Angiotensin II Receptor Antagonists and Angiotensin-Converting Enzyme Inhibitors Lower *In Vitro* the Formation of Advanced Glycation End Products: Biochemical Mechanisms

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Abstract. The implication of advanced glycation end products (AGE) in the pathogenesis of atherosclerosis and of diabetic and uremic complications has stimulated a search for AGE inhibitors. This study evaluates the AGE inhibitory potential of several well-tolerated hypotensive drugs. Olmesartan, an angiotensin II type 1 receptor (AIIR) antagonist, as well as temocaprilat, an angiotensin-converting enzyme (ACE) inhibitor, unlike nifedipine, a calcium blocker, inhibit *in vitro* the formation of two AGE, pentosidine and N^{ϵ}-carboxymethyllysine (CML), during incubation of nonuremic diabetic, nondiabetic uremic, or diabetic uremic plasma or of BSA fortified with arabinose. This effect is shared by all tested AIIR antagonists and ACE inhibitors. On an equimolar basis, they are more efficient than aminoguanidine or pyridoxamine. Unlike the latter two compounds, they do not trap reactive carbonyl precursors for AGE, but impact on the production of reactive carbonyl precursors for AGE by chelating transition metals and inhibiting various oxidative steps, including carbon-centered and hydroxyl radicals, at both the pre- and post-Amadori steps. Their effect is paralleled by a lowered production of reactive carbonyl precursors. Finally, they do not bind pyridoxal, unlike aminoguanidine. Altogether, this study demonstrates for the first time that widely used hypotensive agents, AIIR antagonists and ACE inhibitors, significantly attenuate AGE production. This study provides a new framework for the assessment of families of AGE-lowering compounds according to their mechanisms of action.

Advanced glycation and oxidation irreversibly modify proteins over the years and thus contribute to aging phenomena (1). Their local or generalized acceleration is associated with atherosclerosis (2–6) as well as with various diabetic (7–10) and uremic complications (11–13). Inhibition of advanced glycation end products (AGE) formation has thus become a therapeutic goal.

Aminoguanidine, the first AGE inhibitor discovered in 1986 (14), and ($\pm$)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide (OPB-9195) (15) are both hydrazine-derivatives. They inhibit *in vitro* the formation of AGE, pentosidine (16), and N^{ϵ}-carboxymethyllysine (CML) (17) from a variety of individual precursors, such as ribose, glucose, and ascorbate, as

well as that of advanced lipoxidation end products (ALE), malondialdehyde-lysine and 4-hydroxynonenal-protein adduct (18), from arachidonate (19). They also inhibit pentosidine generation in diabetic and uremic plasma incubated for 4 wk (20).

As expected, both compounds correct several biologic effects that are associated with AGE formation. In murine thymocyte and fibroblasts, they inhibit the phosphorylation of tyrosine residues of a number of intracellular proteins induced by cell surface Schiff base formation (21). Given to diabetic animal models, such as Otsuka-Long-Evans-Tokushima-Fatty (OLETF) or streptozotocin-treated rats, they reduce urinary albumin excretion and improve glomerular morphology (15,22). Oral administration of OPB-9195 to rats after balloon injury of their carotid arteries effectively reduces neointima proliferation in arterial walls (23). Unfortunately, clinical benefits of both compounds given to diabetic patients have been hampered by side effects related to their characteristic trapping of pyridoxal with an attendant vitamin B6 deficiency (24).

We therefore searched for other drugs with tolerance that had been demonstrated in clinical conditions and that might exhibit AGE-inhibiting characteristics. Highly sensitive and specific chemical methodologies for AGE determination were

utilized rather than more disputable physicochemical (fluorescence and cross-linkage on electrophoresis) and immunologic assays. We discovered that angiotensin II type 1 receptor (AIIR) antagonists as well as angiotensin-converting enzyme (ACE) inhibitors, unlike a calcium blocker, lowered pentosidine as well as CML formation. We further elucidated the mechanism of their action and propose a comprehensive approach of the various mechanisms implicated in the AGE-lowering effects of presently available drugs.

Materials and Methods

In Vitro Incubation Experiments

Fresh heparinized plasma samples were obtained with informed consent from healthy subjects, non–insulin-dependent nonuremic diabetic patients with normal renal function and no proteinuria, and nondiabetic as well as diabetic hemodialysis patients before the dialysis session. To achieve adequate volumes of plasma and to obtain a stable baseline in experiments generating multiple results, the in vitro experiments were performed with plasma pooled from several donors ($n = 3$ to 5).

Two types of incubation media containing AGE precursors were used. First, essentially fatty acid-free grade bovine serum albumin (BSA; Sigma, St. Louis, MO) (30 mg) was incubated in the dark with 10 mM arabinose in 1.0 ml of 0.1 M sodium phosphate buffer (pH 7.4). Second, pooled plasma (900 μl) was incubated under air at 37°C.

The tested compounds included 6 AIIR antagonists (Olmesartan [25], candesartan, irbesartan, losartan, telmisartan, valsartan) and four ACE inhibitors (temocaprilat, enalaprilat, captopril, perindprilat) provided by Pharmacology and Molecular Biology Research Laboratories, Sankyo Pharmaceutical (Tokyo, Japan), nifedipine (Sigma), aminoguanidine (Tokyo Chemical Industry, Tokyo, Japan), pyridoxamine (Sigma), and OPB-9195 (Fujii Memorial Research Institute, Otsuka Pharmaceutical, Ohtsu, Japan). They were dissolved in 100 μl of DMSO to obtain a stock solution of 50 mM and further diluted to required concentrations. DMSO at the final concentration of 10% does not influence the oxidation process.

One milliliter of the medium was incubated under air at 37°C for 7 d in the presence of the tested compounds (final concentrations: 0.8, 2.0, and 5.0 mM). In an additional set of experiments, lower drug concentrations were used (0.01, 0.05, 0.26, 1.28, 6.4, 32, 160, and 800 μM). At the end of the incubation, pentosidine and CML content were measured as described below.

Pentosidine Measurement by HPLC

For quantitation of pentosidine, each sample of the incubation mixtures (50 μl) was mixed with equal volume of 10% TCA and centrifuged at 5000 $\times$ g for 5 min. The supernatant was discarded, and the pellet was washed with 300 μl of 5% TCA. The pellet was then dried under vacuum, lyophilized, and hydrolyzed by 100 μl of 6 N HCl for 16 h at 110°C under nitrogen. The acid hydrolysates were subsequently neutralized with 100 μl of 5 N NaOH and 200 μl of 0.5 M phosphate buffer (pH 7.4), filtered through a 0.5-μm-pore filter, and diluted with phosphate-buffered saline (PBS).

The pentosidine content was analyzed on a reverse-phase HPLC as described previously (26). In brief, a 50-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. The detection limit was 0.1 picomole of pentosidine per milligram of proteins.

CML Measurement by GC/MS

For quantitation of CML, samples (100 μl) were diluted with 100 μl of 0.2 M sodium borate (pH 9.1) before the addition of 20 μl of 1 M NaBH4 in 0.1 N NaOH. Reduction was performed for 4 h at room temperature. Protein was then precipitated by addition of an equal volume of 20% TCA and pelleted by centrifugation at 2000 $\times$ g for 5 min. The supernatant was discarded, and the pellet was washed with 500 μl of 10% TCA. After the addition of heavy-labeled internal standards (d4-CML), the sample was hydrolyzed in 0.3 ml of 6 N HCl at 110°C for 16 h. The hydrolysate was dried under a stream of nitrogen. CML content was measured as its N,O-trifluoroacetyl methyl esters by selected-ion monitoring gas chromatography/mass spectrometry (GC/MS) (27). The CML and d4-CML standards were gifts from Dr. John W. Baynes (University of South Carolina, Columbia, SC). Limit of detection was 1.0 picomole of CML per milligram of protein.

