for early markers of diabetic nephropathy, and our study shows that at least a part of the increase seems to be related to Ang II–induced renal oxidative stress. Because microalbuminuria has also been shown to be a risk factor for cardiovascular events, renal oxidative stress may also be involved in cardio renal risk. Additional studies are required to determine the best sensitive markers for detection of diabetic nephropathy and prediction of cardiovascular events.

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