Original Article

Effect of Olmesartan on Oxidative Stress in Hemodialysis Patients

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The effect of olmesartan, an inverse angiotensin II type 1 receptor blocker (ARB), on oxidative stress in hemodialysis (HD) patients is not fully understood, and has not been widely investigated *in vitro* or *in vivo*. We determined the amount of oxidized albumin and albumin hydroperoxides formed during incubation in the absence and presence of olmesartan by high-performance liquid chromatography (HPLC) and by a ferrous oxidation xylenol assay in an *in vitro* study. Six hypertensive HD patients were treated with 40 mg of olmesartan once daily, and blood pressure monitoring (BPM) was performed after 0, 4, and 8 weeks of treatment. The ratio of oxidized to unoxidized albumin was also determined. The oxidized albumin ratios and levels of albumin hydroperoxides were significantly decreased in a concentration-dependent manner in the presence of olmesartan, compared with the absence of olmesartan ($p<0.05$) in *in vitro* studies. In HD patients, olmesartan also significantly reduced systolic and diastolic blood pressure after 4 weeks, with a further significant decrease after 8 weeks. The ratio of oxidized to unoxidized albumin was markedly decreased after 4 weeks and these lower levels were maintained at 8 weeks. Olmesartan effectively lowered the extent of oxidation of albumin in both *in vitro* and *in vivo* studies, and this effect might confer benefits beyond a reduction in blood pressure. (*Hypertens Res* 2007; 30: 395–402)

***Key Words*: olmesartan, blood pressure, oxidative stress, hemodialysis, albumin**

Introduction

The renin-angiotensin-aldosterone system (RAAS) plays an important role in regulating blood pressure (BP). Angiotensin II type 1 (AT1) receptor blockers (ARBs) inhibit the RAAS and have been shown to be effective for treating hypertension (*1*, *2*). Independent of their ability to lower BP, these compounds have also been reported to reduce the progression of nephropathy in patients with diabetes mellitus (DM) and chronic kidney disease (CKD) (*3*–*5*). Although much of the renal protective effects of ARBs might be due to the lowering of BP, some protection may be due to their effects in reducing oxidative stress. In support of this idea, the blocking of AT1 receptors in hypertensive patients has been shown to reduce oxidative stress, inflammation, and endothelial dysfunction (*6*).

The mechanisms associated with hypertension in hemodialysis (HD) patients are complex, but the RAAS is generally thought to be an important contributor. Angiotensin II, *via* AT1 receptor, stimulates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and enhances the production of reactive oxygen species (*7*), which in turn contributes to endothelial dysfunction and vascular inflammation (*8*, *9*). Thus, the combination of hypertension and oxidative stress induced by stimulation of the RAAS results in the accelerated progression of atherosclerosis in HD patients (*10*).

Olmesartan is an orally active nonpeptide ARB that lowers

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BP when administered daily. Due to its long duration of action, BP control is maintained throughout 24 h. The antihypertensive efficacy and excellent tolerability of olmesartan have been demonstrated in short-term and long-term controlled trials (*11–14*). Miyata *et al*. recently reported that olmesartan, unlike a calcium channel blocker (CCB), inhibited the formation of advanced glycation end-products (AGE) in an *in vitro* study. Olmesartan is a biphenyl tetrazole derivative with a common core structure, 5-(4′-methylbiphenyl-2-yl)-1H-tetrazol, and this core structure is probably responsible for the inhibitory effect on oxidative stress (*15*). Thus, this effect suggests that olmesartan exhibits antioxidant activity in addition to reducing BP. However, the antioxidant effects of olmesartan have not been extensively studied either *in vitro* or *in vivo*.

The aim of this study was to examine the possible antioxidant and free radical-scavenging properties of olmesartan in *in vitro* studies. We also evaluated the effect of once daily administration of 40 mg of olmesartan on BP and on oxidized serum albumin, a marker of protein oxidation, in HD patients (*16–18*).

Methods

Patients

The study protocol was approved by the Institutional Review Board of Kumamoto University. Patients who met each of the following criteria were included in the study: 1) predialysis BP > 140/90 mmHg for 6 consecutive dialysis sessions; 2) no prior treatment with RAAS inhibitors; 3) stable weight for at least 3 months before enrollment; 4) weight gain of less than 5% between dialysis sessions. Written informed consent was obtained from each of 6 stable HD patients (4 men, 2 women) aged 37–80 (mean, 55.5±6.4) years with a duration of dialysis under 1 year. The cause of end-stage renal disease was glomerulonephritis in all cases. At enrollment, all patients were on regular bicarbonate HD for 4–5 h 3 times weekly using high-flux polysulfone hollow-fiber dialyzers. They were not treated with antioxidants such as vitamin E and C or with intravenous iron supplements during the 3 months before inclusion in the study.