Inhibition of Dicarbonyl Formation by Olmesartan

Arabinose (10 mM) was incubated with various concentrations of olmesartan in 0.1 M phosphate buffer (pH 7.4) at 37°C for 1 wk. At the end of the incubation period, two major arabinose degradation products, glyoxal and methylglyoxal, for which pure standards are available, were identified and quantitated by HPLC (28). The standards were used for calibration purposes. One hundred microliters of sample was mixed with 100 μl of 20 μM 2,3-butanedione (internal standard), 80 μl of 2 M perchloric acid, and 40 μl of 1% o-phenylenediamine. After the mixture was incubated at 25°C for 3 h, quinoxaline derivatives were analyzed by reverse-phase HPLC, using a Waters Purecil C18 column (5 μm; 4.6 $\times$ 250 mm) and absorbance detection at 315 nm. The buffer gradient used for elution was 15% to 30% buffer B (85% to 70% buffer A) in 25 min: buffer A, 0.10% trifluoroacetic acid; buffer B, 80% acetonitrile containing 0.08% trifluoroacetic acid.

Dicarbonyl Entrapment

The tested compounds (0.8, 2, 5 mM) were incubated at 37°C in the presence of glyoxal (50 μM) in 0.1 M phosphate buffer (pH 7.4) for 20 h. At the end of the incubation period, the glyoxal content was determined after derivatization with o-phenylenediamine by the HPLC methods described above.

Free Radical Scavenging Activity

The scavenging activities of olmesartan, temocaprilat, aminoguanidine, and pyridoxamine for sugar-derived free radical and hydroxyl radical were evaluated according to a previously described method with some modifications (29). Sugar-derived free radical was generated by the autoxidation of ribose. In some experiments, the phosphate buffer (0.2 M, pH 7.4) was treated with Chelex-100 (Sigma) resin (wet weight, 7 g/L) to remove contaminating trace amount of metal ions (30). Ten millimolar ribose (Wako Pure Chemical, Osaka, Japan), 1 mM of N-t-butyl-α-phenylnitrone (PBN; Wako Pure Chemical), and various drug concentrations in phosphate buffer were incubated for 75 min at 100°C in tightly sealed tubes (total, 500 μl). The resulting solution was cooled to room temperature and sucked into a flat quartz cell. The sugar-derived radical adduct of PBN was measured on an electron-spin resonance (ESR) spectrometer (JES-FE2XG; JEOL, Tokyo, Japan) under the following conditions: microwave frequency, 9.42 GHz; magnetic field, 334.5 $\pm$ 5 mT; time

constant, 0.3 s; microwave power, 8 mW; field modulation width, 0.2 mT; amplitude, 1.6×1000; sweep time, 2 min/10 mT. The amount of nitroxide radical was determined as the relative signal height to the third signal of external instrumental standard (Mn^{2+} in MgO).

Hydroxyl radical was generated according to the Fenton reaction. Hydroxyl radical was trapped by 5,5'-dimethyl-1-pyrroline-*N*-oxide (DMPO) to produce DMPO-OH spin adduct, which should decrease if any hydroxyl radical scavenger was present in the solution. Various concentrations of the tested drugs, 1 mM DMPO, 100 μM diethylenetriaminepentaacetic acid (DTPA), and 1 mM H_2O_2, were mixed in this order in 0.2 M phosphate buffer. The Fenton reaction was initiated by the addition of ferrous ammonium sulfate solution in distilled water (finally, 50 μM) at 25°C. The resulting DMPO-OH was measured with the ESR spectrometer. Every ESR measurement was started 45 s after the initiation of reaction.

Inhibitory Effect on Leukocyte-Derived Superoxide Production

The inhibitory effect of olmesartan on the superoxide production by polymorphonuclear leukocytes (PMN) was examined. PMN were isolated from heparinized venous blood of healthy volunteers by gradient centrifugation ($400 \times g$ for 30 min) using Mono-Poly resolving medium (Dainippon Pharmaceutical, Osaka, Japan) at room temperature. The final cell suspension contained more than 95% PMN and was >95% viable as examined by trypan blue exclusion test. The mixture of PML (1×10^5 cells/ml) with 2-methyl-6-phenyl-3,7-dihydroimidazo [1,2-a] pyrazine-3-one (1 μM) as a chemiluminescent probe for superoxide in Hanks' balanced salt solution buffered with 40 mM HEPES (pH 7.4) was incubated at 37°C in the presence or absence of olmesartan (5 mM). Chemiluminescence of the solution was monitored by a C1230 photon counter with an R550 photomultiplier (Hamamatsu Photonics, Shizuoka, Japan). Superoxide production of PMN was stimulated with phorbol-12-myristate-13 acetate (1 μM). After the photon count peaked, recombinant human superoxide dismutase (300 U/ml; Nippon Kayaku, Tokyo, Japan) was added to extract net superoxide-based chemiluminescence.

Metal Chelating Activity

The chelating activity of the tested compounds for transition metal ions was measured by the method of Price *et al.* (30) with some modifications. Briefly, 15 μl of 50 μM $CuCl_2$ and 30 μl of tested compound solution were preincubated in 1.38 ml of phosphate buffer at 30°C for 5 min. The reaction was initiated by the addition of 75 μl of 10 mM ascorbic acid (final concentrations of $CuCl_2$ and ascorbic acid were 500 nM and 500 μM, respectively). Incubation lasted from 0 to 60 min at 30°C to allow kinetic reaction studies. Ascorbic acid content was determined by reverse phase HPLC, using a C18 reverse-phase column (5 μm, 4.6×250 mm) with absorbance detection at 244 nm. The buffer gradient used for elution was 0 to 50% buffer B for 5 min; buffer A, 0.10% trifluoroacetic acid; buffer B, 80% acetonitrile containing 0.08% trifluoroacetic acid.

Inhibition of Post-Amadori AGE Formation

Amadori-rich proteins were prepared according to the method of Booth *et al.* (31) with some modifications. Glycation was carried out by first incubating BSA (10 mg/ml) with arabinose or ribose (0.5 M) at 37°C in 0.1 M phosphate buffer (pH 7.4) for 24 h. Glycation was then interrupted to remove excess and reversibly bound sugar by extensive dialysis against frequent cold buffer changes at 4°C. The glycated BSA was then incubated with the tested compounds at 37°C

in 0.1 M phosphate buffer (pH 7.4) for 1 wk. The content of pentosidine was determined by HPLC.

