

Renoprotective Properties of Angiotensin Receptor Blockers beyond Blood Pressure Lowering

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Clinical studies have demonstrated that some antihypertensive agents provide renoprotection independent of BP lowering. Recent *in vitro* and *in vivo* studies evaluated the mechanisms involved in this protection. First, the *in vitro* effects of several angiotensin II type 1 receptor blockers (ARB), calcium channel blockers (CCB), and β blockers (BB) on various mediators were compared: Formation of pentosidine (an advanced glycation end product), hydroxyl radical-induced formation of *o*-tyrosine, and transition metals-induced oxidation of ascorbic acid (the Fenton reaction). All of the six tested ARB but neither the six CCB nor the nine BB inhibited pentosidine formation. ARB, as well as BB but not CCB, inhibited hydroxyl radicals-mediated *o*-tyrosine formation. ARB but neither BB nor CCB inhibited efficiently transition metals-catalyzed oxidation of ascorbic acid. Second, the *in vivo* consequences for the kidney of these various *in vitro* effects were evaluated. Hypertensive, type 2 diabetic rats with nephropathy, SHR/NDmcr-cp, were given for 20 wk either olmesartan (ARB) or nifedipine (CCB), or atenolol (BB). Despite similar BP reduction, only ARB significantly reduced proteinuria and prevented glomerular and tubulointerstitial damage (mesangial activation, podocyte injury, tubulointerstitial injury, and inflammatory cell infiltration). It is interesting that only ARB prevented abnormal iron deposition in the interstitium, corrected chronic hypoxia, reduced expressions of heme oxygenase and p47phox (a subunit of NADPHoxidase), and inhibited pentosidine formation (which correlates well with proteinuria). These observations confirm unique renoprotective properties of ARB, independent of BP lowering but related to decreased oxidative stress (hydroxyl radicals scavenging and inhibition of the Fenton reaction), correction of chronic hypoxia, and inhibition of advanced glycation end product formation and of abnormal iron deposition. These benefits of ARB may contribute to the renoprotection observed beyond BP lowering.

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Several clinical studies, mainly but not only in diabetic patients, have provided evidence that some antihypertensive agents that inhibit the renin-angiotensin system (RAS), namely angiotensin II type 1 receptor blockers (ARB) and angiotensin-converting enzyme inhibitors (ACEI), are renoprotective (1–4). Recently, the renoprotection provided by these drugs seems at least partly independent of BP lowering and related perhaps to the inhibition of the RAS (5–8). ARB and ACEI thus now are part of the standard treatment of patients with diabetic nephropathy, regardless of the presence of systemic hypertension. A similar renoprotective effect has been claimed for nifedipine or other calcium channel blockers (CCB) acting independent of the RAS (9,10), but results of clinical studies remain disputed (5).

The nature of the renoprotection provided by some antihypertensive agents independent of BP lowering and inhibition of

the RAS thus has become a topic of major interest. We previously demonstrated *in vitro* that ARB and ACEI but not nifedipine inhibit, in diabetic or uremic serum, the formation of advanced glycation end products (AGE) (11), previously implicated in the pathology of diabetic complications and atherosclerosis (12–16). The AGE inhibitory effect of ARB, unlike that of ACEI, is linked to a common core structure, 5-(4'-methylbiphenyl-2-yl)-1H-tetrazol. It thus is a class effect (11). By contrast, ACEI have no common core structure and it is not conclusive whether the AGE inhibitory effect of ACEI is a class effect.

We now extend this *in vitro* approach to several other renoprotective mechanisms and compare the effects of ARB, CCB, and β blockers (BB). In this model, the effects of the various antihypertensive drugs are independent of BP lowering or RAS modifications. Clearly, only ARB combined unique *in vitro* properties, possibly involved in renoprotection (*e.g.*, hydroxyl radicals scavenging, inhibition of AGE formation and of the Fenton reaction by transition metal chelation).

The *in vivo* relevance of these *in vitro* results is confirmed, at least in part, in a hypertensive, type 2 diabetic rat model with nephropathy, SHR/NDmcr-cp. Despite similar BP-lowering effects, only an ARB significantly reduces proteinuria and pre-

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vents glomerular and tubulointerstitial damage (mesangial activation, podocyte injury, tubulointerstitial injury, and inflammatory cell infiltration). These benefits coincide with reduced AGE formation. Of great interest, they are concomitant with the prevention of abnormal iron deposition and of expressions of heme oxygenase-1 (HO-1) and p47phox (a subunit of NADPH oxidase) and with the correction of chronic hypoxia in the tubulointerstitium.

Materials and Methods

Reagents

The tested compounds included six ARB (valsartan, olmesartan, candesartan, irbesartan, losartan, telmisartan), six CCB (nicardipine, amlodipine, nifedipine, nisoldipine, nitrendipine, and felodipine), nine BB (atenolol, betaxolol, bisoprolol, carteolol, nadolol, pindolol, propranolol, metoprolol, and alprenolol), and three AGE inhibitors (aminoguanidine, pyridoxamine, and OPB-9195). They were purchased from Sigma (St. Louis, MO) or purified in our laboratory. They were dissolved in DMSO to obtain a stock solution of 50 mM and further diluted to required concentrations. We have confirmed that DMSO at the final concentration of 10% does not influence the following assays (11).

In Vitro Studies

Pentosidine Measurement by HPLC. Fresh heparinized plasma samples were obtained after informed consent from hemodialysis patients before the dialysis session. Pooled plasma ($n = 4$) was incubated with the tested reagents (final concentration of 0.8, 2.0, and 5.0 mM) for 1 wk under air at 37°C. The pentosidine content was analyzed on a reverse-phase HPLC as described previously (17). Synthetic pentosidine was used as a standard.