Study Design

The study consisted of a 4-week placebo baseline period followed by an 8-week, open-label active treatment period during which the patients received olmesartan once daily, in the morning, at a dose of 40 mg. In patients already on CCB therapy, olmesartan was added to the previous drug. After 0, 4, and 8 weeks of olmesartan therapy, blood samples were obtained from each patient before the first HD session of the week for measurement of the ratio of oxidized to unoxidized albumin. In addition, blood pressure monitoring (BPM) was performed for each patient after 0, 4, and 8 weeks. To avoid any bias with respect to body fluid condition and dialysis efficiency, BPM was performed on the first non-dialysis day after the first HD session of the week. The drugs, dialysis conditions and dry weight of each patient were not changed during either the 4-week placebo period or 8-week treatment period.

Materials and Reagents

Human serum albumin (HSA; Cohn fraction V, fat free) and catalase (EC 1.11.1.6; 65,000 U/mg) were supplied by Boehringer-Mannheim (Mannheim, Germany). Xylenol orange (*o*-cresosulfonaphthalein-3,3-bis-[sodium methyliminodiacetate]) was from Sigma (St. Louis, USA). Olmesartan was obtained from Sankyo Pharmaceutical (Tokyo, Japan).

Chromatography of Serum Albumin

High-performance liquid chromatography (HPLC) was used to analyze serum albumin as described previously (*16*). Samples obtained from the *in vitro* study and from each patient were immediately frozen and stored at −80°C until used for analysis. Then 5 μL aliquots of serum were analyzed on a Shodex Asahipak ES-502N column (Showa Denko Co., Ltd., Tokyo, Japan). From the HPLC profile, the content of each albumin fraction (human mercaptalbumin, f[HMA]; human nonmercaptalbumin-1, f[HNA-1]; human nonmercaptalbumin-2, f[HNA-2]) was estimated as the area of the HNA fraction divided by the HMA fraction of the serum albumin peak (*17*).

Antioxidant Activity of Olmesartan *In Vitro*

Incubation Assay

Fresh heparinized plasma samples were obtained from uremic patients, with informed consent, before the dialysis session. To achieve adequate volumes of plasma and to obtain a stable baseline in experiments in which multiple results were generated, *in vitro* experiments were performed with plasma pooled from several donors ($n=3$ to $n=5$). Pooled plasma (900 μL) was incubated with olmesartan in the presence of air at 37°C. The drug was dissolved in ethanol to obtain a stock solution of 200 μmol/L and further diluted to the required concentrations. One milliliter samples of the plasma were incubated in the presence of air at 37°C for 7 days in the presence of the tested compounds (final concentrations: 2.0 and 20 μmol/L). At the end of the incubation, the ratio of oxidized to control albumin was measured, as described before.

Protein Hydroperoxides

Protein hydroperoxides were generated by irradiating 20 μmol/L HSA solutions with ^{60}Co γ-rays at a dose rate of 36 Gy/min. Protein hydroperoxides were measured by the perchloric acid–xylenol orange assay (*19*). The radiation-generated H_2O_2 was removed by treatment with catalase (154 U/mL). After the addition of the assay reagents and standing at

