Anti-Hypertensive Agents Inhibit \textit{In Vivo} the Formation of Advanced Glycation End Products and Improve Renal Damage in a Type 2 Diabetic Nephropathy Rat Model

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Abstract. Prevention or retardation of diabetic nephropathy (DN) includes anti-hypertensive treatment with angiotensin-converting enzyme inhibitors (ACEI) and angiotensin II type 1 receptor blockers (ARB) on the premises that these drugs have an added protective effect beyond their influence on BP. The present study used a strain of spontaneously hypertensive/NIH-corpulent rats [SHR/NDmc-cp (fat/fat)] as a model of type II DN to unravel the renoprotective effects of anti-hypertensive drugs. Olmesartan (1 or 5 mg/kg per d), an ARB, and hydralazine (5mg/kg per d), an anti-hypertensive drug without effect on the renin-angiotensin system (RAS), were given for 20 wk. BP, renal function, glucose and insulin levels, and proteinuria were monitored. Glomerular lesions and kidney pentosidine content were assessed at the end of the study. Olmesartan (1 and 5 mg) significantly reduced BP and kidney pentosidine content and improved histologic renal damage and proteinuria. The changes were dose-dependent. The effect of hydralazine (5 mg) was similar to that of olmesartan (1 mg) but reached statistical significance only for kidney pentosidine content. The similarity of both drugs’ effects on kidney damage and proteinuria suggest that renoprotection does not hinge on manipulation of RAS in these rats. By contrast, the inhibition of renal pentosidine formation assessed both by immunohistochemistry and HPLC suggests a critical role of advanced glycation end product (AGE) formation together with hypertension in the genesis of diabetic nephropathy. This view is supported by the correlation found between renal pentosidine content and proteinuria. The unsuspected AGE-lowering effect of hydralazine was further confirmed \textit{in vitro} and elucidated; it is due to both reactive carbonyl compounds trapping and modifications of the oxidative metabolism. It is concluded that AGE inhibition should be included in the therapeutic strategy of DN.

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Diabetic nephropathy (DN) increases dramatically worldwide and is now the first cause of end-stage renal failure requiring renal replacement therapy (reviewed in references 1 and 2). Its prevention or retardation has thus become an important issue in biomedical research.

Several large clinical trials have recently demonstrated that control of hypertension by angiotensin-converting enzyme inhibitors (ACEI) and angiotensin II type 1 receptor blocker (ARB) significantly delayed the onset and progression of DN (3–5). This effect is due to the reduction of BP together with as-yet undefined additional mechanisms. Only studies of adequate experimental models will allow an in-depth exploration of DN pathogenic mechanisms and thus the development of newer therapeutic tools.

In the present study, we use a strain of a spontaneously hypertensive/NIH-corpulent rat [SHR/NDmc-cp (fat/fat)] as a model of type 2 DN (6) to unravel the mechanisms of the ARB-induced renoprotection against DN. We demonstrate a dose-dependent, renoprotective effect of olmesartan, which is an ARB associated with the inhibition of advanced glycation end product (AGE) formation (7). Hydralazine, the effect of which does not involve the renin-angiotensin system (RAS), has anti-hypertensive, renoprotective, and AGE-inhibitory effects similar to those of low-dose olmesartan, an observation suggesting that renoprotection does not hinge on the manipulation of the RAS. Taken together, the present data suggest the possibility that prevention of DN should rely not only on the reduction of BP but also on the inhibition of protein modifications induced by advanced glycation.
Table 1. Body weight, blood glucose, and lipid levels of the experimental rats at the end of the study (mean ± SE)

<table>
<thead>
<tr>
<th></th>
<th>SHR/NDmc-cp (fat/fat)</th>
<th>SHR</th>
<th>WKY</th>
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<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Olmesartan 1 mg/kg</td>
<td>Olmesartan 5 mg/kg</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>650 ± 14a</td>
<td>663 ± 14</td>
<td>651 ± 12</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>173 ± 29</td>
<td>142 ± 14</td>
<td>254 ± 33b</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>195.7 ± 31.9a</td>
<td>186.5 ± 46.1</td>
<td>271.4 ± 35.6</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>128 ± 6a</td>
<td>115 ± 7</td>
<td>102 ± 7</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>872 ± 130a</td>
<td>596 ± 94</td>
<td>533 ± 74</td>
</tr>
<tr>
<td>Phospholipid (mg/dl)</td>
<td>387 ± 19a</td>
<td>336 ± 19</td>
<td>310 ± 12c</td>
</tr>
</tbody>
</table>