Inhibition of Hydroxyl Radical-Mediated Phenylalanine Modification. Phenylalanine (1 mM) and the tested compound (final concentration of 0.1, 0.5, and 2.5 mM) were dissolved in 200 mM of phosphate buffer (pH 7.4) and incubated at room temperature for 4 h with H₂O₂ (5 mM) in the presence of 0.1 mM CuSO₄ as a catalyst. After the metal-catalyzed oxidation reactions were quenched by addition of 1 mM diethylenetriamine pentaacetic acid (DTPA) and 260 units of catalase, *o*-tyrosine was measured by reverse-phase HPLC using a C18 reverse-phase column with fluorescence detector at an excitation of 275 nm, emission 305 nm (18). The very low water solubility of some antihypertensive agents prevented their evaluation in this assay.

Metal Chelating Activity. The chelating activity of the tested compounds for transition metal ions was measured by the method described previously (11).

In Vivo Studies

Animals. The previously used (19) male subline of spontaneously hypertensive/NIH-corpulent rat (SHR/NDmcr-cp) was investigated. Rats, aged 13 wk, were randomly divided into four groups and allocated to various regimens for an additional 20 wk as follows: 10 rats on vehicle; 10 rats on an ARB, olmesartan (5 mg/kg per d); 10 rats on a CCB, nifedipine (45 mg/kg per d); and 10 rats on a BB, atenolol (20 mg/kg per d). Ten control WKY rats were on vehicle. Carboxymethylcellulose that contained the antihypertensive agent was given orally to rats by gavage. Taking into account their plasma half-lives, olmesartan and atenolol were given once and nifedipine was three times a day. The protocol was in accordance with the Animal Experimentation Guidelines of Tokai University.

BP, Urine Collection, and Blood Sampling. Systolic BP was determined in conscious rats by the tail-cuff method at the beginning of

the study and every 2 wk thereafter until the rats were killed at 33 wk. At the end of the study, each rat was weighed and placed in a metabolic cage for a 24-h urine collection. Blood samples were obtained before death.

Biochemical Measurements in Blood and Urine. Total cholesterol, triglyceride, urea nitrogen (BUN), and albumin concentrations were determined in plasma, and protein concentrations were determined in urine with an automatic analyzer (Synchro CX7; Beckman Coulter Inc., Fullerton, CA). Plasma insulin was measured with commercially available kits (Morinaga Biochemistry Lab, Tokyo, Japan). Hemoglobin A_{1c} was measured using the immunoassay technique by DCA2000 (Bayer HealthCare, Tarrytown, NY).

Renal Pentosidine Content. Kidney tissue (100 mg) was minced, rinsed with 10% TCA, dried under vacuum, and acid hydrolyzed. Its pentosidine content then was quantified as described above.

Morphologic Analysis

Coronal sections of renal tissue (3 to 4 μ m thick) were stained with periodic acid-Schiff and examined by light microscopy in a blinded manner for morphologic analysis. Glomerular sclerosis was semiquantitatively evaluated according to a previous paper (19). The severity of glomerular sclerosis was graded according to the percentage of sclerotic area expressed as a percentage of total area (0, no lesions; 1+, 1 to 25%; 2+, 25 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 that displayed the same degree of injury and summing these scores.

Immunohistochemistry

Desmin, a marker of podocyte injury; α -smooth muscle actin, a marker of mesangial injury; vimentin, a marker of tubulointerstitial injury; and monocytes/macrophages infiltration were detected in tissue sections (4 μ m) of kidneys that were fixed with methyl Carnoy's. For HO-1, tissue samples were fixed with formalin. The sections first were incubated with the appropriate mouse mAb (desmin by D33, 4.6 μ g/ml [Dako, Carpinteria, CA]; vimentin by V9, 7.2 μ g/ml [Dako]; α -smooth muscle actin by asm-1, 0.5 μ g/ml [Neomarkers, Fremont, CA]; monocytes/macrophages by ED-1, 5 μ g/ml [Serotec, Oxford, UK]), or rabbit polyclonal antibody to HO-1 (1:200 dilution; StressGen, Victoria, BC, Canada). Subsequently, the sections were incubated with biotinylated horse anti-mouse IgG polyclonal antibody (Vector Laboratories Inc., Burlingame, CA; 1:400 dilution). Development was performed with peroxidase-conjugated avidin (Vector Laboratories Inc.) and 3,3'-diaminobenzidine tetrahydrochloride (Wako, Osaka, Japan).

For semiquantitative analysis, desmin staining was graded as follows: 0, no staining; 1+, 1 to 25% of the glomerular tufts positive; 2+, 25 to 50%; 3+, 50 to 75%; 4+, 75 to 100%. Fifty glomeruli were selected randomly in each animal, and an overall score per animal was obtained by multiplying each score (0 to 4+) with the percentage of glomeruli that displayed the same degree of injury and summing these scores. Vimentin-positive tubules were counted in 10 randomly selected cortical fields with a $\times 10$ objective. ED-1-positive cells that infiltrated the tubulointerstitium were counted in 20 randomly selected cortical fields with $\times 20$ objective.

Detection of Tissue Iron Deposition

Prussian blue staining was used to detect iron deposition (20). After deparaffinization, the sections (4 μ m) of formalin-fixed tissues were washed in deionized water and stained with 2% HCl/potassium ferrocyanide solution for 10 to 20 min. The sections were counterstained with nuclear fast red.

Detection of Hypoxia

Hypoxia was detected in renal tissues of SHR/NDmcr-cp rats that were treated with olmesartan. A hypoxic probe, pimonidazole (60 mg/kg; Chemicon, Temecula, CA), was injected intravenously 2 h before the rats were killed (21). Renal tissues then were obtained, fixed in formalin, and paraffin embedded. Accumulation of pimonidazole in hypoxic cells was detected by a specific mAb, Hypoxyprobe-1 Mab 1 (Hypoxyprobe-1 kit for the detection of tissue hypoxia; Chemicon) according to the method recommended by the manufacturer.