Entrapment of Pyridoxal 5'-Phosphate

The tested compounds (0.5 mM) were incubated with pyridoxal 5'-phosphate (50 μM) in PBS at 37°C under air. To perform kinetic studies, incubations lasted between 0 and 20 h. Aliquots were removed at various times, and pyridoxal 5'-phosphate content was assayed by HPLC (24). In brief, a 10-μl solution of reaction mixture was injected into an HPLC system and separated on a Waters Purecil C18 column (5 μm, 4.6×250 mm). The effluent was monitored with a fluorescence detector (RF-10A; Shimadzu, Kyoto, Japan) at excitation-emission wavelength of 300/400 nm. The buffer gradient used for elution was 0 to 3% buffer B (100 to 97% buffer A) for 25 min at a
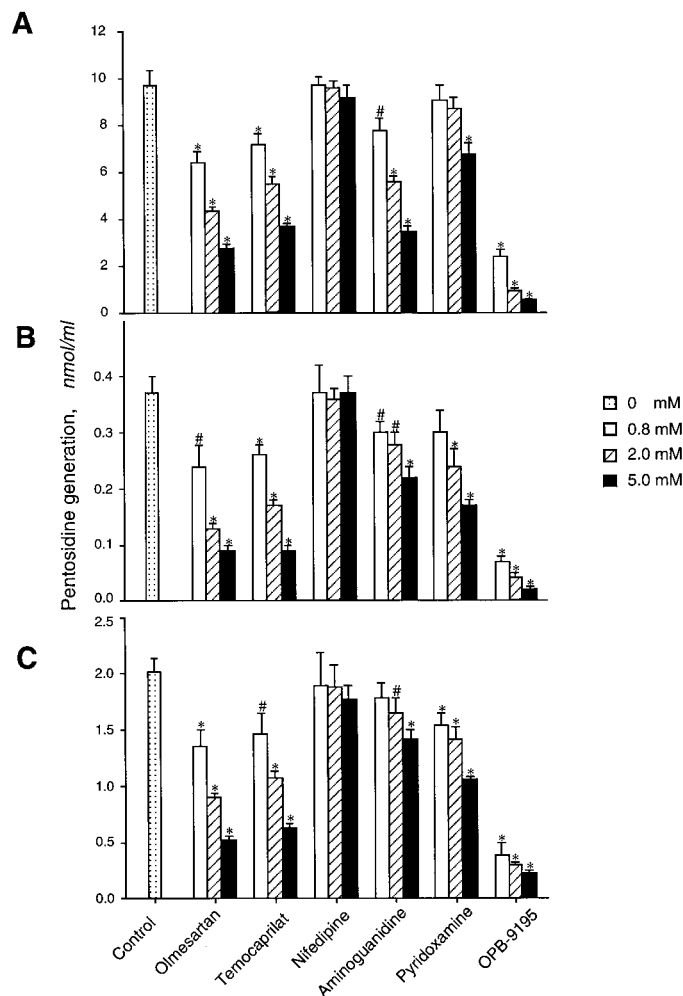

Figure 1. Inhibition of pentosidine formation. (A) Inhibition of arabinose-derived pentosidine formation. Bovine serum albumin (30 mg/ml) was incubated at 37°C with 10 mM arabinose in 0.1 M phosphate buffer (pH 7.4) in the presence of several concentrations of the tested compounds. (B) Inhibition of pentosidine formation on incubation of diabetic plasma. (C) Inhibition of pentosidine formation on incubation of uremic plasma. Diabetic plasma or predialysis uremic plasma was incubated at 37°C for 1 wk in the presence of several concentrations of the tested compounds. The yields of pentosidine in the incubation mixtures were determined by HPLC. Data are expressed as mean $\pm$ SD. $^\#P < 0.05$ and $^*P < 0.01$ compared with control.

Table 1. Half-maximal inhibition (IC50) value of pentosidine or N^ϵ-carboxymethyllysine (CML) formation during incubation of diabetic, uremic, or diabetic uremic plasma[a]

| Compound | IC50 (mM) | | | | |
| | Pentosidine | | | CML | |
	Diabetic Plasma	Uremic Plasma	Diabetic Uremic Plasma	Diabetic Plasma	Uremic Plasma
Olmesartan	1.39	1.71	1.99	3.70	4.54
Temocaprilat	1.80	2.40	2.12	4.65	6.63
Nifedipine	>20	>20	>20	>20	>20
Aminoguanidine	6.75	8.27	6.70	2.65	5.43
Pyridoxamine	4.41	5.42	3.31	5.31	11.70
OPB-9195	0.49	0.50	0.50	0.60	1.48

[a] Diabetic plasma, predialysis uremic plasma, or predialysis diabetic uremic plasma was incubated at 37°C for 1 wk in the presence of several concentrations of the tested compounds. The yields of pentosidine and CML in the incubation mixtures were determined by HPLC and gas chromatography/mass spectrometry (GC/MS), respectively.

flow rate of 0.6 ml/min: buffer A, 0.10% trifluoroacetic acid; buffer B, 80% acetonitrile containing 0.08% trifluoroacetic acid.

Statistical Analyses

Statistical differences were tested using the *t* test. $P < 0.05$ was considered statistically significant. Data are expressed as the mean ± SD.

Results

AGE Inhibition

In vitro production of two AGE, pentosidine and CML, is inhibited in a dose-dependent manner by olmesartan, an AIIR antagonist (Figure 1), and by temocaprilat, an ACE inhibitor, but not by nifedipine, a calcium blocker. This phenomenon is demonstrated in incubation media containing arabinose, non-uremic diabetic plasma, nondiabetic uremic plasma, or diabetic uremic plasma as the sources for AGE (Table 1). It is similar for the three tested AGE sources but is more striking for olmesartan and temocaprilat than for aminoguanidine or pyridoxamine and lower than for OPB-9195 at the tested concentrations. The dose-effect relationship was further delineated for low drug concentrations ranging from 0.01 to 800 μM. As shown in Figure 2, the dose response is curvilinear.

The effect on AGE formation is common to all six tested AIIR antagonists, *e.g.*, olmesartan, candesartan, irbesartan, losartan, telmisartan, valsartan, including their common core structure, 5-(4'-methylbiphenyl–2–yl)–1H–tetrazol (Table 2). A similar but milder effect on AGE formation is observed with all four ACE inhibitors tested at similar concentrations, *e.g.*, temocaprilat, enalaprilat, captopril and perindprilat (Table 2). No core structure common to the tested ACE inhibitors was found.

Mechanisms of AGE Inhibition

RCO Trapping. Reactive carbonyl and dicarbonyl compounds (RCO) are critical precursors of AGE. Their trapping might account for the inhibition of AGE formation by several compounds. As shown in Table 3, the inhibitory effect of

olmesartan and temocaprilat is not mediated by the trapping of RCO precursors for AGE. In contrast, aminoguanidine, pyridoxamine, and OPB-9195, efficiently trap RCO such as glyoxal and methylglyoxal.