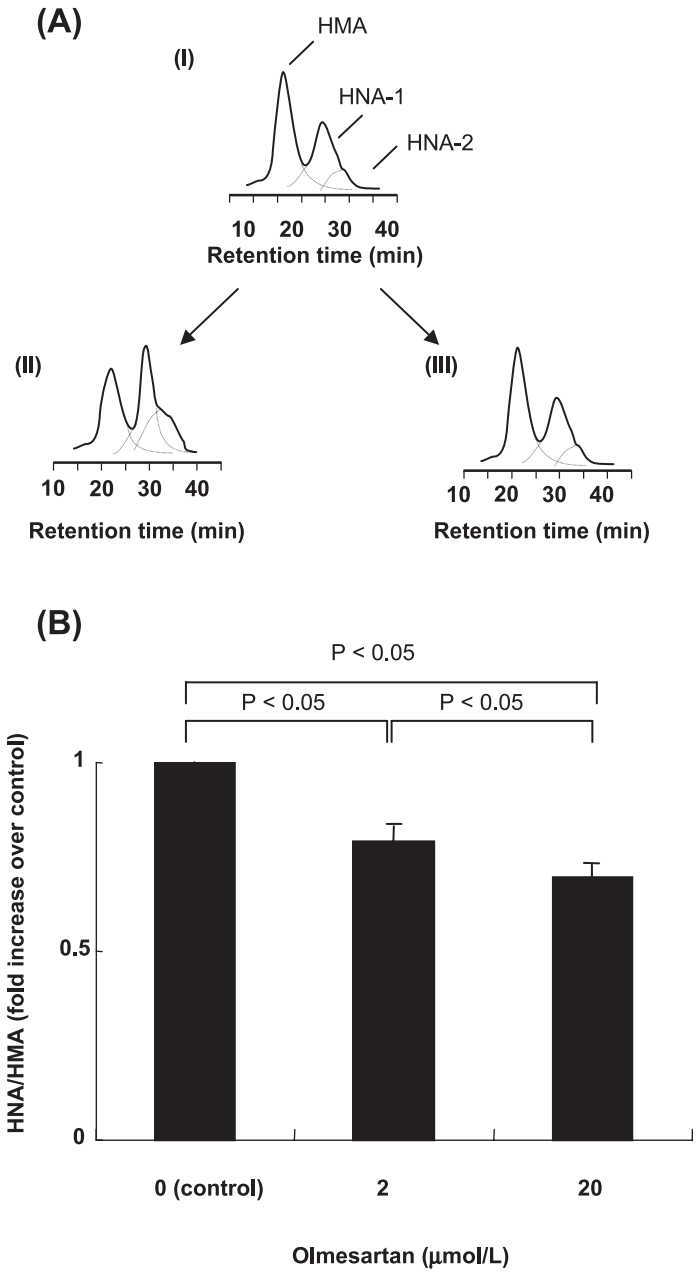

Fig. 1. *A: HPLC profile of in vitro–oxidized serum albumin. HPLC profile of albumin from a uremic subject under control conditions before incubation (I). In II and III, plasma was incubated without or with 20 μmol/L of olmesartan for 7 days. HMA, mercaptalbumin (reduced form); HNA-1, nonmercaptalbumin (disulfide form); HNA-2, nonmercaptalbumin (oxidized form). B: Effect of olmesartan on the HPLC profiles of serum albumin. The calculated ratio of oxidized to reduced albumin ([HNA-1+HNA-2]/[HMA]). Values are expressed as the fold-increase over the control (without olmesartan) (mean±SEM).*

room temperature for 30 min, absorbances were measured at 560 nm and converted to concentrations using the molar absorption coefficient of 3.70×10^4 mol/L/cm (*17*).

Antioxidant Activity of Olmesartan *In Vivo*

Individual Plasma Carbonyl Contents Measurement

The oxidation of individual plasma proteins was measured by Western blot analysis as described by Shacter *et al*. (*20*). Plasma was diluted to 2 mg/mL of total protein with phosphate-buffered saline (PBS) and derivatized with anti-2,4-

dinitrophenylhydrazine (DNP) using an OxyBlot Kit (Serologicals Corporation, Norcross, USA). Samples were diluted to 1 mg/mL of total protein by the addition of an equal volume of nonreducing sample buffer, and 15 μL samples were electrophoresed on duplicate SDS-PAGE gels. Following electrotransfer to a PVDF membrane, one blot was stained for DNP using the OxyBlot Kit reagents. The second blot was stained with Coomassie brilliant blue G for proteins. The bands were visualized with chemiluminescent chemicals and captured on film at 10 min. Each Western blot included samples from both HD patients and healthy controls. These data were recorded as DNP area/protein area, and are reported as densitometry units. The means for each subject group are calculated from each blot.

Statistics

Statistical significance was evaluated by the 2-tailed paired Student's *t*-test for comparison between 2 mean values and by ANOVA followed by the Newman-Keuls test for comparison among >2 mean values. For all analyses, $p<0.05$ was regarded as being statistically significant. The results are reported as the mean±SEM.