Statistical Analyses

All data are expressed as the mean $\pm$ SEM. The differences among the four SHR/NDmcr-cp groups were assessed by one-way ANOVA. Multiple comparisons were performed between the vehicle group and the other three groups of SHR/NDmcr-cp by Dunnett *t* test. The SHR/NDmcr-cp vehicle group was compared with the WKY group by Student *t* test. For histologic data, the differences among the four SHR/NDmcr-cp groups were assessed by Kruskal-Wallis test. Multiple comparisons were performed between the vehicle group and the other three groups of SHR/NDmcr-cp by Mann-Whitney *U* test. Differences between vehicle and WKY were assessed by Mann-Whitney *U* test. All calculations relied on SAS software (SAS Institute, Cary, NC). Values are considered significant at $P < 0.05$.

Results

In Vitro Studies

AGE Inhibition. Pentosidine production, used as a surrogate of the formation of AGE, during incubation of uremic plasma was inhibited in a dose-dependent manner by all six ARB but not by any of six CCB and nine BB tested (Table 1). The same effect was demonstrated during incubation in medium that contained only arabinose and BSA or nonuremic diabetic plasma as sources for AGE (data not shown). ARB inhibited pentosidine formation to a similar or much greater extent than three well-known AGE inhibitors (aminoguanidine, pyridoxamine, and OPB-9195) at the tested concentrations (0.8 to 5 mM). We previously demonstrated an ARB class effect related to a common core structure, 5-(4'-methylbiphenyl-2-yl)-1H-tetrazol (11).

Inhibition of Hydroxyl Radical-Mediated Phenylalanine Modification. Hydroxyl radical-induced formation of *o*-tyrosine then was evaluated in the presence of the drugs. As shown in Table 2, all tested ARB ($n = 3$) as well as, to a slightly lower extent, BB ($n = 3$) inhibited, in a dose dependant manner, *o*-tyrosine formation during hydroxyl radical-mediated phenylalanine modification. By contrast, all CCB ($n = 4$) failed to modify the *o*-tyrosine formation. Note that the very low water solubility of several other compounds prevented their evaluation in this assay.

Inhibition of the Fenton Reaction. The Fenton reaction catalyzed by transition metal ions (copper and iron) is critical in oxidative biochemistry to generate hydroxyl radicals (22). The chelating activity of the tested compounds for transition metal ions therefore was evaluated by determining the concentration required for a 50% inhibition (IC₅₀) of the rate of copper-catalyzed autoxidation of ascorbic acid in phosphate buffer. This concentration was below 5 μ M for all six ARB but above 50 and 2000 μ M for CCB and BB, respectively (Table 3). The effect

Table 1. Half-maximal inhibition (IC₅₀) value of pentosidine formation during incubation of uremic plasma

Compound	IC ₅₀ (mM)
Angiotensin II receptor blockers	
candesartan	2.00
irbesartan	3.57
losartan	4.39
olmesartan	1.78
telmisartan	6.25
valsartan	2.22
5-(4'-methylbiphenyl-2-yl)-1H-tetrazol (core structure)	3.80
Calcium channel blockers	
amlodipine	>20
felodipine	>20
nicardipine	>20
nifedipine	>20
nisoldipine	>20
nitrendipine	>20
β -blockers	
alprenolol	>20
atenolol	>20
betaxolol	>20
bisoprolol	>20
carteolol	>20
metoprolol	>20
nadolol	>20
pindolol	>20
propranolol	>20
AGE inhibitors	
aminoguanidine	7.18
pyridoxamine	4.31
OPB-9195	0.61

Pooled plasma from hemodialysis patients was incubated with the tested reagents (final concentration of 0.8, 2.0, and 5.0 mM) for 1 week under air at 37°C. The pentosidine content was analyzed on a reverse-phase HPLC.

on the autoxidation of ascorbic acid was concentration dependent.

In Vivo Studies

Biochemistry, BP, and Renoprotection. At the end of the study (at 33 wk), all of the SHR/NDmcr-cp, compared with WKY control rats, were obese and had developed hyperglycemia with hyperinsulinemia and hyperlipidemia and a rise in BUN ($P < 0.001$): Body weight (687 ± 18 g in SHR/NDmcr-cp at 33 wk *versus* 499 ± 5 g in WKY), HbA_{1c} ($4.2 \pm 0.8\%$ in SHR/NDmcr-cp *versus* $2.5 \pm 0.1\%$ in WKY), serum insulin (96.9 ± 21.6 ng/dl in SHR/NDmcr-cp *versus* 1.4 ± 0.2 ng/dl in WKY), serum triglyceride (382 ± 67 mg/dl in SHR/NDmcr-cp *versus* 47 ± 10 mg/dl in WKY), serum total cholesterol (189 ± 9 mg/dl in SHR/NDmcr-cp *versus* 139 ± 2 mg/dl in WKY), and BUN (24.7 ± 2.0 mg/dl in SHR/NDmcr-cp *versus* 15.7 ± 0.5

Table 2. Half-maximal inhibition (IC₅₀) value of *o*-tyrosine formation during hydroxyl radical-mediated phenylalanine modification

Compound	IC ₅₀ (mM)
Angiotensin II receptor blockers	
losartan	0.38
olmesartan	0.32
valsartan	0.31
Calcium channel blockers	
felodipine	>10
nifedipine	>10
nisoldipine	>10
nitrendipine	>10
β-blockers	
atenolol	1.45
metoprolol	0.41
propanolol	0.50

Phenylalanine and the tested compound (final concentration of 0.1, 0.5, 2.5 mM) dissolved in phosphate buffer (pH 7.4) were incubated at room temperature for 4 h with H₂O₂ in the presence of CuSO₄. After the metal-catalyzed oxidation reactions were quenched by addition of diethylenetriamine pentaacetic acid (DTPA) and catalase, *o*-tyrosine was measured on a reverse-phase HPLC.

mg/dl in WKY). Albumin rose slightly (4.7 ± 0.1 mg/dl in SHR/NDmcr-cp *versus* 4.3 ± 0.1 mg/dl in WKY). Three types of antihypertensive agents were used: The RAS-dependent olmesartan (ARB) and the RAS-independent nifedipine (CCB) and atenolol (BB). When compared with control SHR/NDmcr-cp that were given vehicle, all three drugs failed to modify body weight and HbA_{1c}, insulin, total cholesterol, triglycerides, BUN, and albumin levels, except for olmesartan, which decreased plasma total cholesterol (151.7 ± 3.4 mg/dl; *P* < 0.05), and for atenolol, which increased plasma triglycerides (646.6 ± 32.9 mg/dl; *P* < 0.05) and decreased body weight (620 ± 14 g; *P* < 0.05) and plasma albumin (3.8 ± 0.1 mg/dl; *P* < 0.001).