* a P < 0.01 versus SHR (vehicle) by Student t test.
* b P < 0.05 versus SHR/NDmc-cp (fat/fat) (olmesartan, 1 mg/kg) by Tukey test.
* c P < 0.01 versus SHR/NDmc-cp (fat/fat) (vehicle) by Tukey test.
* d P < 0.01 versus SHR (vehicle) by Student t test.

Materials and Methods
Anti-Hypertensive Agents
Two anti-hypertensive agents, olmesartan (Pharmacology and Molecular Biology Research Laboratories, Sankyo Pharmaceutical, Tokyo, Japan) and hydralazine (Sigma, St. Louis, MO) were used.

Animals
Male subline of spontaneously hypertensive/NIH-corpulent rat [SHR/NDmc-cp (fat/fat)] established in Disease Model Cooperative Research Association (Kyoto, Japan), spontaneously hypertensive rats (SHR), and Wistar-Kyoto rats (WKY) were purchased from SLC (Shizuoka, Japan). SHR/NDmc-cp (fat/fat), aged 13 wk, were randomly divided into four groups and allocated to various regimens as follows: ten rats on vehicle (group 1), nine rats on low-dose olmesartan (1 mg/kg per d) (group 2), ten rats on high-dose olmesartan (5 mg/kg per d) (group 3), and nine rats on hydralazine (5 mg/kg per d) (group 4). SHR (hypertensive, non-obese), aged 13 wk, were divided into two groups: ten rats on vehicle (group 5) and ten rats on high-dose olmesartan (5 mg/kg per d) (group 6). Ten control WKY were on vehicle (group 7). Drug treatment lasted 20 wk. The protocol was in accordance with the Animal Experimentation Guidelines of Research Laboratories, Sanyo Co., Ltd.

BP, Urine Collection, and Blood Sampling
Systolic BP was determined in conscious rats by the tail-cuff method at the beginning of the study, 2 wk, and every 4 wk subsequently until euthanasia. At the end of the study, each rat was weighted and placed in a metabolic cage for a 24-h urine collection. Blood samples were obtained before death.

Biochemical Measurements in Blood and Urine
Glucose, total cholesterol, triglycerides, phospholipids, creatinine, and urea nitrogen (BUN) concentrations were determined in plasma, and protein and creatinine concentrations in urine with an automatic analyzer (Synchron CX7, Beckman Coulter Inc., Fullerton, CA). Plasma insulin was measured with a commercially available kit (Morinaga Biochemistry Lab, Tokyo, Japan).

Table 2. Systolic BP levels (mmHg) of the experimental rats (mean ± SE)

<table>
<thead>
<tr>
<th>Weeks</th>
<th>SHR/NDmc-cp (fat/fat)</th>
<th>SHR</th>
<th>WKY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Olmesartan 1 mg/kg</td>
<td>Olmesartan 5 mg/kg</td>
</tr>
<tr>
<td>Pre</td>
<td>153 ± 4ab</td>
<td>152 ± 4</td>
<td>151 ± 5c</td>
</tr>
<tr>
<td>2</td>
<td>152 ± 4ab</td>
<td>132 ± 4d</td>
<td>126 ± 5d</td>
</tr>
<tr>
<td>4</td>
<td>149 ± 4ab</td>
<td>131 ± 6e</td>
<td>120 ± 5d</td>
</tr>
<tr>
<td>8</td>
<td>157 ± 3ab</td>
<td>138 ± 4d</td>
<td>128 ± 3d</td>
</tr>
<tr>
<td>16</td>
<td>168 ± 5ab</td>
<td>135 ± 8d</td>
<td>115 ± 5d</td>
</tr>
<tr>
<td>20</td>
<td>170 ± 9ab</td>
<td>133 ± 8f</td>
<td>118 ± 6d</td>
</tr>
</tbody>
</table>

