

Fluvastatin suppresses oxidative stress and fibrosis in the interstitium of mouse kidneys with unilateral ureteral obstruction

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Fluvastatin suppresses oxidative stress and fibrosis in the interstitium of mouse kidneys with unilateral ureteral obstruction.

Background. Recently, we demonstrated increased oxidative stress in the interstitium of ureteral obstructed kidneys based on the increased expression of heme oxygenase-1 and immunohistochemical detection of advanced glycation end products (AGE) in the interstitium. Antioxidant therapy may have a therapeutic potential toward interstitial fibrosis of unilateral ureteral obstruction (UUO) kidneys. Fluvastatin is an HMG-CoA reductase inhibitor and has been demonstrated to have an antioxidant activity *in vitro*.

Methods. The effects of fluvastatin on UUO kidneys from the viewpoints of antioxidant action *in vivo* and antifibrosis action were studied. To investigate the antioxidant action and its therapeutic efficacy of fluvastatin in UUO kidneys, AGE accumulation and fibrosis in the obstructed kidneys was compared among vehicle-, pravastatin-, or fluvastatin-treated (10 or 40 mg/kg/day) groups.

Results. Tubulointerstitial fibrosis was significantly attenuated in fluvastatin-treated animals. Fluvastatin significantly suppressed the degree of immunostaining of AGE in UUO kidneys.

Conclusions. These results provide evidence for the antioxidant action of fluvastatin *in vivo*. The decreased interstitial fibrosis along with a decreased oxidative stress marker in the interstitial lesion strongly suggests the existence of a causal relationship between them. Fluvastatin may have therapeutic value in slowing or preventing interstitial fibrosis in progressive renal disease.

Renal interstitial fibrosis is one of the common histopathological features of progressive renal disease of diverse etiology. Chronic unilateral ureteral obstruction (UUO) is a well-characterized experimental model of

renal injury leading to tubulointerstitial fibrosis that was pioneered by Klahr and Purkerson [1, 2]. The UUO is an excellent model of interstitial fibrosis because it is normotensive, nonproteinuric, and nonhyperlipidemic without any apparent immune or toxic renal insult. The molecular and cellular mechanism(s) of interstitial fibrosis in UUO kidney is beginning to be elucidated. The mechanical disturbances resulting from ureteral ligation (tension stress) [3], hypoxia induced by a marked decline in renal plasma flow [2], up-regulation of monocyte chemoattractant peptide [4], osteopontin [5], heat shock protein 47 (HSP47) [6, 7], intercellular adhesion molecule 1 [8], macrophage influx into the interstitium [4, 9], and production of macrophage-derived cytokines, especially transforming growth factor- β (TGF- β) [10, 11], have been shown to play important roles in the tubulointerstitial damage in UUO kidney. We have been investigating the molecular mechanisms of interstitial cell activation in UUO from the viewpoint of stress responses [6, 7].

While oxidative stress has been implicated in the pathogenesis of various forms of renal injury, little is known about its involvement in the setting of ureteral obstruction. We have recently found that the tubulointerstitium of obstructed kidneys is under increased oxidative stress [12]. Detection of oxidative stress *in vivo* is not easy because of the unstable and reactive nature of reactive oxygen species (ROS). We used heme oxygenase-1 (HO-1) expression and tissue accumulation of *N*^ε-(carboxymethyl) lysine (CML) as indicators of oxidative stress *in vivo*. CML is an integrative biomarker of the cumulative protein damage induced by glycooxidation [13]. HO-1 mRNA and protein expression, which is a sensitive and reliable indicator of oxidative stress [14], were also examined. This observation led us to investigate the therapeutic efficacy of a compound having antioxidant activity toward increased oxidative stress and, if possible, against fibrosis. We chose fluvastatin, an HMG-CoA reductase

Key words: HMG-CoA reductase inhibitor, kidney injury, antioxidant therapy, tubulointerstitial fibrosis, advanced glycation end products, heme oxygenase-1.