Results

Inhibition of Oxidized Albumin Formation by Olmesartan

We determined the HPLC profile of serum albumin with or without olmesartan before and after the *in vitro* incubation. The HPLC profile of plasma for mercaptalbumin (HMA) and nonmercaptalbumin-1 and -2 (HNA-1 and HNA-2) in uremic patients before incubation is shown in Fig. 1A (I). One week after the incubation, in the absence of olmesartan, HMA was reduced and both the HNA-1 and HNA-2 fractions were further increased (Fig. 1A (II)). In the presence of olmesartan, the oxidation of albumin was decreased (Fig. 1A (III)). The ratio of the HNA fraction (HNA-1 and HNA-2) to the HMA fraction was calculated and the results are summarized in Fig. 1B. Treatment with olmesartan caused a significant decrease (21.7±4.1%) in the HNA/HMA ratio at 2 μmol/L of olmesartan ($p<0.05$ *vs*. control), with a further reduction (31.2±3.7%) at 20 μmol/L of olmesartan. These results demonstrate that olmesartan inhibits the oxidation of serum albumin in a concentration-dependent manner.

Inhibition of Albumin Hydroperoxides Formation by Olmesartan

The ability of olmesartan to inhibit the generation of protein hydroperoxides by hydroxyl radicals was measured by irradiating HSA with or without olmesartan by a γ-source, followed by an assay for hydroperoxides. The results showed that olmesartan lowered the amount of HSA hydroperoxides generated and that the effect was concentration- and time-dependent (Fig. 2). These results are consistent with albumin oxidation being inhibited by olmesartan, as shown in Fig. 1.

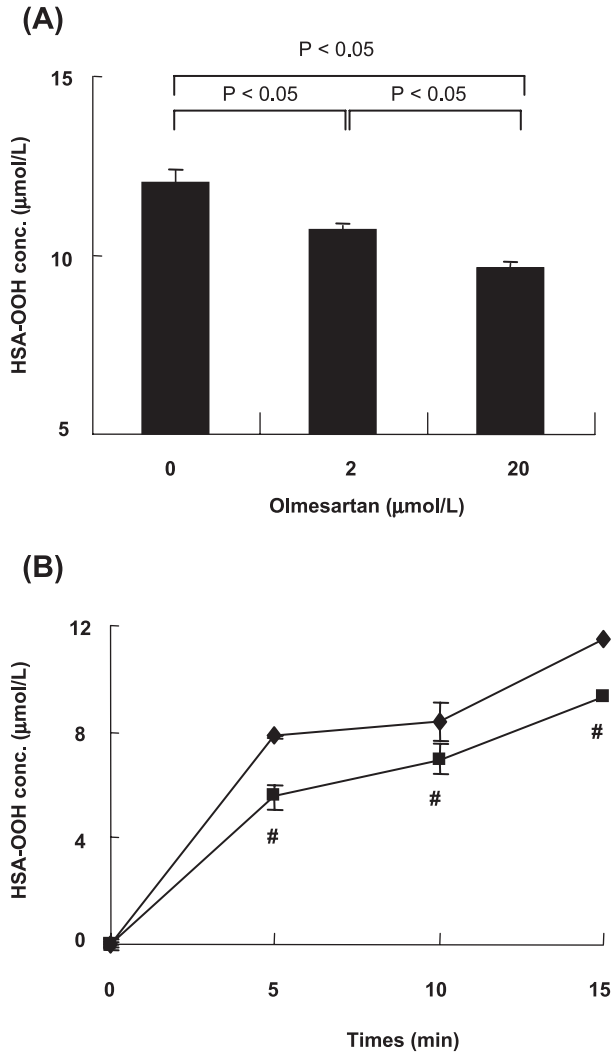

Fig. 2. *Effect of the presence of olmesartan on HSA-OOH formation after γ-irradiation. Concentration (A)- and time (B)- dependent formation of HSA-OOH with olmesartan (20 μmol/L) (■) and with olmesartan (2 μmol/L) (◆). The concentration of HSA was 20 μmol/L and the cobalt-60 radiation dose rate was 36 Gy/min. Hydroperoxide concentrations were measured using the perchloric acid–xylenol orange assay method. Each bar represents the mean±SEM from triplicate samples. HSA-OOH, human serum albumin hydroperoxide. #p < 0.05 vs. 2 μmol/L olmesartan.*

Carbonylation of Plasma Protein from HD Patients with or without Olmesartan

We also investigated the antioxidant effects of olmesartan *in vivo*. As shown in Fig. 3, oxidized proteins were derivatized