* a P < 0.01 versus SHR (vehicle) by Student t test.
* b P < 0.01 versus WKY (vehicle) by Student t test.
* c P < 0.05, d P < 0.01 versus SHR/NDmc-cp (fat/fat) (vehicle) by Tukey test.
* e P < 0.01 versus SHR (olmesartan, 5 mg/kg) by Student t test.
* f P < 0.05 versus SHR/NDmc-cp (fat/fat) (olmesartan, 1 mg/kg) by Tukey test.
Table 3. Renal functions and urinary protein excretion of the experimental rats at the end of the study (mean ± SE)

<table>
<thead>
<tr>
<th></th>
<th>SHR/NDmc-cp (fat/fat)</th>
<th>SH</th>
<th>WKY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle 1 mg/kg</td>
<td>Olmesartan 1 mg/kg</td>
<td>Hydralazine 5 mg/kg</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.36 ± 0.05</td>
<td>0.42 ± 0.04</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>2.64 ± 0.60</td>
<td>1.89 ± 0.29</td>
<td>2.16 ± 0.32</td>
</tr>
<tr>
<td>Urinary total protein (mg/kg per d)</td>
<td>276.2 ± 31.2</td>
<td>185.5 ± 31.9</td>
<td>117.9 ± 26.5</td>
</tr>
</tbody>
</table>

\* P < 0.05, \* P < 0.01 versus SHR (vehicle) by Student t test.
\* P < 0.01 versus SHR/NDmc-cp (fat/fat) (vehicle) by Tukey test.
\* P < 0.01 versus WKY (vehicle) by Student t test.

Table 4. Morphologic evaluation of glomerular damage in the experimental rats at the end of the study (mean ± SE)

<table>
<thead>
<tr>
<th></th>
<th>SHR/NDmc-cp (fat/fat)</th>
<th>SH</th>
<th>WKY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle 1 mg/kg</td>
<td>Olmesartan 1 mg/kg</td>
<td>Hydralazine 5 mg/kg</td>
</tr>
<tr>
<td>Glomerular sclerosis score</td>
<td>0.75 ± 0.06a</td>
<td>0.61 ± 0.07</td>
<td>0.39 ± 0.02b-c</td>
</tr>
</tbody>
</table>

\* P < 0.01 versus SHR (vehicle) by Wilcoxon test.
\* P < 0.01 versus SHR/NDmc-cp (fat/fat) (vehicle) by Tukey test.
\* P < 0.05 versus SHR/NDmc-cp (fat/fat) (hydralazine, 5 mg/kg) by Steel-Dwass test.
\* P < 0.01 versus WKY (vehicle) by Wilcoxon test.

Morphologic Analysis

Coronal sections of renal tissue (3 to 4-μm-thick) were stained with periodic acid-Schiff (PAS) and examined by light microscopy in a blinded fashion. Glomerular sclerosis was semiquantitatively evaluated according to criteria developed by Uehara et al. (8). Briefly, 50 glomeruli were selected randomly in each animal for morphometric analysis. The severity of glomerular sclerosis was graded according to the damaged glomerular area expressed as a percentage of total area: 0, no lesions; 1+, 1% to 25%; 2+, 5% to 50%; 3+, 50% to 75%; 4+, 75% to 100%. An overall glomerular sclerosis score per animal was obtained by multiplying each severity score (0 to 4+) with the percentage of glomeruli displaying the same degree of injury and summing these scores.

Immunohistochemistry

Pentosidine, an AGE, and another oxidative protein product generated during lipid peroxidation, 4-hydroxynonenal protein adduct, were detected in frozen kidney sections fixed with ethanol: acetone. The avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) was utilized to reduce background staining. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in PBS for 15 min at room temperature before application of the first antibodies. The sections were stained with rabbit anti-pentosidine antibody (9) or mouse monoclonal anti-4-hydroxynonenal antibody, NA59 (10) (generously provided from Dr. Witztum JL) using a 3,3’-diaminobenzidine detection system. Non-immune or mouse rabbit IgG was used as a negative control.

AGE Measurement

The pentosidine content of the kidney was measured. Kidney tissue (100 mg) was minced, rinsed with 10% TCA, dried under vacuum, and acid hydrolyzed in 500 μl of 6 N HCl for 16 h at 110°C under nitrogen. Pentosidine was analyzed on a reverse-phase HPLC as described previously (11). In brief, a 20-μl aliquot of the acid hydrolysate diluted by PBS was injected into an HPLC system and separated on a C18 reverse-phase column (Waters, Tokyo, Japan). The effluent was monitored with a fluorescence detector (RF-10A; Shimadzu, Kyoto, Japan) at an excitation-emission wavelength of 335/385 nm. Synthetic pentosidine was used as a standard.