In comparison with control WKY rats, SHR/NDmcr-cp rats were hypertensive at the end of the study (*P* < 0.001). The three antihypertensive agents significantly decrease systolic BP to control WKY levels throughout the experiment (Table 4). No significant difference was observed in the level of BP achieved with each drug, although animals that were given olmesartan tended to have slightly lower levels.

At the end of the study, SHR/NDmcr-cp rats had a markedly higher proteinuria than WKY controls (Table 4). Olmesartan but neither nifedipine nor atenolol reduced urinary protein excretion (*P* < 0.001) to the level of WKY controls. Urinary protein excretion of WKY remained normal throughout the study.

AGE Inhibition. The renal pentosidine content of the kidneys, expressed as pmol/mg protein (Table 4), was significantly (*P* < 0.001) higher in the SHR/NDmcr-cp vehicle group than in the WKY group. Olmesartan but not nifedipine or

Table 3. Half-maximal inhibition (IC₅₀) value of copper-catalyzed oxidation of ascorbic acid by the tested compounds

Compound	IC ₅₀ (μM)
Angiotensin II receptor blockers	
candesartan	<5
irbesartan	<5
losartan	<5
olmesartan	<5
telmisartan	<5
valsartan	<5
5-(4'-methylbiphenyl-2-yl)-1H-tetrazol (core structure)	<5
Calcium channel blockers	
amlodipine	>50
azelnidipine	>50
clinidipine	>50
felodipine	>50
nicardipine	>50
nifedipine	>50
β-blockers	
alprenolol	>2000
atenolol	>2000
betaxolol	>2000
bisoprolol	>2000
carteolol	>2000
metoprolol	>2000
nadolol	>2000
pindolol	>2000
propranolol	>2000

CuCl₂ and tested compound solution were preincubated in phosphate buffer (pH 7.4) at 30°C for 5 min. The reaction was initiated by the addition of ascorbic acid (final concentrations of CuCl₂ and ascorbic acid were 500 nM and 500 μM, respectively), followed by the subsequent incubation for 60 min at 30°C. Ascorbic acid content was determined by reverse-phase HPLC; the concentration required for a 50% inhibition (IC₅₀) of the rate of copper-catalyzed autooxidation of ascorbic acid in phosphate buffer was evaluated.

atenolol returned renal pentosidine content toward control level (*P* < 0.001). The renal pentosidine content (pmol/mg of tissue proteins) observed in all SHR/NDmcr-cp rats, whether given vehicle or any of the three antihypertensive agents, was significantly correlated (*P* < 0.01) with proteinuria (mg/d; *n* = 40, *R*² = 0.3522, *y* = 0.0004*x* + 0.0530).

Renal Histology and Immunohistochemistry

On light microscopy, segmental sclerosis with attachment of the capillary tuft to the Bowman's capsule (Figure 1A) and tubulointerstitial injury (tubular dilation, atrophy of tubular epithelial cells, fibrosis, and infiltration of inflammatory cells) were observed in SHR/NDmcr-cp rats that were given vehicle. Glomerular (Table 5) and tubulointerstitial injury (data not shown) was markedly ameliorated (*P* < 0.05) but not fully

Table 4. Systolic BP, renal damage, and pentosidine content in the kidney of type 2 diabetic rats given the three antihypertensive agents^a

	SHR/NDmcr-cp				WKY
	Vehicle	Olmesartan (5 mg/kg per day)	Nifedipine (45 mg/kg per day)	Atenolol (20 mg/kg per day)	
Systolic BP (mmHg)	211±7 ^{b,f}	147±5 ^{b,c}	158±3 ^{b,c}	154±2 ^{b,c}	155±2
Urinary total protein (mg/d)	139.1±26.8 ^{b,f}	63.4±5.8 ^{b,e}	139.8±14.5 ^b	195.5±28.4 ^b	8.6±0.7
Pentosidine content in the kidney (pmol/mg)	0.126±0.015 ^{b,f}	0.050±0.009 ^{b,d}	0.122±0.015 ^b	0.129±0.017 ^b	0.039±0.001

^aData are expressed as mean±SE.

^b $P < 0.001$ by ANOVA (vehicle, olmesartan, nifedipine, atenolol).

^c $P < 0.001$, ^d $P < 0.01$, ^e $P < 0.05$ versus vehicle by Dunnett *t* test.

^f $P < 0.001$ versus WKY by Student *t* test.

corrected in SHR/NDmcr-cp rats that were given olmesartan. No effect was noted in rats that were given nifedipine or atenolol.

On immunohistochemistry, podocytes in SHR/NDmcr-cp rats that were given vehicle, nifedipine, or atenolol expressed desmin (Figure 1B), a marker of podocyte injury (23). This expression was prevented only in the animals that were given olmesartan. Vimentin (Figure 1C), a marker of tubular injury (24), was expressed in many tubules of SHR/NDmcr-cp rats that were given vehicle, nifedipine, or atenolol. By contrast, its expression was completely prevented by olmesartan. α -Smooth muscle actin (Figure 1D), a marker of mesangial injury and activation (25), was expressed in three to five glomeruli per section in SHR/NDmcr-cp rats that were given vehicle, nifedipine, or atenolol. These changes were improved by olmesartan: No α -smooth muscle actin-positive mesangial cells were detected in these animals. Macrophage infiltration, assessed with ED-1 (Figure 1E), was present in the tubulointerstitium of SHR/NDmcr-cp rats that were given vehicle, nifedipine, or atenolol but markedly diminished in rats that were given olmesartan. Semiquantitative analysis of desmin staining as well as the count of vimentin-positive tubules and ED-1 positive infiltrating cells confirmed these observations (Table 6).