Effect on Oxidative Metabolism and Dicarbonyl Production. The production of RCO precursors for AGE depends on oxidative metabolism. We therefore evaluated the impact of
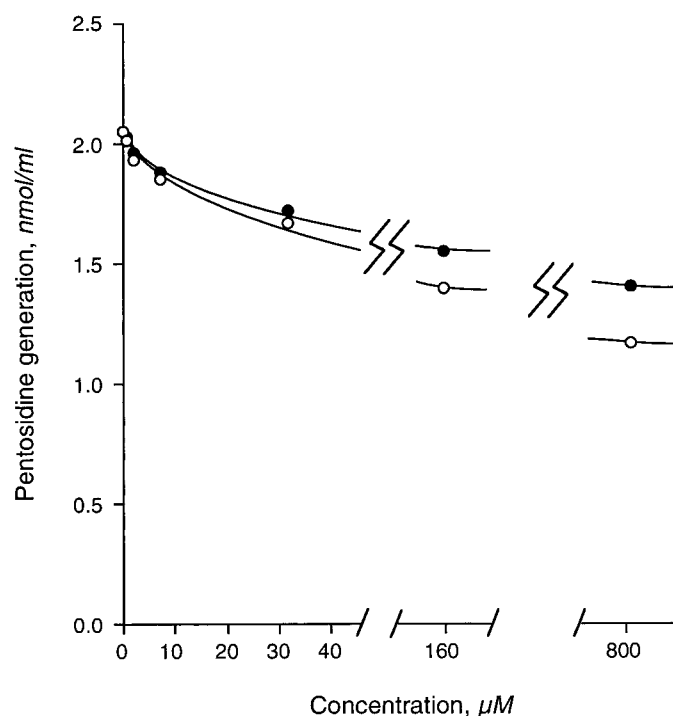

Figure 2. Inhibition of pentosidine formation at lower drug concentrations. Predialysis uremic plasma was incubated at 37°C for 1 wk in the presence of several concentrations of olmesartan (○) or temocaprilat (●). The yields of pentosidine in the incubation mixtures were determined by HPLC. Olmesartan: $y = -0.215 \log \chi + 1.962$; $r^2 = 0.906$. Temocaprilat: $y = -0.140 \log \chi + 1.903$; $r^2 = 0.957$.

Table 2. Half-maximal inhibition (IC50) value of pentosidine formation during incubation of bovine serum albumin (BSA) and arabinose[a]

Compound	IC50 (mM)	Compound	IC50 (mM)
1H-Tetrazole	10.37	Temocaprilat	2.43
5-Phenyl-1H-tetrazole	9.38	Enalaprilat	4.47
5-(4'-Methylbiphenyl-2-yl)-1H-tetrazol	5.64	Captopril	9.15
Olmesartan	1.72	Perindprilat	14.80
Candesartan	1.65	Nifedipine	>20
Irbesartan	6.03	Aminoguanidine	2.37
Losartan	6.29	Pyridoxamine	8.43
Telmisartan	6.25	OPB-9195	0.52
Valsartan	1.58		

[a] BSA (30 mg/ml) was incubated at 37°C with 10 mM arabinose in 0.1 M phosphate buffer (pH 7.4) for 1 wk in the presence of several concentrations of the tested compounds. The yields of pentosidine in the incubation mixtures were determined by HPLC.

Table 3. Half-maximal entrapment value for glyoxal by the tested compounds[a]

Compound	Half-Maximal Entrapment Value (mM)
Olmesartan	>100
Temocaprilat	>100
Aminoguanidine	8.73
Pyridoxamine	20.02
OPB-9195	5.76

[a] Various concentrations of the test compounds were incubated with 50 μM glyoxal in 0.1 M phosphate buffer (pH 7.4) for 20 h at 37°C. The content of residual glyoxal was determined by HPLC at the end of incubation.

the tested compounds on the formation of carbon-centered radicals, hydroxyl radicals as well as of superoxide radicals. Temocaprilat and especially olmesartan decreased in a dose-dependent manner the level of carbon-centered radicals, unlike aminoguanidine (Figure 3A). This decrease was similar whether or not the phosphate buffer had been pretreated with Chelex-100 resin, a chelator of trace metals, an observation suggesting that contaminating trace amounts of metal ions contributed little to this phenomenon. Both drugs also reduced the level of hydroxyl radicals, a characteristic shared to a minor extent by aminoguanidine and pyridoxamine (Figure 3B). By contrast, olmesartan did not modify the peak photon count, an expression of polymorphonuclear leukocyte-derived superoxide production (data not shown).

Interestingly, the inhibition of the oxidative metabolism by olmesartan is matched by a decreased production of dicarbonyls, glyoxal (Figure 4A), and methylglyoxal (Figure 4B), confirming the above findings.

Transition Metal Chelation. The formation of two AGE, CML and pentosidine, is closely related to the presence of transition metal ions (30,32). We therefore assessed the chelating activities of the tested compounds for transition metal ions by determining the concentration required for 50% inhibition of the rate of copper-catalyzed autoxidation of ascorbic acid in phosphate buffer. Among the tested compounds, 5-(4'-methylbiphenyl–2–yl)–1H–tetrazol (a core structure of AIIR antagonist) and olmesartan as well as OPB-9195 chelated copper and inhibited the autoxidation of ascorbic acid in a concentration-dependent manner to a much greater extent than pyridoxamine and aminoguanidine (Table 4). Temocaprilat and nifedipine have an intermediary effect.

Post-Amadori AGE Formation. Several oxidative steps lead to the formation of AGE. Some have been linked with the pre- and others with post-Amadori events. To ascertain at which step our drugs act, we prepared *in vitro* Schiff base-modified BSA. During its subsequent incubation, we monitored the formation of pentosidine, which thus relied on post-Amadori events. Indeed, arabinose- and ribose-derived Schiff base-modified BSA yield pentosidine upon a 7-d incubation. Olmesartan and temocaprilat as well as aminoguanidine, pyridoxamine and OPB-9195 inhibited pentosidine formation (Table 5). They have thus an impact on post-Amadori events.

Pyridoxal Binding. Finally, the ability of the tested compounds to bind pyridoxal has also been evaluated. Olmesartan, temocaprilat, and pyridoxamine do not react with pyridoxal, in contrast with both aminoguanidine and OPB-9195 (Figure 5).

Discussion

These results demonstrate an unexpected characteristic of two widely used hypotensive agents, olmesartan (an AIIR antagonist) and temocaprilat (an ACE inhibitor). Both significantly attenuate *in vitro* the production of AGE, such as pentosidine and CML. Nifedipine (a calcium-blocking hypotensive drug) has no such effect. A similar AGE-lowering effect has been identified in all tested AIIR antagonists, all of which are biphenyl tetrazole derivatives. Their common core structure, 5-(4'-methylbiphenyl-2-yl)-1H-tetrazol, is equally effective and is thus probably responsible for the inhibitory effect. By contrast, all four tested ACE inhibitors have an AGE-lowering potential despite the lack of a common core structure.
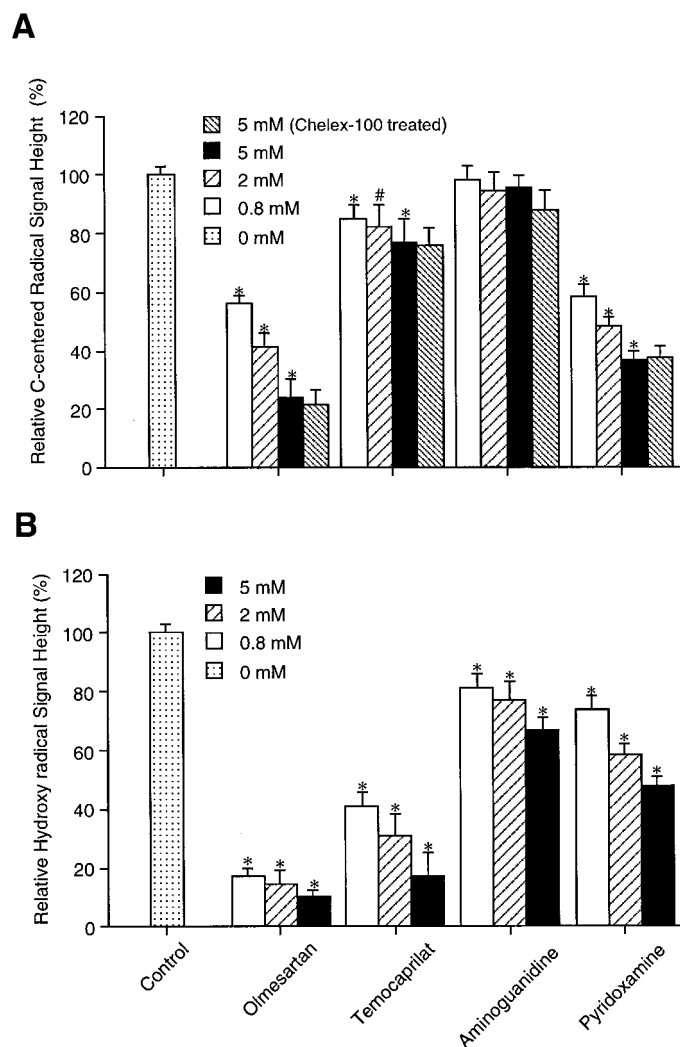
A