In Vitro AGE-Inhibitory Effects

The inhibitory effect of hydralazine on AGE formation was assessed in vitro. In brief, fresh heparinized pooled plasma sample (900 μl), obtained with informed consent from nondiabetic hemodialysis patients before the dialysis session, was incubated with 100 μl of 50 mM hydralazine dissolved in DMSO. The generation of pentosidine during incubation was then determined by the HPLC assay according to our previous method (11). The mechanisms of AGE inhibition, i.e., dicarbonyl (glyoxal and methylglyoxal) entrainment, pyridoxal 5'-phosphate entrainment, scavenging activities for sugar-derived free radical and hydroxyl radical, and chelating activity for transition metal ions, were also investigated as described previously (7).
Statistical Analyses
All data are expressed as the mean ± SEM. The statistical significance of the differences between the SHR/NDmc-cp (fat/fat) vehicle group and the other three groups of SHR/NDmc-cp (fat/fat) was determined by the Tukey test. The dose-dependency of the olmesartan effects was assessed by linear regression analysis. The differences between the SHR group and the SHR treated with olmesartan group, the differences between the SHR/NDmc-cp (fat/fat) group and SHR group, and the difference between SHR group and WKY group were determined by t test. For histologic data, the statistical significance of the differences was determined by the Steel-Dwass test or the Wilcoxon test. All calculations relied on SAS software (SAS Institute, Cary, NC). Values are considered significant at P < 0.05.

Results
Body Weight, Blood Glucose, and Lipid Levels
At the end of the study, all the SHR/NDmc-cp (fat/fat) were obese and had developed hyperglycemia with hyperinsulinemia and hyperlipidemia in contrast with SHR (Table 1). Two types of anti-hypertensive agents were used: the RAS-dependent olmesartan, an ARB, and the RAS-independent hydralazine. When compared with control SHR/NDmc-cp (fat/fat) given vehicle, both drugs failed to modify SHR/NDmc-cp (fat/fat) body weight and plasma levels of glucose, insulin, total cholesterol, and phospholipids except for high-dose olmesartan, which significantly (P < 0.05) decreased the plasma levels of total cholesterol and phospholipid in SHR/NDmc-cp (fat/fat).

Systolic BP
In comparison with control WKY rats, all SHR (P < 0.01) and SHR/NDmc-cp (fat/fat) rats (P < 0.01) were hypertensive at the onset of the study, the former more severely than the latter (P < 0.01). Olmesartan and hydralazine significantly decreased systolic BP throughout the experiment (Table 2). The anti-hypertensive effect of high-dose (5 mg) olmesartan was significantly more marked than that of hydralazine (P < 0.005). The anti-hypertensive effect of high-dose olmesartan was similar in SHR and SHR/NDmc-cp (fat/fat).
Urinary Protein Excretion and Renal Function

At the end of the study, SHR/NDmc-cp (fat/fat) had an elevated proteinuria (Table 3). Olmesartan reduced urinary protein excretion in a dose-dependent fashion ($P < 0.005$). The fall averaged 33 and 57% in the 1 and 5 mg group. The fall in proteinuria observed in the hydralazine group (25%) was similar to that of the low-dose olmesartan group but failed to reach statistical significance. Control SHR developed a mild proteinuria, which was significantly reduced by high-dose olmesartan ($P < 0.01$). Urinary protein excretion of WKY remained

Figure 2. Immunohistochemical analysis of 4-hydroxynonenal protein adduct (A and B), an oxidative protein product generated during lipid peroxidation, and of pentosidine (C through E), an AGE. (A) The accumulation of 4-hydroxynonenal protein adduct was observed in glomeruli of SHR/NDmc-cp (fat/fat) given the vehicle. (B) 4-hydroxynonenal protein staining was abolished in SHR/NDmc-cp (fat/fat) given high-dose olmesartan. (C) Pentosidine accumulated in the tubules of SHR/NDmc-cp (fat/fat) given the vehicle. (D) Pentosidine accumulation was observed in the arterial walls of SHR/ND mc-cp (fat/fat) rats. (E) Pentosidine staining was abolished in SHR/NDmc-cp (fat/fat) given high dose olmesartan.
normal. Renal function remained normal in all experimental groups.