Tissue Iron Deposition

Prussian blue staining revealed abnormal iron deposition in tubules and interstitial cells of SHR/NDmcr-cp rats that were given vehicle, whereas staining was absent in nondiabetic WKY rats (Figure 2). Olmesartan but neither nifedipine nor atenolol inhibited iron deposition.

Presence of Chronic Hypoxia

The degree of renal hypoxia was evaluated with pimonidazole, a compound incorporated into hypoxic cells, thus serving as a hypoxic marker (21). In WKY control rats, pimonidazole was incorporated in tubular cells, mainly in the medulla and medullary rays, two areas that are known to be mildly hypoxic under physiologic conditions. In the diabetic rats that were given vehicle, pimonidazole accumulated markedly in the cor-

tical tubules, an abnormality completely prevented by olmesartan treatment (Figure 3).

Oxidative Stress

HO-1, one of the antioxidative renoprotective genes that are regulated by hypoxia (26,27), was expressed in a small number of tubules in WKY rats. The number of HO-1-positive tubules increased markedly in SHR/NDmcr-cp rats (Figure 4), an abnormality abolished by olmesartan.

A semiquantitative PCR analysis of the mRNA expression of p47phox, a subunit of NADPHoxidase (28), was performed in kidney tissues. The p47phox expression in SHR/NDmcr-cp rats that were given vehicle increased approximately three times above that in control WKY rats but returned to control levels in rats that were given olmesartan (data not shown).

Discussion

This study provides new insights into the mechanisms of renoprotection linked with some antihypertensive agents and might identify new targets and agents for the treatment and prevention of diabetic nephropathy. The advantage of *in vitro* studies of antihypertensive agents is that their results cannot be mediated by cellular intermediates, the RAS, or BP. In this model, we previously demonstrated that all ARB that share a common core structure have the ability to inhibit advanced glycation. We now establish that this characteristic is absent in a series of six CCB and nine BB. ARB thus constitute a unique class of therapeutic agents.

ARB also inhibit oxidative metabolism as now demonstrated directly by their ability to block *o*-tyrosine formation by hydroxyl radicals. This effect is shared to a minor extent by BB but is absent in CCB. Previous *in vitro* data (11) already hinted that ARB reduce oxidative metabolism as olmesartan, an ARB, scavenges carbon-centered and hydroxyl-radicals and decreases, accordingly, the formation of dicarbonyls such as glyoxal or methylglyoxal. Taken together, these data establish conclusively a direct antioxidant effect of ARB.

All ARB also share the chelating activity for transition metals

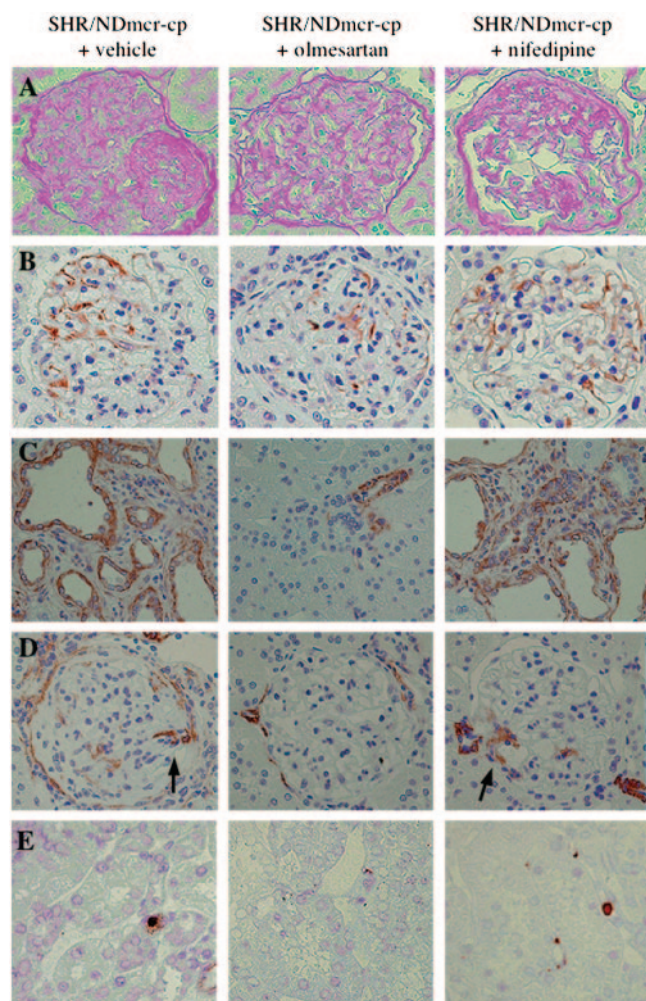


Figure 1. (A) Light microscopy analysis. Representative figures of glomerular damage of SHR/NDmcr-cp rats that were given antihypertensive agents. Segmental glomerulosclerosis is observed in glomeruli in SHR/NDmcr-cp rats that were given vehicle. Glomerular injury is ameliorated in SHR/NDmcr-cp rats that were given olmesartan but not nifedipine. (B through E) Immunohistochemical analysis of glomerular and tubulointerstitial injury in SHR/NDmcr-cp rats that were given antihypertensive agents. Representative figures of podocyte damage with anti-desmin antibody (B), of tubulointerstitial injury with anti-vimentin antibody (C), of mesangial injury with anti- α -smooth muscle actin antibody (D), and of infiltration of macrophage with ED-1 (E). Podocyte damage, mesangial injury, tubulointerstitial injury, and macrophage infiltration were improved by olmesartan but not by nifedipine. Normal podocytes expressed vimentin, and α -smooth muscle actin in afferent and efferent arterioles of glomeruli. Magnification, $\times 400$ in A, B, and D; $\times 200$ in C and E.

involved in the Fenton reaction generating hydroxyl radicals. Neither the CCB nor the BB have this effect.