B

Figure 3. Free radical-scavenging activity. (A) The scavenging effect on ribose-derived carbon-centered radical. Vertical axis shows relative electron-spin resonance (ESR) signal height of *N-t*-butyl-α-phenylnitrone (PBN) spin adduct of ribose-derived carbon-centered radical at various drug concentrations and in the presence of chelex, a trace metal chelator. (B) The scavenging effect on hydroxyl radical. Vertical axis shows relative ESR signal height of 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 are expressed as means ± SEM. #$P < 0.05$ and *$P < 0.01$ compared with control.

The mechanism of the AGE-lowering effect of AIIR antagonists and ACE inhibitors remains to be evaluated. The fact that AGE inhibition is demonstrated in a cell-free system suggests that it does not involve cellular intermediates and that it is independent of the activity of angiotensin II. Of course, additional effects may accrue *in vivo* from cellularly related mechanisms. AGE production depends on the concentration and on the production of precursors, many of which are reactive carbonyl and dicarbonyl compounds (4,5,18,33–37). AGE inhibitors might thus act either as trapping agents for RCO or, alternatively, as inhibitors of RCO production. In this study,
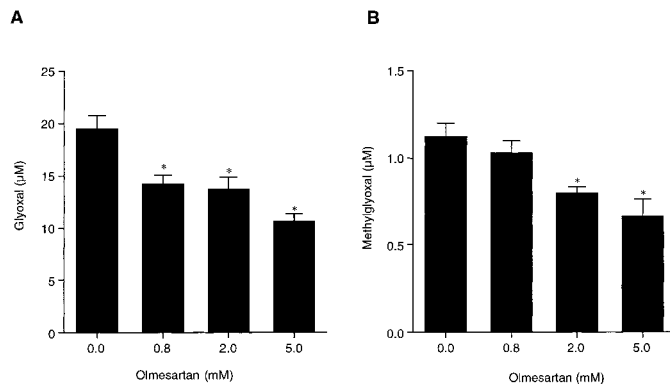
A   **B**

Figure 4. Inhibition of dicarbonyl compounds formation by olmesartan during autoxidation of arabinose. Arabinose (10 mM) was incubated with various concentrations of olmesartan in 0.1 M phosphate buffer (pH 7.4) at 37°C for 1 wk. The generation of glyoxal (A) or methylglyoxal (B) was determined at the end of incubation by HPLC. Data are expressed as means ± SD. *$P < 0.01$ compared with control.

Table 4. Half-maximal inhibition (IC50) value of copper-catalyzed oxidation of ascorbic acid by the tested compounds[a]

Compound	IC50 (μM)
1H-Tetrazole	7600
5-Phenyl-1H-tetrazole	120
5-(4'-Methylbiphenyl-2-yl)-1H-tetrazol	2
Olmesartan	5
Temocaprilat	110
Nifedipine	280
Aminoguanidine	4600
Pyridoxamine	1100
OPB-9195	3

[a] Ascorbic acid (500 μM) was incubated at 30°C with 500 nM CuCl$_2$ in 50 mM phosphate buffer (pH 7.4) in the presence of several concentrations of the tested compounds. The contents of ascorbic acid in the incubation mixtures were determined by HPLC.

we demonstrate that neither olmesartan, an AIIR antagonist, nor temocaprilat, an ACE inhibitor, entrap and lower *in vitro* the concentration of two RCO, glyoxal and methylglyoxal. This finding stands in contrast with aminoguanidine and OPB-9195, both of which, at equimolar concentrations, markedly reduce the levels of both RCO, confirming previous observations (28). Pyridoxamine has an intermediary effect. The mechanism of the AGE-lowering effect of olmesartan and temocaprilat differs markedly from that of aminoguanidine and OPB-9195.

AGE inhibitors may also decrease RCO production. The oxidative metabolism is significantly involved in the generation of RCO; hence its inhibition might lower RCO and thus AGE formation. Our results support such a mechanism of action for both AIIR antagonists and ACE inhibitors. Both olmesartan and temocaprilat interfere with at least two oxida-

Table 5. Half-maximal inhibition (IC50) value of the post-Amadori pentosidine formation[a]

Compound	IC50 (mM)	
	Preglycation with Arabinose	Preglycation with Ribose
Olmesartan	6.22	8.81
Temocaprilat	10.80	11.20
Nifedipine	>50	>50
Aminoguanidine	1.58	6.94
Pyridoxamine	13.00	11.20
OPB-9195	0.61	4.90

[a] BSA was incubated with 0.5 M arabinose or ribose for 24 h at 37°C, followed by extensive dialysis at 4°C. AGE formation was initiated in the presence of various concentrations of the tested compounds by subsequently incubating the Amadori-rich proteins (30 mg/ml) in 0.1 M phosphate buffer (pH 7.4) at 37°C for 1 wk. The content of pentosidine generated upon incubation was determined by HPLC.
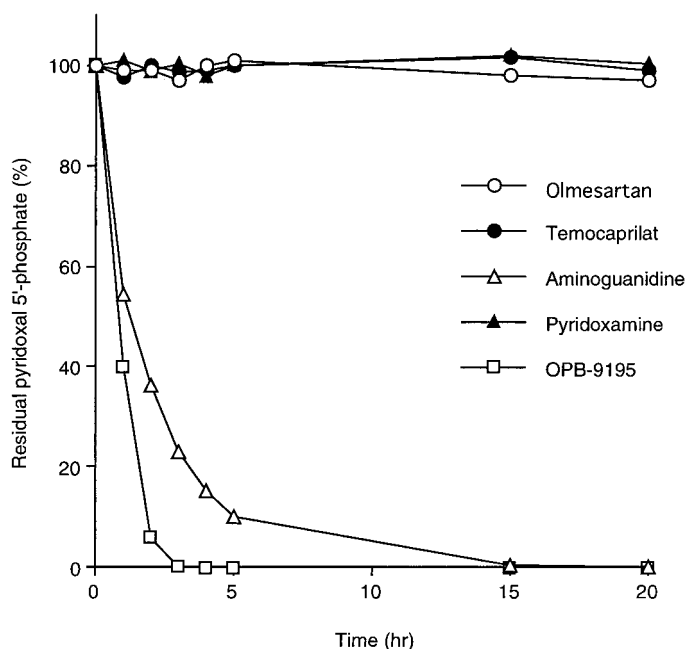

Figure 5. Kinetics of pyridoxal 5'-phosphate (PLP) entrapment by the tested compounds during incubation. PLP (50 μM) was incubated with the tested compounds (500 μM) in phosphate-buffered saline (PBS) at 37°C. Aliquots were removed at various times and assayed for residual PLP by HPLC.

tive steps, the formation of carbon-centered radicals and hydroxyl radicals, here again in contrast with aminoguanidine. Of note, the impact on oxidative processes does not extend to the cellular release of superoxide. This drug-induced alteration of oxidative metabolism is associated with a decreased formation of RCO from arabinose as demonstrated by the effect of olmesartan on glyoxal and methylglyoxal production.