Renal Histology

Glomerular damage developed mainly in SHR/NDmc-cp (fat/fat) rats (Table 4). At the end of the study, segmental glomerulosclerosis was markedly elevated ($P < 0.01$) in SHR/NDmc-cp (fat/fat) given the vehicle (Figure 1C). Olmesartan reduced glomerular damage in a dose-dependent manner ($P < 0.001$). The decrease averaged 19 and 48% in the 1 and 5 mg groups, respectively. (Figure 1D). The fall in glomerular damage observed in the hydralazine group (16%) was similar to that of the low-dose olmesartan group. Segmental glomerulosclerosis was present only in a small number of glomeruli in the SHR group (Figure 1A) and absent in the WKY group (Figure 1B).

Immunohistochemistry for Oxidative Protein Product and AGE

The oxidative protein product generated during lipid peroxidation, 4-hydroxynonenal-protein adduct, accumulated in glomeruli of SHR/NDmc-cp (fat/fat) given the vehicle (Figure 2A). It was not suppressed by low-dose olmesartan or hydralazine, but it disappeared in the high-dose olmesartan group (Figure 2B). We did not detect 4-hydroxynonenal-protein adduct in either control WKY or SHR rats.

Pentosidine was absent in glomeruli of all groups. It was detected in the arterial walls in the SHR/NDmc-cp (fat/fat) vehicle group (Figure 2D). It was suppressed only in the high-dose olmesartan group (Figure 2E). Pentosidine was not found in the arterial walls of WKY or SHR rats.

Pentosidine Content of the Kidney

Immunohistochemistry does not allow a sensitive, precise quantification of the AGE protein modification. Pentosidine content in the kidney was therefore measured chemically by HPLC analysis (Table 5). The renal pentosidine, expressed as pmol/mg protein, was significantly ($P < 0.01$) higher in the SHR/NDmc-cp (fat/fat) vehicle group than in both SHR and WKY groups. Olmesartan reduced renal pentosidine content in a dose-dependent way ($P < 0.005$). Hydralazine also decreased renal pentosidine content ($P < 0.05$).

In SHR/NDmc-cp (fat/fat) rats, the renal pentosidine content was significantly correlated ($P < 0.001$) with proteinuria (Figure 3A). Systolic BP was significantly ($P < 0.05$) correlated with either proteinuria (Figure 3B) or renal pentosidine content (Figure 3C).

In Vitro AGE Inhibition

We were surprised by the AGE-lowering effect of hydralazine. We tested its mechanism in vitro, as we have previously done for a series of compounds including olmesartan (7). In vitro production of two AGE, pentosidine (Figure 4A) and CML (Figure 4B), in nondiabetic hemodialysis plasma is inhibited in a dose-dependent manner by hydralazine (IC50 = 0.52 and 0.77 mM for pentosidine and CML, respectively). A similar effect is demonstrated in plasma from non-uremic diabetic patients and from diabetic hemodialysis patients, and in bovine serum albumin incubated with 10 mM ascorbine in 0.1 M sodium phosphate buffer (pH 7.4) (data not shown).

The mechanisms of the AGE inhibitory effect of hydralazine have been further documented. In contrast with olmesartan (7), but like aminoguanidine, hydralazine efficiently traps reactive carbonyl compound (RCO) precursors for AGE such as glyoxal and methylglyoxal (Figure 4C) as well as pyridoxal (Figure 4D). Hydralazine also impairs oxidative metabolisms. In contrast with aminoguanidine but like olmesartan, hydralazine decreases in a dose-dependent manner the level of carbon-centered radicals (Figure 4E). It also reduces the level of hydroxyl radicals, a characteristic shared to a minor extent by aminoguanidine (Figure 4F). Finally, hydralazine interferes with the Fenton reaction as it chelates copper and inhibited the autoxidation of ascorbic acid (IC50 = 1.3 μM), as shown in Figure 4G.