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inhibitor, as a test compound since fluvastatin is known to have antioxidant activity in vitro [15–17]. Pravastatin, another HMG-CoA reductase inhibitor that lacks antioxidant activity, was used as a control compound.

In the present study, we studied the effect of fluvastatin on the tubulointerstitial lesion of obstructed kidneys from the viewpoint of oxidative stress. Immunohistological detection of advanced glycation end product (AGE) was performed using anti-AGE monoclonal antibody, which recognizes mainly CML, and the effects of fluvastatin and pravastatin on the accumulation of AGE were compared. HO-1 mRNA expression was also studied one day after ureteral ligation. We found significant amelioration of interstitial fibrosis along with decreased accumulation of AGE in the interstitium of the obstructed kidneys. HO-1 mRNA induction was blunted by fluvastatin treatment. These findings demonstrate the antioxidant activity of fluvastatin in vivo and suggest the therapeutic value of fluvastatin in the treatment of interstitial fibrosis.

METHODS

Experimental protocol and disease model

This study was designed to determine whether fluvastatin reduces oxidative stress in the interstitium of obstructed kidneys and improves the tubulointerstitial fibrosis of the affected kidneys. Pravastatin was used as a control compound because it is an HMG-CoA reductase inhibitor without antioxidant activity. Two experimental protocols were performed. The first experiment was undertaken as a pilot study to determine the effective dosage and actual consumption of the fluvastatin. The summary of the first experiment is described in the **Results** section. Based on the first experiment, the following doses of the fluvastatin were chosen for the second experiment. Mice were given a standard diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) containing 0.004% (10 mg/kg body weight) or 0.016% (40 mg/kg body weight) test compounds from three days before (–3) the UUO operation to the day of sacrifice. Male B6C3 F1 mice, weighing 20 to 22 g, were used in these experiments. The general procedure of the mouse UUO operation is the same as described previously [7]. After induction of general anesthesia by the intraperitoneal injection of pentobarbital (50 mg/kg body weight), all mice underwent left proximal ureteral ligation. The left ureter was identified through a small suprapubic incision and was ligated with 4-0 silk at two points. Both the obstructed and contralateral kidneys were harvested from UUO animals at 1 (vehicle, $N = 7$; fluvastatin 10 mg/kg, $N = 8$; fluvastatin 40 mg/kg, $N = 8$; pravastatin, $N = 7$), 4 (vehicle, $N = 7$; fluvastatin 10 mg/kg, $N = 8$; fluvastatin 40 mg/kg, $N = 6$; pravastatin, $N = 7$), and 10 days (vehicle, $N = 6$; fluvastatin 10 mg/kg, $N = 8$; fluvastatin 40 mg/kg, $N = 8$; pravastatin, $N = 7$) after ureteral obstruction. Midcoronal sections of the kidneys were also taken for immunohistochemical labeling.

Tissue preparation

For morphological examination, the mice were anesthetized with pentobarbital, and the kidneys were perfused in situ with cold phosphate-buffered saline (PBS) to remove circulating blood cells. The kidneys were removed, sliced transversely in 2 to 3 mm thick sections, immersed in a cold 4% paraformaldehyde (PFA) or 70% ethanol solution for 16 to 24 hours, and embedded in paraffin. Four micrometer thick paraffin sections were subjected to Masson's trichrome staining.

Immunohistochemical study

Antibodies. α -Smooth muscle actin (α -SMA) was identified with mouse anti- α -SMA monoclonal antibody (1A4; EPOS, peroxidase-conjugated; Dako, Glostrup, Denmark). Mouse anti-AGE monoclonal antibody (4D12; peroxidase-conjugated) was purchased from Wako Chemical Company (Osaka, Japan).