The finding that AIIR antagonists and ACE inhibitors are able to chelate transition metals is of great interest. Indeed,

metal-catalyzed glucose autoxidation and oxidation of glycated residues have been proposed as potent sources of free radicals, which accelerate the browning and cross-linking of proteins and lead to the formation of AGE (30,32,38,39). The transition metal-chelating activity of AIIR antagonists far exceeds that of aminoguanidine and pyridoxamine, a result that is in agreement with a recent report by Price *et al.* (30).

The relationship between the transition metal chelation and the inhibition of oxidative steps produced by AIIR antagonists remains to be clarified. The effect of the tested drugs on oxidative steps may reflect not only an impaired production of oxygen species by metal chelation but also the scavenging of released oxygen species. The latter hypothesis is supported by our observation that prior chelation of transition metals fails to prevent the effect of the various tested drugs on carbon-centered or hydroxyl radicals. Furthermore, we have demonstrated that nifedipine and temocaprilat have a similar potential for chelation of transition metals despite the former drug's inability to attenuate the formation of AGE. Still, the chelation of transition metals likely inhibits oxygen species production, but the extent of its influence on AGE formation remains to be ascertained.

As shown in Figure 6, oxidation influences AGE formation at different stages, *i.e.*, before and after the formation of Amadori products (31,40). To identify the steps at which various AGE-lowering compounds interfere, we studied their effect on Schiff base–modified BSA; upon incubation, this *in vitro*–modified BSA generates pentosidine through post-Amadori steps. We thus demonstrated that both olmesartan and temocaprilat, but not nifedipine, decreased the post-Amadori pentosidine production. Aminoguanidine and OPB-9195 had, on an equimolar basis, a more conspicuous effect, whereas pyridoxamine had an effect similar to that of the hypotensive compounds. The overall effect of olmesartan and temocaprilat on pentosidine generation is markedly higher than that of aminoguanidine and OPB-9195; these data therefore suggest that their influence is exerted mainly on the pre-Amadori and only to a more limited extent on the post-Amadori formation of AGE.

Our approach provides for the first time a comprehensive outline of the mechanisms involved in the prevention of AGE formation. It further shows that available compounds may simultaneously act at different levels. So aminoguanidine traps RCO, chelates mildly transition metals, inhibits mildly or little the formation of carbon-centered and hydroxyl radicals, and is also endowed with a post-Amadori effect. By contrast, both AIIR antagonists and ACE inhibitors have no trapping effect but strongly chelate transition metals, markedly inhibit the formation of oxygen species, and reduce AGE formation, mainly at the pre- but also at the post-Amadori levels.

The discovery that AIIR antagonists and ACE inhibitors reduce AGE formation raises prospects for new therapeutic interventions. Both families of compounds have been used for many years and are well tolerated, unlike the first AGE-lowering agents, aminoguanidine and OPB-9195, which trap not only noxious RCO but also pyridoxal and induce vitamin B6 deficiency. The demonstration that pyridoxal binding is
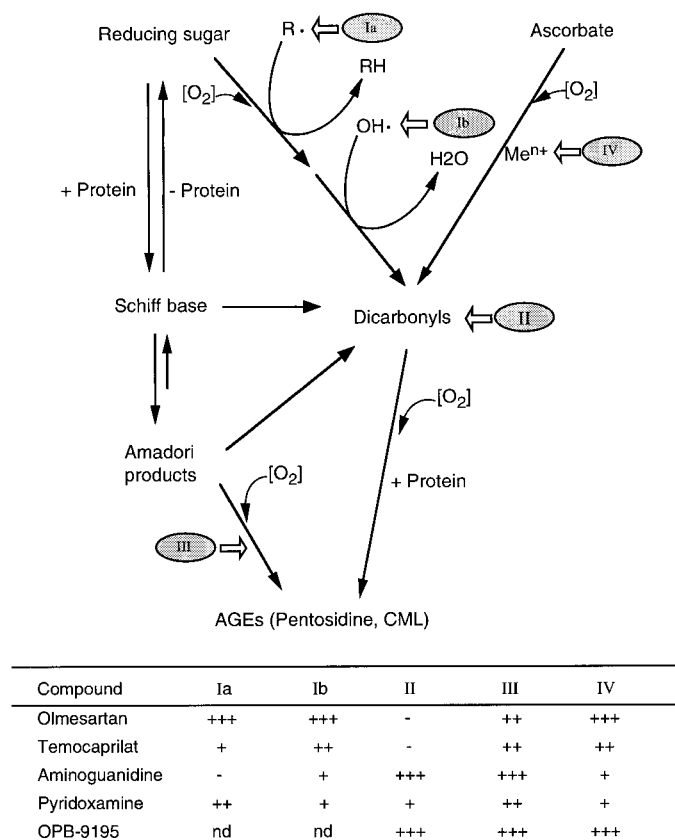
Compound	Ia	Ib	II	III	IV
Olmesartan	+++	+++	-	++	+++
Temocaprilat	+	++	-	++	++
Aminoguanidine	-	+	+++	+++	+
Pyridoxamine	++	+	+	++	+
OPB-9195	nd	nd	+++	+++	+++

Figure 6. Mechanisms of drug-induced advanced glycation end products (AGE) inhibition. Reducing sugars are autoxidized to their carbonyls or dicarbonyls and reduce molecular oxygen to form superoxide/carbon-centered/hydroxyl radical. These radicals further promote the chain reaction of sugar oxidation. Some compounds trap carbon-centered radicals (Ia) and hydroxyl radicals (Ib) to inhibit whole sugar-autoxidation process and to consequently suppress carbonyl/dicarbonyl and the attendant AGE formation. Some compounds trap carbonyls by themselves (II) or inhibit the post-Amadori AGE formation (III). In addition, these compounds chelate transition metal ions, potent catalysts of the AGE formation (IV). ND, not determined due to the low solubility of OPB-9195 in the reaction mixture.

restricted to aminoguanidine and OPB-9195 but does not occur with olmesartan or temocaprilat confirms this view.

Our *in vitro* observations have been made at drug concentrations far above those achieved under clinical circumstances. Still, the dose effect relationship depicted in Figure 2 demonstrates an effect at far lower concentrations that those used in most of the present assays. In this context, it is of interest that *in vitro* concentrations of 800 and 2000 μM of either pyridoxamine or aminoguanidine are necessary to demonstrate inhibition of pentosidine and CML generation, whereas *in vivo* levels of 106 and 87 μM, respectively, lower AGE levels in rat skin collagen (41). Such discrepancies are not unusual in pharmacology. They may be related to a much longer drug of exposure *in vivo* than in *in vitro* assays. Still, more data are needed to ascertain whether the unexpected AGE-lowering effect of AIIR antagonists and some ACE inhibitors contribute to some of the recently demonstrated clinical and experimental benefits of

these drugs (42–51). An interaction between AGE, endothelial cell function, and nitric oxide release (29,49–51) provides a useful framework for the exploration of these systemic effects, which are often unaccounted for by BP lowering.