Discussion

The present data demonstrate that olmesartan, an ARB given to rats with type II like diabetes, reduces in a dose-dependent fashion the development of diabetic nephropathy (DN) as evidenced by a decrease in proteinuria and in pathologic evidence of diabetic glomerulosclerosis. Proteinuria decreased by 33 and 57%, and glomerular damage by 19 and 48% in the 1 and 5 mg/kg per d groups, respectively. These results are consistent with recent clinical trials in patients with type II DN given ARB (3–5). The renal benefits accruing from BP lowering did not fully account for the observed protection so that additional benefits derived from the blockade of the RAS, its lowering of glomerular capillary pressure, and/or the reduced

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**Table 5.** Pentosidine content in the kidney (pmol/mg) of the experimental rats at the end of the study (mean ± SE)

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<thead>
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<th>SHR/NDmc-cp (fat/fat)</th>
<th>SHR</th>
<th>WKY</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Kidney               | 0.101 ± 0.009<sup>a</sup>b 0.089 ± 0.015 0.058 ± 0.006<sup>d</sup> 0.061 ± 0.006<sup>c</sup> 0.040 ± 0.003 0.039 ± 0.002 0.042 ± 0.004

<sup>a</sup> $P < 0.01$ versus SHR (vehicle) by Student t test.
<sup>b</sup> $P < 0.01$ versus WKY by Student t test.
<sup>c</sup> $P < 0.05$.
<sup>d</sup> $P < 0.01$ versus SHR/ND mc-cp (fa/fa) (vehicle) by Tukey test.
fibro-proliferative actions of aldosterone and angiotensin (12) were postulated.

To evaluate whether the additional benefit of olmesartan derived from its interference with the RAS, we compared the efficacy of olmesartan with another anti-hypertensive agent, acting independently of the RAS, hydralazine (13). The effect of hydralazine at 5 mg/kg per d on proteinuria and glomerular damage, −25 and −16%, respectively, was similar to that observed with the 1 mg/kg per d low olmesartan dosage, the BP reduction being similar in the two groups. Although the observed changes did not reach statistical significance, their similarity raises the possibility that the added benefits of ARB in clinical trials are not necessarily due to their ability to interfere with the RAS. Of note, hydralazine was not used in the comparative clinical trials, which concluded that ARB had additional renoprotective effects. Alternatively, the fact that hydralazine’s reduction of proteinuria and glomerular damage did not reach significance might suggest a lack of renoprotection. Taken together with the striking hydralazine-induced inhibition of intrarenal formation of pentosidine, this interpretation might point to dissociation between AGE and the development of DN. Although unlikely, this view points to the necessity of more experimental evidence to delineate the respective roles of AGE and RCO and of the vasomotor consequences of RAS inhibition in the protection against DN.

It should be pointed out that the drug dosage used in the present study is clinically relevant. The plasma levels achieved in rats are close to the pharmacologically effective levels in healthy human volunteers. Olmesartan (10 mg/kg body wt per d) and hydralazine (12 mg/kg body wt per d) yield peak plasma levels of 1.3 µg/ml and 0.6 to 1.1 µg/ml, respectively, in rats to be compared with 0.4 to 0.5 µg/ml and 0.10 to 0.15 µg/ml peak plasma levels for olmesartan and hydralazine, respectively, given to human volunteers at pharmacologically effective doses (Sada T, unpublished observation and [14]).

If intrarenal hemodynamic changes are not at the root of the additional benefits derived from ARB, what other mechanism should be contemplated? We previously demonstrated in vitro that olmesartan inhibits in a dose-dependent fashion the formation of AGE and raised the possibility that this effect contributed to the renoprotective effect of ARB (7). The present results confirm in vivo the inhibitory effect of ARB on advanced glycation. In the SHR/NDmc-cp (fat/fat) rats, olmesartan reduces in a dose-dependent fashion the pentosidine content of renal tissue measured by HPLC and, at high dosage, suppresses the formation of pentosidine in the renal arterial walls detected by immunohistochemistry. Surprisingly, hydralazine also significantly reduced (~40%) the renal tissue pentosidine content. The similarity of the effects of hydralazine and olmesartan on renal pentosidine content supports the possibility that AGE inhibition contributes to the renoprotective action of ARB. Further evidence in favor of this hypothesis has been recently reported by Forbes et al. (15) and Wilkinson-Berka et al. (16) in streptozotocin-induced diabetic rats. In the former study, ramipril and the AGE inhibitor, aminoguanidine, reduced to the same extent renal AGE accumulation measured by immunohistochemistry and fluorospectrometry. In the latter

![Figure 3](image-url)
study, two AGE inhibitors, aminoguanidine and ALT-946, decreased AGE immunolabeling and provided renal protection. These results are in agreement with our observations based on a highly specific HPLC methodology for tissue pentosidine measurement.