Paraffin sections obtained from obstructed kidneys and contralateral control kidneys were deparaffinized by xylene. All sections were incubated in a 99:1 methanol/ H_2O_2 solution for 30 minutes at room temperature to inactivate the endogenous peroxidase activity, then washed in PBS for 10 minutes three times, and preincubated at room temperature in blocking solution (10% goat serum and 1% bovine serum albumin (BSA), 0.01% Triton x-100) for 30 minutes. The blocking solution was then blotted onto a paper, and the first antibody was applied to the sections. Incubation with the first antibody was performed for 16 hours at 4°C. After removal of the first antibody and three serial washes in ice-cold PBS (10 min each), the sections for α -SMA and AGE were incubated with solution containing 0.35 mg/mL p-dimethyl aminobenzaldehyde and in 50 mmol/L Tris-HCl (pH 7.6) to visualize the peroxidase activity on the section. After counterstaining in methyl green, the sections were mounted. As a negative control for AGE or α -SMA staining, normal mouse serum was used instead of the anti-AGE or α -SMA antibody. No significant staining was observed in the sections incubated with normal mouse serum instead of the first antibody.

RNA isolation and Northern blotting

For RNA extraction, the kidneys were perfused in situ with cold PBS, were removed and snap frozen in liquid nitrogen, and were kept at -80°C until use. A whole kidney was homogenized with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) in TRIzol reagent (3 mL for a whole kidney; GIBCO BRL, Gaithersburg, MD, USA), and the total cellular RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction procedure according to the manufacturer's instructions [18]. Twenty micrograms of RNA were size fractionated on 1% agarose-formaldehyde gel and transferred to a

nylon membrane filter (Hybond N+; Amersham, Boston, MA, USA) and cross-linked by ultraviolet wave irradiation. Prehybridization of the membranes was done for two hours at 42°C in a buffer containing $5 \times$ SSPE [$1 \times$ SSPE = 150 mmol/L NaCl, 11 mmol/L NaH_2PO_4 , 2 mmol/L ethylenediaminetetraacetic acid (EDTA)], $10 \times$ Denhardt's (100 \times Denhardt's = 2% Ficoll, 2% polyvinylpyrrolidone, 2% BSA), 0.5% sodium dodecyl sulfate (SDS), 100 mg/mL denatured salmon sperm DNA, and 50% (vol/vol) formamide. The membranes were hybridized with 10^6 cpm/mL cDNA probes (described later in this article) in hybridization buffer (same as the prehybridization buffer) for 24 hours at 42°C. After hybridization, the filters were washed three times for 15 minutes in $1 \times$ standard sodium citrate (SSC; $20 \times$ SSC = 3 mol/L NaCl, 0.3 mol/L sodium citrate, pH 7.0) with 0.1% SDS at 60°C. The membranes were then autoradiographed with the intensifying screens (Du Pont, Wilmington, DE, USA) at -70°C for one to three days. The density of the bands was quantitated with the computing densitometer Image Quant (Molecular Dynamics, Sunnyvale, CA, USA).

The following ^{32}P multiprime-labeled DNA probes were prepared by a *rediprime* DNA labeling system (Amersham, Buckinghamshire, UK). Rat HO-1 cDNA (pRHO-1) was kindly provided by Dr. Shibahara (Tohoku University School of Medicine, Sendai, Japan) [19]. ApaI/EcoRI fragment of pRHO-1 (649 bp) was used as a probe for Northern blotting. Rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA [20] was used to monitor the variation of sample loading.

Measurement of serum lipids

Fasting vein blood sample was collected from each mouse just before nephrectomy. Serum levels of total cholesterol, triglyceride, were determined by using commercially available enzymatic methods (EIKEN Chemical Co., Tokyo, Japan).

Morphometric analysis of the interstitial fibrosis in Masson's trichrome or immunohistochemically stained sections by a computer-aided manipulator

The area of the fibrotic lesion in the cortical interstitium was determined on sections stained by Masson's trichrome method to stain the collagen fibers (stained in light blue) as already described [7]. The interstitial area positive for immunohistochemical detection was analyzed and quantitated as described [21]. Under high-power magnification ($\times 400$), five randomly selected nonoverlapping fields from the cortical region were analyzed. The fibrotic areas stained in light blue in the interstitium and tubular basement membrane or immunostaining positive area in the interstitium stained in brown were picked up on the digital images using a computer-aided manipulator (image-operating computer program, Mac-

scope®; Mitani Corporation, Fukui, Japan), and the percentage of the fibrotic area or immunostaining positive area relative to the whole area of the field was calculated (percentage area). Glomeruli and large vessels were not included in the microscopic fields for image analysis. The scores of the five fields of each kidney were averaged. The scores of six separate animals were then averaged.