The *in vivo* relevance of these three effects (inhibition of advanced glycation and oxidative metabolism and the chelation of transition metals) as well as their respective contributions to renoprotection was evaluated further in a hypertensive type 2 diabetic rat model with nephropathy, the SHR/

NDmcr-cp strain (29). In this model, rats develop obesity, hyperglycemia with hyperinsulinemia, and hyperlipidemia. Renal involvement is expressed by proteinuria and pathologic lesions of the glomeruli and as we now demonstrate the tubulointerstitium (19). Striking, olmesartan, an ARB, but neither nifedipine, a CCB, nor atenolol, a BB, reduces proteinuria and kidney lesions. The effect on BP, body weight, glucose levels, and lipid disorders is similar for ARB, CCB, and BB and thus does not mediate ARB-induced renoprotection. By contrast, the *in vitro* demonstrated characteristics of ARB have their *in vivo* counterpart in the diabetic kidneys.

Inhibition of advanced glycation translates into a marked reduction of the renal pentosidine content observed only in olmesartan-treated rats. The pathologic relevance of this phenomenon is illustrated by the tight correlation observed between proteinuria and renal pentosidine content when all results of the experimental groups are combined. This already published relationship (19) remains highly significant after inclusion of the data of the nifedipine-treated animals.

The *in vivo* impact of ARB on oxidative stress and transition metal chelation within the diabetic kidney remains to be ascertained. It is difficult to measure directly the degree of the Fenton reaction and the generation of hydroxyl radicals within renal tissues. The iron deposits observed within diabetic kidneys and their disappearance only after olmesartan treatment is of interest. Iron deposition has harmful consequences for the kidney (30–32). The extent of iron accumulation in proximal tubular cells, both in human chronic renal disease (33) and in the rat hemosiderosis model (34), correlates with proteinuria but not with GFR. Conversely, the iron chelator, desferrioxamine, prevents iron deposition in tubular cells of rats that are given angiotensin II and reduces proteinuria (35). Iron staining therefore may provide investigators with an easy tool to ascertain *in vivo* the renoprotective effects of various treatments. The exact relationship among *in vitro* metal chelation, the *in vivo* reduction of iron deposits, and the direct inhibition of oxidative metabolism is still to be investigated.

Iron deposition might reflect an increased activity of the RAS. Indeed, angiotensin II infusion provokes marked iron deposition in the rat kidney (35). However, an exclusive role of angiotensin is unlikely as hydralazine, a RAS-independent antihypertensive agent, is also able to reduce iron deposition in rats (35). Noteworthy, iron deposition in the tubulointerstitium parallels the infiltration of inflammatory cells. Apoptotic degradation of inflammatory cells might release intracellular iron into the interstitial space. The simultaneous correction of abnormal iron deposition, tubulointerstitial fibrosis, and inflammatory cell infiltration by ARB renders moot the identification of a primary phenomenon.

Indirect evidence for *in vivo* ARB protection against oxidative stress accrues also from our observations on the antioxidative renoprotective genes in the kidney. On immunohistochemistry, expression of HO-1, one of the renal antioxidative proteins, was upregulated in diabetic rat kidney but returned to normal levels by olmesartan. Similarly, the mRNA of p47phox, a subunit of NADPH oxidase, was upregulated in the rat diabetic kidney but returned to normal by ARB. HO-1 expression is

Table 5. Semiquantitative analysis of glomerular damage^a

Vehicle	SHR/NDmcr-cp			WKY
	Olmesartan (5 mg/kg per day)	Nifedipine (45 mg/kg per day)	Atenolol (20 mg/kg per day)	
0.58±0.07 ^{b,d}	0.28±0.06 ^{b,c}	0.68±0.07 ^b	0.68±0.13 ^b	0.07±0.03

^aThe severity of glomerular sclerosis was graded according to the percentage of sclerotic area expressed as a percentage of total area: 0, no lesions; 1+, 1 to 25%; 2+, 25 to 50%; 3+, 50 to 75%; 4+, 75 to 100%. Data are expressed as mean±SE.

^b*P* < 0.05 by Kruskal-Wallis test (vehicle, olmesartan, nifedipine, atenolol).

^c*P* < 0.05 versus vehicle by Mann-Whitney U test.

^d*P* < 0.01 versus WKY by Mann-Whitney U test.

Table 6. Semiquantitative analysis of immunohistochemical studies^a

	SHR/NDmcr-cp				WKY
	Vehicle	Olmesartan (5 mg/kg per day)	Nifedipine (45 mg/kg per day)	Atenolol (20 mg/kg per day)	
Desmin (scores 0 to 4)	1.6±0.1 ^{b,f}	0.8±0.1 ^{b,d}	1.5±0.1 ^b	1.6±0.2 ^b	0.03±0.02
Vimentin (positive tubules/field)	3.6±0.5 ^{c,f}	0.3±0.2 ^{c,e}	3.7±0.4 ^c	4.3±0.3 ^c	0.06±0.03
ED-1 (positive cells/field)	11.2±1.4 ^{c,f}	3.4±0.6 ^{c,e}	8.3±1.4 ^c	10.4±0.8 ^c	0.84±0.16

^aData are expressed as mean±SE. Desmin staining was graded as follows: 0, no staining; 1+, 1% to 25% of the glomerular tufts positive; 2+, 25% to 50%; 3+, 50% to 75%; 4+, 75% to 100%. Fifty glomeruli were selected randomly in each animal, and an overall score per animal was obtained by multiplying each score (0 to 4+) by the percentage of glomeruli displaying the same degree of injury and adding these scores. Vimentin-positive tubules were counted in 10 randomly selected cortical fields with a ×10 objective. ED-1-positive cells infiltrating the tubulointerstitium were counted in 20 randomly selected cortical fields with a ×20 objective.

^b*P* < 0.05, ^c*P* < 0.001 by Kruskal-Wallis test (vehicle, olmesartan, nifedipine, atenolol).

^d*P* < 0.01, ^e*P* < 0.001 versus vehicle by Mann-Whitney U test.