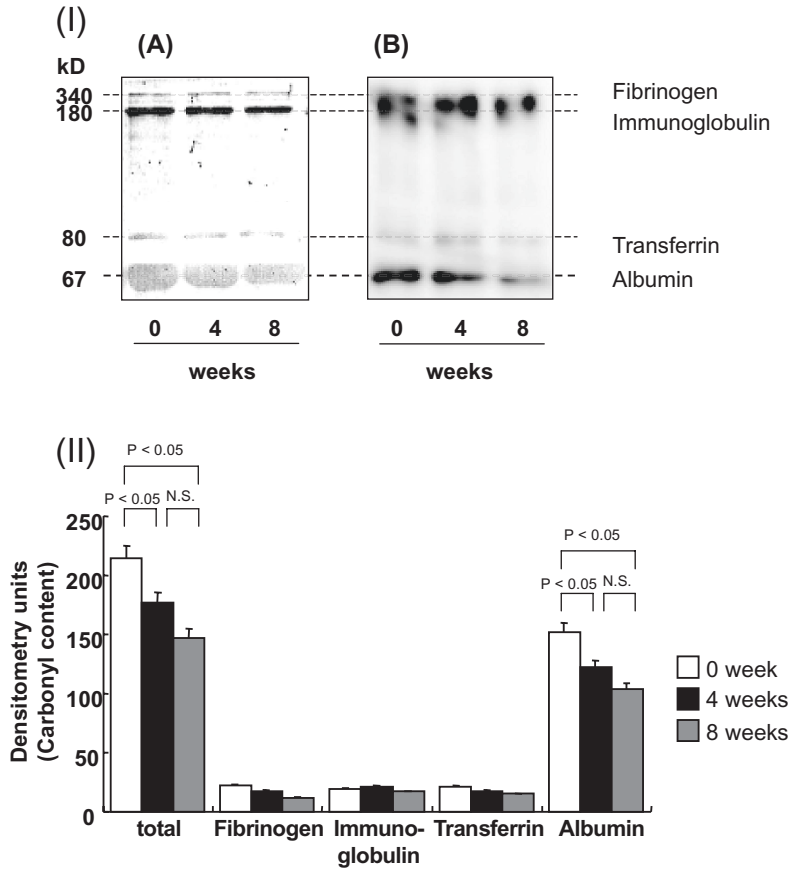

Fig. 3. *Carbonyl content of major plasma proteins from HD patients treated with olmesartan at different times. (I) Plasma samples from HD patients with or without olmesartan were derivatized with DNP after 4 and 8 weeks of treatment and subjected to duplicate SDS-PAGE gels. Following electrotransfer, one blot was stained with Coomassie brilliant blue G for protein (A) and the second blot was stained for DNP using OxiBlot kit reagents (B). (II) Carbonyl formation of major plasma proteins (albumin, transferrin, immunoglobulin, and fibrinogen) was determined as the densitometry ratio of the DNP area and the protein area, and is reported in densitometry units. Values are expressed as the mean±SEM; n = 6 patients per group.*

with DNP, separated by SDS-gel electrophoresis, and screened with antibodies against dinitrophenyl groups. HSA was the only major plasma protein that was significantly oxidized in HD patients without olmesartan and, in the group treated with the drug, the oxidation of HSA was decreased. There was no significant difference in the carbonyl contents of the other plasma proteins (transferrin, immunoglobulin, and fibrinogen). These findings show that the decrease in plasma protein carbonyl contents in HD patients was largely due to a decrease in the level of oxidized HSA. Therefore, it would be expected that characterization of the oxidation status of serum albumin might provide useful information regarding the redox state of the human body, prompting us to examine the effect of olmesartan on the oxidation of albumin.

Oxidation of HSA from HD Patients with or without Olmesartan

The ratio of each HSA fraction to total HSA (f[HMA], f[HNA-1], and f[HNA-2]) was calculated and the results are summarized in Fig. 4. Treatment with olmesartan caused a significant decrease (20.5±3.3%) in the HNA/HMA ratio at 4 weeks ($p<0.05$ *vs*. ratio at 0 weeks), and this effect was maintained up to 8 weeks.