The highly significant correlation (P < 0.001) observed in SHR/NDmc-cp (fat/fat) rats between proteinuria, taken as an index of renal damage, and renal levels of pentosidine support the existence of a link between renal damage and AGE formation. Still, it should not be forgotten that systolic BP is also correlated, although less strongly, with both proteinuria and renal pentosidine content. In SHR rats, despite a marked elevation of systemic BP [much higher than in SHR/NDmc–cp (fat/fat)], renal levels of pentosidine are not higher than in WKY and pentosidine was not detected by immunohistochemistry in the arterial walls. It is thus unlikely that hypertension by itself enhances the formation of AGE. Of course, in SHR/NDmc-cp (fa/fa) rats, hypertension combined with metabolic derangement of diabetes, e.g., hyperglycemia and oxidative stress, could influence pentosidine formation and proteinuria.

The beneficial effects of both olmesartan and hydralazine on diabetic renal damage are especially noteworthy, as they are manifest despite sustained hyperglycemia. A similar observation has been reported in other experimental diabetic rats given AGE inhibitors without anti-hypertensive effect, e.g., aminoguanidine, ATL-946, (±)-2-isopropylidenehydradono-4-oxothiazolidin-5-ylacetanilide (OPB-9195), and pyridoxamine (16–19). These data thus suggest a direct role of AGE formation in the development of diabetic renal complications.

The discovery that hydralazine treatment was associated with a lowered pentosidine formation in our diabetic rat model, raised the possibility that hydralazine affected directly advanced glycation, just as olmesartan and a series of other ACEI and ARB (7). Hydralazine was thus characterized in vitro as described previously (7). Indeed, it reduces in vitro pentosidine and CML production through a combination of characteristics identified in several other AGE inhibitors. They include the trapping of RCO precursors for AGE, e.g., GO and MGO, just as aminoguanidine and OPB-9195 as well as the inhibition of oxygen radical production and the chelation of transition metals accelerating the Fenton reaction, just as ACEI and ARB.

Our in vitro results were obtained at drug concentrations above those achieved under clinical circumstances. We have therefore delineated the dose-effect relationship of lower concentrations of olmesartan and hydralazine, from 0.01 to 800 μM, on pentosidine generation. The dose responses are curvilinear for both olmesartan (7) and hydralazine (data not shown) demonstrating thus an effect at far lower concentrations than those used in the present assays. These data confirm previous conclusions drawn for temocaprilat and olmesartan (7). Such discrepancies between in vivo and in vitro data are not unusual in pharmacology and may be related to a much longer drug exposure in vivo than in in vitro assays.

The significant fall of cholesterol and phospholipids observed in the high-dose olmesartan group remains to be discussed. It might result from the marked fall in proteinuria observed in this group as suggested by the positive correlation reported by Zoa et al. (20) between serum cholesterol and proteinuria, in a model of passive Heymann nephritis. AGE formation has been linked with lipid metabolism in both experimental and clinical studies. In streptozotocin diabetic rats (19), the group of Baynes has reported that an AGE inhibitor, pyridoxamine, reduced both AGE formation and hyperlipidemia. In diabetic humans, another AGE inhibitor, aminoguanidine, lowers lipids (21), perhaps through a decreased advanced glycation of apolipoprotein B (22). In a group of diabetic patients, plasma levels of cholesterol proved positively correlated with plasma pentosidine levels (23). Finally, we recently observed in hemodialysis patients that lowering of pentosidine levels was accompanied by improved triglyceride levels (Miyata T, unpublished observation). The biochemical basis of these relationships is yet to be ascertained.