Chelation of transition metals by some ACE inhibitors and AIIR antagonist with an attendant effect on oxidative protein products (48,52,53) and probably on lipoxidation may also contribute to their beneficial effects on atherosclerosis progression (32).

In conclusion, we demonstrate that AIIR antagonists and ACE inhibitors markedly reduce AGE formation. Unlike aminoguanidine, these compounds do not act through the trapping of RCO precursors of AGE. Rather, they reduce the production of RCO by interfering with the oxidative metabolism, mainly by lowering the formation of carbon-centered radicals and of hydroxyl-radicals, which also contrasts with aminoguanidine. This effect is paralleled by their ability to chelate transition metals. Inhibition of AGE formation occurs both at the pre- and, to a lesser extent, at the post-Amadori steps. Finally, neither AIIR antagonist olmesartan nor ACE inhibitor temocaprilat trap pyridoxal.

References

1. Brownlee M, Cerami A, Vlassara H: Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med* 318: 1315–1321, 1988
2. Palinski W, Koschinsky T, Butler SW, Miller E, Vlassara H, Cerami A, Witztum JL: Immunological evidence for the presence of advanced glycosylation end products in atherosclerotic lesions of euglycemic rabbits. *Arterioscler Thromb Vasc Biol* 15: 571–582, 1995
3. Palinski W, Miller E, Witztum JL: Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. *Proc Natl Acad Sci USA* 92: 821–825, 1995
4. Anderson MM, Hazen SL, Hsu FF, Heinecke JW: Human neutrophils employ the myeloperoxidase-hydrogen peroxide-chloride system to convert hydroxy-amino acids into glycolaldehyde, 2-hydroxypropanol, and acrolein: A mechanism for the generation of highly reactive α-hydroxy and α, β-unsaturated aldehydes by phagocytes at sites of inflammation. *J Clin Invest* 99: 424–432, 1997
5. Anderson MM, Requena JR, Crowley JR, Thorpe SR, Heinecke JW: The myeloperoxidase system of human phagocytes generates N^ϵ-(carboxymethyl)lysine on proteins: a mechanism for producing advanced glycation end products at sites of inflammation. *J Clin Invest* 104: 103–113, 1999
6. Kirstein M, Brett J, Radoff S, Ogawa S, Stern D, Vlassara H: Advanced protein glycosylation induces transendothelial human monocyte chemotaxis and secretion of platelet-derived growth factor: Role in vascular disease of diabetes and aging. *Proc Natl Acad Sci USA* 87: 9010–9014, 1990
7. Sell DR, Lapolla A, Odetti P, Forgarty J, Monnier VM: Pentosidine formation in skin correlates with severity of complication

in individuals with long standing IDDM. *Diabetes* 41: 1286–1292, 1992

8. McCance DR, Dyer DG, Dunn JA, Bailie KE, Thorpe SR, Baynes JW, Lyons TJ: Maillard reaction products and their relation to complications in insulin-dependent diabetes mellitus. *J Clin Invest* 91: 2470–2478, 1993

9. Beisswenger PJ, Moore LL, Brink-Johnsen T: Increased collagen-linked pentosidine levels and advanced glycosylation end products in early diabetic nephropathy. *J Clin Invest* 92: 212–217, 1993

10. Sugiyama S, Miyata T, Ueda Y, Tanaka H, Maeda K, Kawashima S, van Ypersele de Strihou C, Kurokawa K: Plasma level of pentosidine, an advanced glycation end product, in diabetic patients. *J Am Soc Nephrol* 9: 1681–1688, 1998

11. Miyata T, Oda O, Inagi R, Iida Y, Araki N, Yamada N, Horiuchi S, Taniguchi N, Maeda K, Kinoshita T: β2-Microglobulin modified with advanced glycation end products is a major component of hemodialysis-associated amyloidosis: *J Clin Invest* 92: 1243–1252, 1993

12. Miyata T, Hori O, Zhang J, Yan SD, Ferran L, Iida Y, Schmidt AM: The receptor for advanced glycation end products (RAGE) is a central mediator of the interaction of AGE-β2microglobulin with human mononuclear phagocytes via an oxidant-sensitive pathway. *J Clin Invest* 98: 1088–1094, 1996

13. Miyata T, van Ypersele de Strihou C, Kurokawa K, Baynes JW: Alterations in non-enzymatic biochemistry in uremia: Origin and significance of "carbonyl stress" in long-term uremic complications. *Kidney Int* 55: 389–399, 1999

14. Brownlee M, Vlassara H, Kooney A, Ulrich P, Cerami A: Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science* 232: 1629–1632, 1986

15. Nakamura S, Makita Z, Ishikawa S, Yasumura K, Fujii W, Yanagisawa K, Kawata T Koike T: Progression of nephropathy in spontaneous diabetic rats is prevented by OPB-9195, a novel inhibitor of advanced glycation. *Diabetes* 46: 895–899, 1997

16. Sell DR, Monnier VM: Structure elucidation of a senescence cross-link from human extracellular matrix. *J Biol Chem* 264: 21597–21602, 1989

17. Ahmed MU, Thorpe SR, Baynes JW: Identification of N^ϵ-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J Biol Chem* 261: 4889–4894, 1986

18. Esterbauer H, Schuer RJ, Zollner H: Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde and related aldehyde. *Free Radic Biol Med* 11: 81–128, 1991

19. Miyata T, Ueda Y, Asahi K, Izuhara Y, Inagi R, Saito A, van Ypersele de Strihou C, Kurokawa K: Mechanism of the inhibitory effect of OPB-9195 [(±)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide] on advanced glycation end product and advanced lipoxidation end product formation. *J Am Soc Nephrol* 11: 1719–1725, 2000

20. Miyata T, Ueda Y, Yamada Y, Saito A, Jadoul M, van Ypersele de Strihou C, Kurokawa K: Carbonyl stress in uremia: Accumulation of carbonyls accelerate the formation of pentosidine, an advanced glycation end product. *J Am Soc Nephrol* 9: 2349–2356, 1998

21. Ahkand AA, Kato M, Suzuki H, Hamaguchi M, Miyata T, Kurokawa K, Nakashima I: Carbonyl compounds cross-link cellular proteins and activate protein-tyrosine kinase p60^{c-src}. *J Cell Biochem* 72: 1–7, 1999

22. Soulis-Liparota T, Cooper M, Papazoglou D, Clarke B, Jerums G: Retardation by aminoguanidine of development of albumin-uria, mesangial expansion, and tissue fluorescence in streptozocin-induced diabetic rat. *Diabetes* 40: 1328–1334, 1991

23. Miyata T, Ishikawa S, Asahi K, Inagi R, Suzuki D, Horie K, Tatsumi K, Kurokawa K: 2-Isopropylidenehydrazono-4-oxo-thiazolidin-5ylacetanilide (OPB-9195) inhibits the neointima proliferation of rat carotid artery following balloon injury: Role of glycoxidation and lipoxidation reactions in vascular tissue damage. *FEBS Lett* 445: 202–206, 1999

24. Taguchi T, Sugiura M, Hamada Y, Miwa I: In vivo formation of a Schiff base of aminoguanidine with pyridoxal phosphate. *Biochem Pharmacol* 55: 1667–1671, 1998