BPM Profile from HD Patients with or without Olmesartan

We investigated the mean BPM profiles (systolic blood pressure [SBP] and diastolic blood pressure [DBP]) after 0, 4, and 8 weeks of treatment with olmesartan. Olmesartan therapy significantly reduced the SBP and DBP at 8 weeks *vs*. the baseline ($p<0.05$) (data not shown). These findings strongly

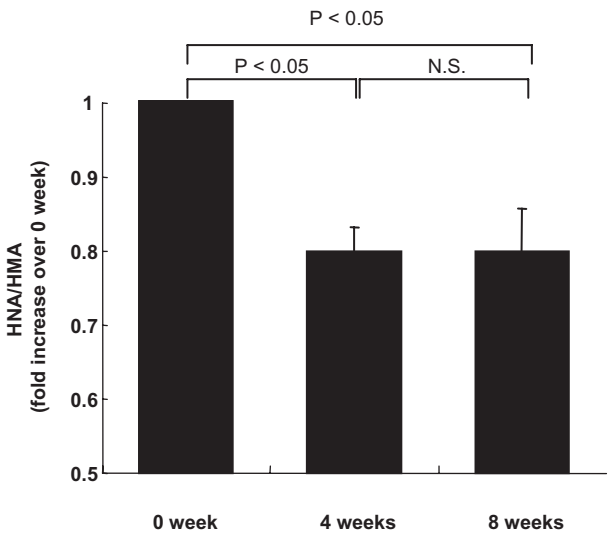

Fig. 4. *Effect of olmesartan on HPLC profiles of serum albumin in vivo. Aliquots (5 μL) of serum were obtained at 0, 4, and 8 weeks after the start of olmesartan therapy and subjected to HPLC using a Shodex Asahipak ES-502N column. The ratio of oxidized albumin to reduced albumin was then calculated ([HNA-1+HNA-2]/[HMA]). Values are expressed as the fold-increase over the control (0 week) (mean±SEM).*

suggest that olmesartan has a significant long-acting BP-lowering effect in HD patients at a daily dose of 40 mg and that ≥8 weeks are required to reach the maximum antihypertensive effect. Olmesartan levels were not significantly different during the experimental periods, suggesting that olmesartan did not accumulate in HD patients when administered at a dose of 40 mg daily for 8 weeks (data not shown). These results indicate that, although the maximum antihypertensive effect was reached at 8 weeks, treatment with olmesartan caused a significant decrease (20.5±3.3%) in oxidative stress at 4 weeks. Thus, this effect might result in benefits by the clinical use of olmesartan.

Discussion

Oxidative stress has long been incriminated in the development of dialysis complications, such as β_2-microglobulin amyloid arthropathy and the acceleration of atherosclerosis (*21*). Until recently, direct evidence for *in vivo* oxidative stress in HD patients was almost entirely limited to the measurement of lipid peroxidation by-products such as malondialdehyde and other thiobarbituric acid–reactive substances (*22*). Despite the observation that proteins are highly susceptible to oxidative stress, there have been few reports of the production of oxidatively modified proteins in HD procedures. Measurement of markers of protein oxidation such as advanced oxidation protein products and carbonyl content have recently been performed to assess oxidative stress under pathological conditions (*23–26*). In 2001, Himmelfarb *et al*. (*27*) reported that the oxidation of albumin accounts for almost all of the excess plasma protein oxidation in uremic patients as demonstrated by SDS-PAGE and an immunoassay using a DNP antibody. In this study, we showed that the decrease in plasma protein carbonyl content in HD patients was largely due to a decrease in the level of oxidized albumin, and that olmesartan substantially decreased the plasma protein carbonyl content by oxidizing albumin (Fig. 3). Given the fact that, in extracellular fluids, serum albumin plays a major antioxidant role (*28*, *29*), we expected that characterization of the oxidation status of serum albumin might provide useful information regarding the redox state of the human body, prompting us to examine the effect of olmesartan on the oxidation of albumin *in vivo*. Previously we reported that purified albumin from HD patients triggered oxidative bursts in neutrophils, and thus appeared to act as a true inflammatory mediator (*30*). Furthermore, the binding of ligands to albumins was found to decrease by oxidative modification of albumin (*25*, *30*). Therefore, management of the oxidation status of serum albumin is an important issue in cases of CKD and medicine therapy.

Serum albumin can be separated into HMA and HNA by HPLC (*31*) and is used to determine the redox state under various pathophysiological conditions (*32–35*). We also recently demonstrated by HPLC that serum albumin shows high levels of oxidation in HD patients compared with age- and gender-matched healthy subjects, and that HPLC analysis of serum albumin can be useful for the quantitative and qualitative evaluation of oxidative stress in HD patients (*16*). However, until the present study, HPLC analysis of serum albumin had not been used to determine whether the antioxidant activity of olmesartan is expressed in *in vivo* and *in vitro* systems.