Analytical procedure

All values were expressed as mean \pm SD. The data were analyzed for homogeneity using Bartlett's test. The statistical significance of any intergroup differences was determined using Dunnett's test for homogeneous data or Kruskal-Wallis test followed by Dunnett's type test for heterogenous data. Statistically significant differences between groups were defined as *P* values of less than 0.05.

RESULTS

Summary of the pilot study

Standard mouse chow containing different amounts of fluvastatin (0.002% and 0.02%) was given to mice, and daily food consumption was measured for one cage (6 mice). The body weight of each mouse was measured every day. The average daily food consumption was 5.5 ± 0.8 g for vehicle-treated UUO mice, 5.4 ± 0.7 g for low-dose fluvastatin-treated UUO mice, and 5.0 ± 0.7 g for high-dose fluvastatin-treated UUO mice. The average body weight for each group was approximately 22 g for vehicle and low-dose fluvastatin-treated mice and approximately 20 g for high-dose fluvastatin-treated mice 10 days after commencing drug administration. The calculated dose was approximately 5 mg/kg/day for the low-dose fluvastatin-treated group and approximately 50 mg/kg/day for high-dose treated mice. Based on these measurements, we defined the fluvastatin doses of the first experiment as 5 and 50 mg/kg/day. Fluvastatin administration was started three days before the UUO operation and was continued to the day of sacrifice at 10 postoperative days ($N = 6$ for each group, each time point). The α -SMA-positive area was quantitatively analyzed to explore possible effects of fluvastatin on the interstitial myofibroblast expansion. We found a dose-dependent decrease in α -SMA-positive area in fluvastatin-treated animals (vehicle 25.5 ± 3.7 , fluvastatin 5 mg/kg 19.5 ± 5.0 , $P = \text{NS}$ vs. vehicle; fluvastatin 50 mg/kg 15.7 ± 1.9 , $P < 0.01$ vs. vehicle).

Based on the results of the preliminary experiment, we chose two doses of fluvastatin, 10 and 40 mg/kg/day, for the treatment of interstitial fibrosis in UUO. In addition, we decided to test the effect of pravastatin, an HMG-CoA reductase inhibitor lacking in vitro antioxidant activity.

The second experiment used the body weight of individual mice just before sacrifice to monitor the differ-