^f*P* < 0.001 versus WKY by Mann-Whitney U test.

known to be enhanced by the hypoxia-inducible factor (36) as well as by abnormal iron deposition (37) and angiotensin II infusion (38). It protects against iron-induced tissue injury by generating the antioxidants biliverdin and bilirubin (39) or by modulating the levels of intracellular iron (40). Altogether, these data might reflect an ARB-induced reduction of oxidative stress possibly through the prevention of chronic hypoxia, abnormal iron deposition, and/or RAS activation.

Our results not only identify the consequences for the diabetic kidney of the *in vitro* demonstrated effects of ARB but also delineate a number of other features associated with ARB-induced renoprotection. Olmesartan but neither nifedipine nor atenolol reverses several abnormalities that are present in rat diabetic nephropathy: It decreases glomerular sclerosis, lowers the stimulation of mesangial cells witnessed by α -smooth actin expression, preserves podocytes as illustrated by the prevention of desmin expression, protects against interstitial fibrosis as shown by the absence of vimentin, and inhibits the infiltration of macrophages stained by ED-1 antibody.

In this study, we also demonstrate the presence of hypoxia in diabetic kidneys and its reversal by ARB but not by CCB and BB. Chronic hypoxia plays a crucial role in the progression of renal disease (41–43). Its presence has been documented early in the evolution of diabetic kidneys (42). Its causes are multifactorial. They include constriction of efferent arterioles by

angiotensin II with an attendant decrease in peritubular capillary flow. Norman's group (44) and ours (45) have shown independently that RAS blockade improves tubulointerstitial hypoxia.

Olmesartan is also the only antihypertensive drug that is able to reduce the infiltration of the interstitium by inflammatory cells. This benefit might result from an amelioration of hypoxia, which is known to induce tubulointerstitial injury and an attendant infiltration by inflammatory cells. Alternatively, olmesartan might correct an imbalance between helper T cell subsets followed by an ameliorated tubulointerstitial inflammation, as observed after angiotensin II infusion in another hypertensive kidney injury model (46). Further discussion of the cytokine balance in our model of diabetic nephropathy is beyond our scope.

The phenomena associated with diabetic nephropathy seem to be heterogeneous. They are tentatively integrated in a hypothetical scheme depicted in Figure 5, suggesting an interaction among oxidative stress, AGE formation, chronic hypoxia, iron deposition, and inflammatory cell infiltration. Abnormal iron deposition accelerates the Fenton reaction and eventual hydroxyl radical generation (22), which in turn increases oxidative stress and AGE formation. The last further interacts with the receptor for AGE with an attendant release of reactive oxygen species and eventual chemotactic attraction of macrophages

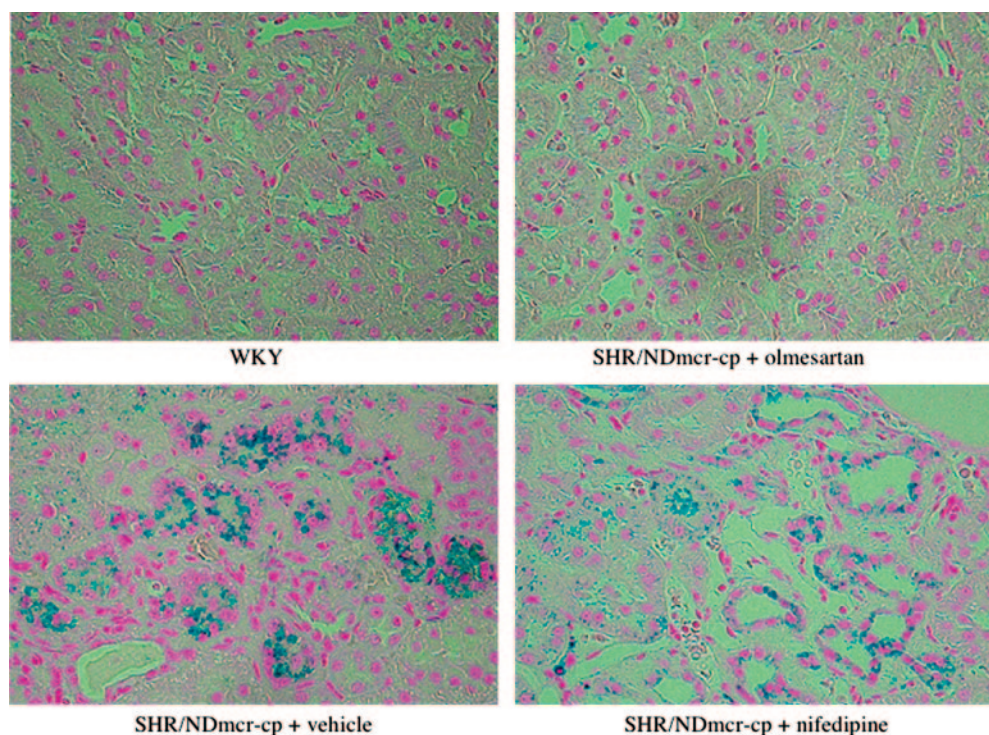


Figure 2. Prussian blue staining of iron in the kidney. Iron deposits were observed in the tubulointerstitium of SHR/NDmcr-cp rats that were given vehicle. Iron deposits were ameliorated in SHR/NDmcr-cp rats that were given olmesartan but not nifedipine. Magnification, $\times 200$.

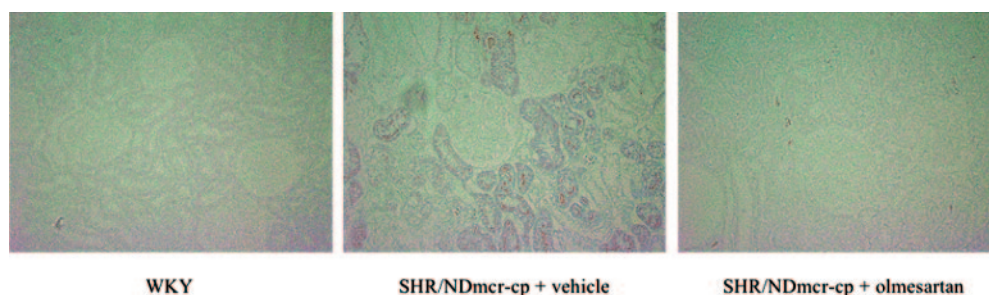


Figure 3. Immunohistochemical analysis of pimonidazole, a hypoxic marker in the kidney. Pimonidazole accumulation was intense and ubiquitous in the cortex of SHR/NDmcr-cp rats that were given vehicle but markedly attenuated by olmesartan treatment. Magnification, $\times 100$.