Despite a BP rise similar to that of SHR/NDmc-cp (fat/fat) rats, nondiabetic SHR rats exhibit only a slight, though significant, increase of proteinuria and glomerular damage. As both rat strains have a common genetic background, this finding points to the critical role of sustained hyperglycemia with high insulin levels and/or obesity-related hypercholesterolemia. The attendant advanced glycation of proteins, identified here by pentosidine, might be a key element of the enhanced susceptibility of the kidneys. It is associated with a number of cellular events, summarized previously (24,25), whose downregulation by AGE inhibitors may prove critical to the protection of the kidney’s integrity. Alternatively, lowering of pentosidine might reflect a decreased oxidative stress with a double consequence: reduced production of RCO precursors of pentosidine and reduced direct oxidative damage to proteins. The evidence that both tested anti-hypertensive agents significantly decrease in vitro the production of oxygen radical species and that high-dose olmesartan reduces in vivo the glomerular accumulation of a lipid peroxidation product, 4-hydroxynonenal-protein adduct, fits with the latter hypothesis.

In nondiabetic SHR rats also, olmesartan induces a minimal, though statistically significant, fall in proteinuria and a marginal, statistically NS decrease in glomerular damage, without changes in renal pentosidine content, all of which probably reflect only the benefits of a lower glomerular filtration pressure.

These considerations provide a framework to understand the additional benefits, beyond those derived from BP lowering, described in clinical studies of ARB in type II diabetics. Indeed our rat model, contrary to other models such as the streptozotocin-induced diabetic rats, is relevant to human type II diabete: obesity, early hypertension, hyperglycemia with hyperinsulinemia, proteinuria, and glomerular lesions resembling human diabetic glomerulosclerosis (6). We suggest that significant renoprotection is derived not only from BP-lowering and antangiotensin inhibition, but also from reduced AGE formation, probably associated with a decreased oxidative stress. This hypothesis fits also with the renoprotection observed in diabetic rat models without hypertension given AGE inhibitors (17–19) or in normotensive human diabetic patients given ACEI endowed with an AGE-inhibiting potential (26,27).

In conclusion, treatment with the anti-hypertensive agents
employed in this study improved functional and morphologic damages of experimental DN. This was associated with a decreased accumulation of AGE in the kidney. These results suggest a potential role for AGE inhibition in future therapeutic strategies to prevent the development of DN. Further studies should elucidate the cause-effect relationship between the AGE inhibition and the improvement of renal function in DN.

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References


Figure 4. Hyaluronic in vitro the formation of advanced glycation end product (AGE). Inhibition of pentosidine (A) and CML (B) formation upon incubation of nondiabetic hemodialysis plasma. Plasma was incubated at 37°C for 1 wk in the presence of several concentrations of hyaluronic. The yields of pentosidine and CML in the incubation mixtures were determined by HPLC and GC/MS, respectively. Data is expressed as mean ± SD. (C) Entrapment of dicarbonyl compounds during incubation. Various concentrations of hyaluronic were incubated at 37°C for 20 h with 50 μM glyoxal and methylglyoxal in 0.1 M phosphate buffer (pH 7.4). The content of residual glyoxal and methylglyoxal was determined by HPLC. Data is expressed as means ± SD. (D) Kinetics of pyridoxal 5’-phosphate (PLP) entrapment by the tested compounds during incubation. PLP (50 μM) was incubated with the tested compounds (500 μM) in PBS at 37°C. Aliquots were removed at various times and assayed for residual PLP by HPLC. (E) The scavenging effect on ribose-derived carbon-centered radical. Vertical axis shows relative electron spin resonance signal (ESR) height of N-t-butyl-a-phenyl nitrotrone (PBN) spin adduct of ribose-derived carbon-centered radical at various drug concentrations and in the presence of chelax, a trace metal chelator. (F) The scavenging effect on hydroxyl radical. Vertical axis shows relative ESR signal height of 5,5’-dimethyl-1-pyrroline-N-oxide (DMPO)OH at various drug concentrations. Details are described in Materials and Methods. Control data obtained in the absence of drug is set to 100%. Data is expressed as means ± SE. (G) Metal chelating activity of hyaluronic. Ascorbic acid (500 μM) was incubated at 30°C with 500 nM CuCl₂ in 50 mM phosphate buffer (pH 7.4) in the presence of various concentrations of hyaluronic. The content of ascorbic acid in the incubation mixture was determined by HPLC.