25. Mizuno M, Sada T, Ikeda M, Fukuda N, Miyamoto M, Yanagisawa H, Koike H: Pharmacology of CS-866, a novel nonpeptide angiotensin II receptor antagonist. *Eur J Pharmacol* 285: 181–188, 1995

26. Miyata T, Taneda S, Kawai R, Ueda Y, Horiuchi S, Hara M, Maeda K, Monnier VM: Identification of pentosidine as a native structure for advanced glycation end products in β2-microglobulin-containing amyloid fibrils in patients with dialysis-related amyloidosis. *Proc Natl Acad Sci USA* 93: 2353–2358, 1996

27. Dunn JA, McCance DR, Thorpe SR, Lyons TJ, and Baynes JW: Age-dependent accumulation of N^ϵ-(carboxymethyl)lysine and N^ϵ-(carboxymethyl)hydroxylysine in human skin collagen. *Biochemistry* 30: 1205–1210, 1991

28. Miyata T, Horie K, Ueda Y, Fujita Y, Izuhara Y, Hirano H, Uchida K, Saito A, van Ypersele de Strihou C, Kurokawa K: Advanced glycation and lipidoxidation of the peritoneal membrane: Respective roles of serum and peritoneal fluid reactive carbonyl compounds. *Kidney Int* 58: 425–435, 2000

29. Asahi K, Ichimori K, Nakazawa H, Izuhara Y, Inagi R, Watanabe T, Miyata T, Kurokawa K: Nitric oxide inhibits the formation of advanced glycation end products. *Kidney Int* 58: 1780–1787, 2000

30. Price DL, Rhett PM, Thorpe SR, Baynes JW: Chelating activity of advanced glycation end-product (AGE) inhibitors. *J Biol Chem* 276: 48967–48972, 2001

31. Booth AA, Khalifah RG, Todd P, Hudson BG: In vitro kinetic studies of formation of antigenic advanced glycation end products (AGEs): Novel inhibition of post-Amadori glycation pathways. *J Biol Chem* 272: 5430–5437, 1997

32. Monnier VM, Transition metals redox: Reviving an old plot for diabetic vascular disease. *J Clin Invest* 107: 799–801, 2001

33. Glomb MA, Monnier VM: Mechanism of protein modification by glyoxal and glycoaldehyde, reactive intermediates of the Maillard reaction. *J Biol Chem* 270: 10017–10026, 1995

34. Thornalley PJ: Advanced glycation and development of diabetic complications: Unifying the involvement of glucose, methylglyoxal and oxidative stress. *Endocrinol Metab* 3: 149–166, 1996

35. Wells-Knecht KJ, Zyzak DV, Litchfield JE, Thorpe SR, Baynes JW: Mechanism of autoxidative glycosylation: Identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. *Biochemistry* 34: 3702–3709, 1995

36. Hayashi T, Namiki M: Role of sugar fragmentation in the Maillard reaction. In: *Amino-Carbonyl Reaction in Food and Biological Systems*, edited by Fujimaki M, Namiki M, Kato H, Amsterdam, Elsevier Press, 1986, pp 29–38

37. Miyata T, Kurokawa K, van Ypersele de Strihou C: Advanced glycation and lipoxidation end products: Role of reactive carbonyl compounds generated during carbohydrate and lipid metabolism. *J Am Soc Nephrol* 11: 1744–1752, 2000

38. Wolff SP, Jiang ZY, Hunt JV: Protein glycation and oxidative stress in diabetes mellitus and ageing. *Free Radic Biol Med* 10: 339–352, 1991

39. Baynes JW: Role of oxidative stress in development of complications in diabetes. *Diabetes* 40: 405–412, 1991

40. Khalifah RG, Bayne JW, Hudson BG: Amadorins: Novel post-Amadori inhibitors of advanced glycation reactions. *Biochem Biophys Res Commun* 257: 251–258, 1999

41. Degenhardt TP, Alderson NL, Arrington DD, Beattie RJ, Basgen JM, Steffes MW, Thorpe SR, Baynes JW: Pyridoxamine inhibits early renal disease and dyslipidemia in the streptozotocin-diabetic rat. *Kidney Int* 61: 939–950, 2002

42. Kilhovd BK, Hjerkinn EM, Seljeflot I, Berg TJ, Reikvam AA: The ACE inhibitor ramipril influences the serum levels of advanced glycation endproducts in high risk patients with coronary artery disease: Results from a HOPE substudy (abstract). *Eur Heart J* 22: 27, 2001

43. Sebekova K, Schinzel R, Munch G, Krivosikova Z, Dzurik R, Heidland A: Advanced glycation end-product levels in subtotally nephrectomized rats: Beneficial effects of angiotensin II receptor 1 antagonist losartan. *Miner Electrolyte Metab* 25: 380–383, 1999

44. Bauer JH, Reams GP, Hewett J, Klachko D, Lau A, Messina C, Knaus V: A randomized, double-blind, placebo-controlled trial to evaluate the effect of enalapril in patients with clinical diabetic nephropathy. *Am J Kidney Dis* 20: 443–457, 1992

45. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD: The effect of angiotensin-converting enzyme inhibition on diabetic nephropathy. *N Engl J Med* 329: 1456–1462, 1993

46. Lewis EJ, Hunsicker LG, Clarke WR, Berl T, Pohl MA, Lewis JB, Ritz E, Atkins RC, Rohde R, Raz I: Renoprotective effect of the angiotensin II receptor antagonist Irbesartan in patients with nephropathy due to type 2 diabetes. *N Engl J Med* 345: 851–860, 2001

47. Heart outcomes prevention evaluation (HOPE) study investigators: Effects of ramipril on cardiovascular and microvascular outcomes in people with diabetes mellitus: Results of the HOPE study and MICRO-HOPE substudy. *Lancet* 355: 253–259, 2000

48. Pitt B, Poole-Wilson PA, Seoul R, Martinez FA, Dickstein K, Camon AJ, Konstam MA, Riegger G, Klinger GH, Neaton J, Sharma D, Thiyagarajan B: Effects of losartan compared with captopril on mortality in patients with symptomatic heart failure: Randomised trail-The losartan heart failure survival study ELITE II. *Lancet* 355: 1582–1587, 2000

49. Heinecke JW: Oxidants and antioxidants in the pathogenesis of atherosclerosis: Implications for the oxidized low density lipoprotein hypothesis. *Atherosclerosis.* 141: 1–15, 1998

50. Miyata T, Devuyst O, Kurokawa K, van Ypersele de Strihou C: Towards better dialysis compatibility: Advances in the biochemistry and pathophysiology of the peritoneal membrane. *Kidney Int* 61: 375–386, 2002

51. Onozato ML, Tojo A, Goto A, Fujita T, Wilcox CS: Oxidative stress and nitric oxide synthase in rat diabetic nephropathy: Effects of ACEI and ARB. *Kidney Int* 61: 186–194, 2002

52. Pennathur S, Wagner JD, Leeuwenburgh C, Litwak KN, Heinecke JW: A hydroxyl radical-like species oxidizes cynomolgus monkey artery wall proteins in early diabetic vascular disease. *J Clin Invest* 107: 853–860, 2001

53. Saxena P, Saxena AK, Cui X-L: Obrenovich, M., Gudipaty K, Monnier, VM: Transition metal-catalized oxidation of ascorbate in human cataract extracts: Possible role of advanced glycation end products. *Invest Ophthalmol Vis Sci* 41: 1473–1481, 2000