(47,48). Chronic hypoxia in the tubulointerstitial tissue transforms tubular cells into myofibroblasts and accelerates tissue fibrosis (49), which is further exacerbated by concomitant inflammatory cells infiltration, oxidative matrix protein damage, and AGE modification. Whatever its truth, this hypothesis provides a useful frame for further investigations.

The interrelationship between these effects may preclude the identification of a single culprit in the alteration of the diabetic kidney. Furthermore, prevention of tubulointerstitial fibrosis, of podocyte injury, of mesangial activation, and of macrophage infiltration implicates, in theory, many more intermediates whose actual contributions to kidney alterations remain to be singled out.

Whatever the sequential mechanisms of diabetic renal injury,

our observations confirm that, among several antihypertensive agents, ARB have unique properties that rely certainly on signs of decreased oxidative stress (hydroxyl radicals scavenging and inhibition of the Fenton reaction); correction of chronic hypoxia; and inhibition of AGE formation, of abnormal iron deposition, and of inflammatory cell infiltration. They confirm clinical evidence that BB have no such effect (5) and support the studies that deny an additional renoprotective role to CCB beyond BP lowering. Finally, our results allow some speculations on the genesis of diabetic nephropathy and identify targets of interest for its prevention. Exploration of the interrelationships among RAS activation, oxidative stress, chronic hypoxia, iron deposition, inflammatory cell infiltration, and diabetic renal injury warrants further studies.

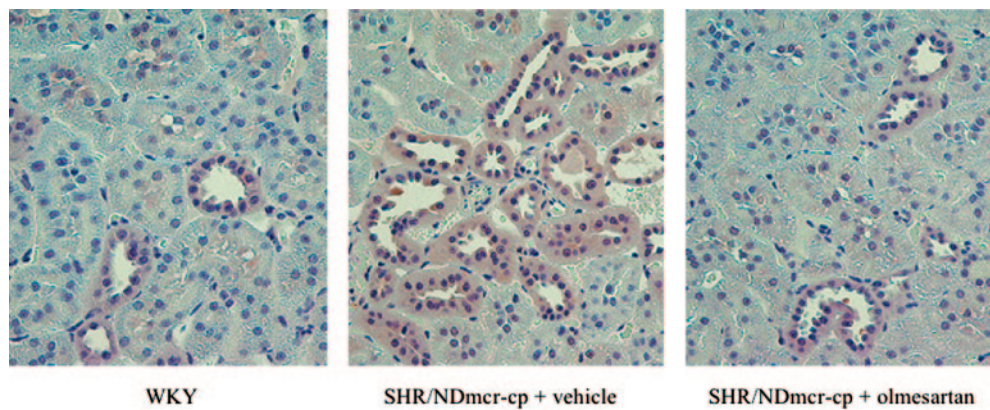


Figure 4. Expression of heme oxygenase-1 (HO-1). HO-1, one of the antioxidative renoprotective genes regulated by hypoxia, was upregulated in diabetic kidneys and returned to normal by olmesartan treatment. Magnification, $\times 200$.

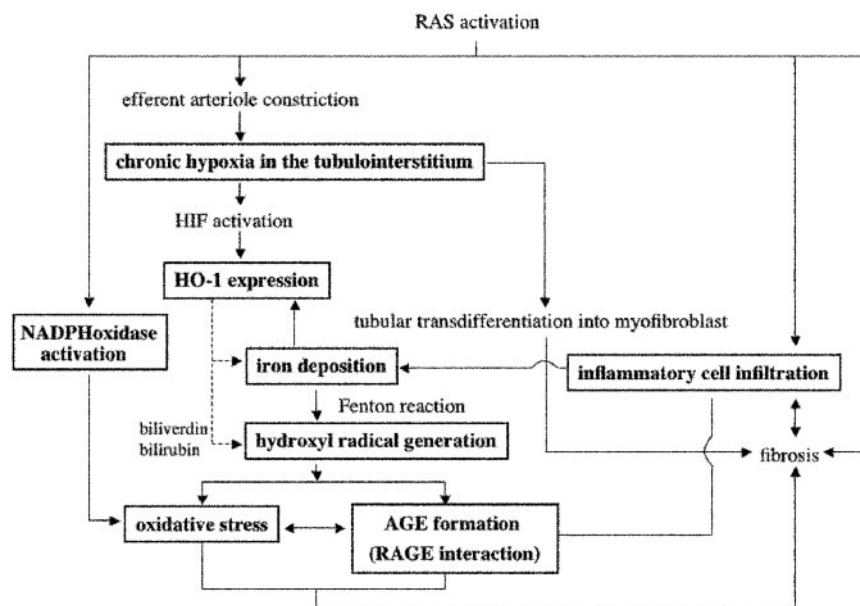


Figure 5. Hypothetical scheme for diabetic renal injury. Among three antihypertensive agents used in this study, angiotensin II type 1 receptor blockers (ARB) only possess all properties that are beneficial for renoprotection (boxed): Inhibition of the renin-angiotensin system (RAS), prevention of abnormal iron deposition in the interstitium, correction of chronic hypoxia, hydroxyl radical scavenging, reduction of expressions of HO-1 and NADPH oxidase, amelioration of inflammatory cell infiltration, and inhibition of pentosidine formation. These interrelated benefits of ARB may contribute to renoprotection: Reduction of proteinuria and improvement of glomerular and tubulointerstitial damage, irrespective of BP lowering. Broken line, inhibitory effect.

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