In a previous study, Miyata *et al*. (*15*) also had suggested that the antagonist olmesartan inhibited the formation of two AGE, pentosidine and carboxymethyllysine, during incubation with uremic plasma or bovine serum albumin. This effect is unlike that of the calcium channel blocker nifedipine. These results suggest that olmesartan has antioxidant activity in HD patients. However, they applied a relatively higher concentration of olmesartan (mmol/L order) to demonstrate the antioxidant effects. Therefore, the anti-oxidant effects of olmesartan at a clinical concentration (around 2 μmol/L) remain to be determined. In the present study, using a highly sensitive HPLC method, a clinical concentration of olmesartan (0–20 μmol/L) was found to attenuate the oxidized albumin ratio *in vitro* based on the redox states of Cys-34 of albumin (Fig. 1) (*36*, *37*). We also examined the possibility that olmesartan can inhibit such damage by preventing the formation of protein peroxides or by decreasing any peroxide groups that are generated by the radicals. The tests showed that irradiated olmesartan did not form stable peroxides (data not shown). However, when HSA (20 μmol/L) was exposed to radiation-generated hydroxyl radicals in the presence of 2.0 or 20 μmol/L of olmesartan, the HSA damage was inhibited, as evidenced

by the amounts of peroxides generated (Fig. 2). These findings suggest that olmesartan, at a clinical concentration, protects HSA against the general oxidation caused by hydroxyl radicals. In fact, olmesartan is a biphenyl tetrazole derivative and its common core structure, 5-(4′-methylbiphenyl-2-yl)-1H-tetrazol, is thus probably responsible for the inhibitory effect on oxidative stress (*15*). This structure is also important for binding to AT1 receptors and is one of the active sites of the inverse agonist. Furthermore, the imidazole ring of olmesartan has carboxyl and hydroxyl groups different from those in other ARBs, which may be the reason for its potent inverse agonist activity.

The findings of our *in vivo* study clearly demonstrated that olmesartan caused a decrease in the levels of oxidized albumin in HD patients after 4 weeks of treatment and this effect was maintained until 8 weeks (Fig. 4), while olmesartan therapy significantly reduced SBP and DBP at 8 weeks (data not shown). Therefore, these results suggest that olmesartan not only reduces SBP but also reduces oxidative stress. Angiotensin II plays an important role in increasing BP by stimulating AT1 receptors. To prevent angiotensin II from acting, ARBs block the binding of angiotensin II to AT1 receptors. Since ARBs block the effects of angiotensin II, they might be expected to decrease the risk of coronary artery disease, cardiac failure, renal dysfunction, and cerebral artery diseases (*38*). Some studies have shown that ARBs do, in fact, significantly reduce these risks, and that their mechanisms of action may involve the blocking of angiotensin II–related functions, such as inducing the production of growth factors and cytokines, in addition to their hypotensive effect. Moreover, angiotensin II has been reported to modulate NADPH oxidase activity in a number of studies, and aldosterone has also been implicated in the generation of reactive oxygen species (*7*, *39*, 40). In theory, then, blocking of the RAAS by ARBs should be effective for reducing oxidative stress. In this work, we showed that olmesartan exhibited antioxidant activity *in vivo*, but this activity might have been a combination of direct and indirect antioxidant effects, such as modulation of NADPH oxidase activity. Moreover, various factors in addition to the above effects may play a role in the antioxidant activity of olmesartan. Therefore, determining the mechanism by which olmesartan decreases the ROS production will require further *in vitro* and *in vivo* studies.

We recently demonstrated that telmisartan effectively lowered the extent of BP and reduced oxidative stress and that it is safe and well-tolerated by HD patients (*17*). Interestingly, the reduction of oxidative stress by olmesartan was slightly higher than that by telmisartan. This effect might have been due to its structure and strong binding to AT1 receptors. However, a long-term study in a large population is required to elucidate the influence of olmesartan therapy on CVD mortality and morbidity in HD patients.

In summary, olmesartan effectively lowered the extent of oxidative damage to HSA in *in vitro* and *in vivo* studies, and this effect might confer benefits beyond simple BP reduction.

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