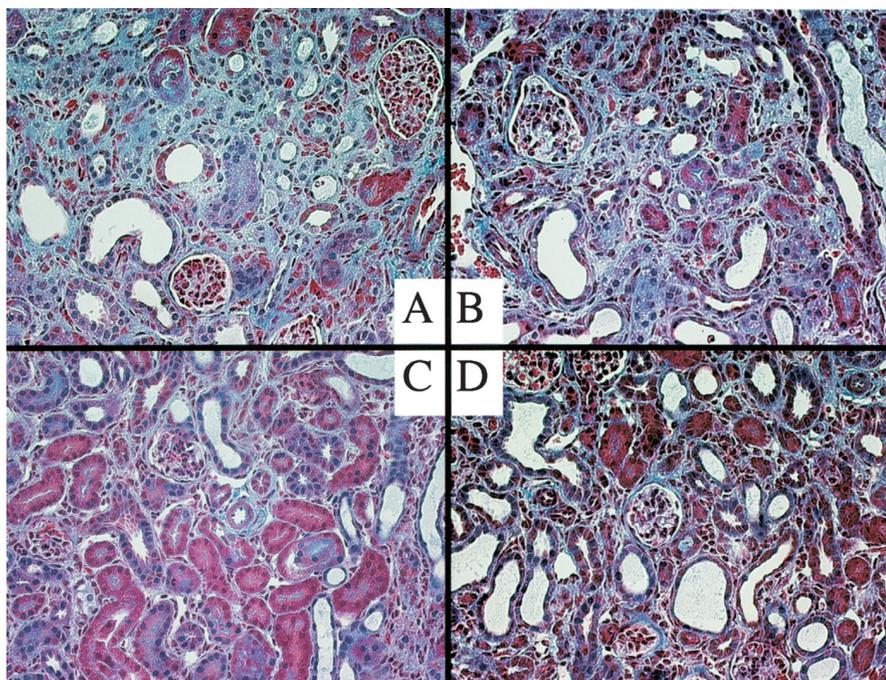


Fig. 1. Masson's trichrome staining of kidneys from four experimental groups at 10 days after ureteral obstruction. Light field photomicrographs of Masson's trichrome-stained sections from (A) vehicle-treated, (B) pravastatin-treated (10 mg/kg/day), (C) fluvastatin-treated (10 mg/kg/day), and (D) fluvastatin-treated (40 mg/kg/day) mouse obstructed kidneys 10 days after ureteral obstruction ($\times 400$).

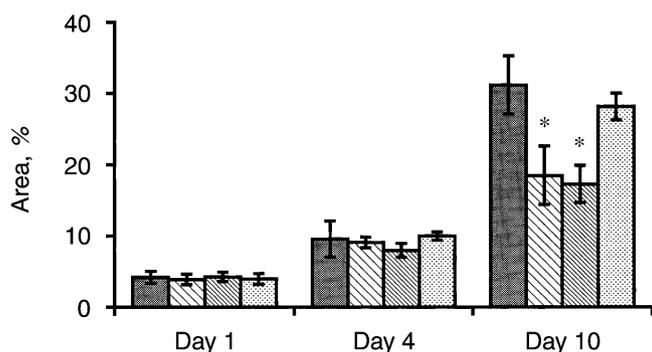


Fig. 2. Quantitative analysis of interstitial fibrosis in kidneys with ureteral obstruction treated with fluvastatin or pravastatin. Interstitial fibrosis was quantitated with a computer-aided image manipulator. Eight animals were analyzed for each point, and five randomly selected high-power fields were quantitated and averaged for the value of each animal. Symbols are: (■) vehicle; (▨) fluvastatin 10 mg/kg/day; (▩) fluvastatin 40 mg/kg/day; (▤) pravastatin 10 mg/kg/day; * $P < 0.01$ vs. vehicle.

ences in terms of food consumption (that is, test compound consumption). The average body weight 10 days after UUO was 22.6 ± 0.9 g for vehicle, 22.0 ± 0.3 g for fluvastatin 10, 20.7 ± 1.1 g for fluvastatin 40, and 22.2 ± 0.8 g for pravastatin 10. There was a slight decrease in body weight gain in fluvastatin 40-treated mice, which was already noticed in the preliminary experiment. No difference in body weight was observed among vehicle- or fluvastatin 10- or pravastatin 10-treated mice, suggesting that a similar amount of fluvastatin and pravastatin was administered in fluvastatin 10- and pravastatin 10-treated mice.

Effects of fluvastatin on interstitial expansion of UUO kidneys

The representative appearance of Masson's trichrome-stained sections of obstructed kidneys from four experimental groups 10 days after ureteral obstruction is shown in Figure 1. Interstitial expansion of obstructed kidneys was prominent in vehicle-treated animals and was also observed to a similar extent in pravastatin-treated animals. In fluvastatin-treated animals, interstitial expansion was less compared with vehicle-treated animals. Quantitative analysis of interstitial fibrosis area in four groups is shown in Figure 2. Fluvastatin treatment at both high (40 mg/kg/day) and low (10 mg/kg/day) doses significantly ameliorated the interstitial expansion in UUO kidneys at 10 days after ureteral obstruction. On the other hand, pravastatin had little effect on the interstitial expansion of UUO kidneys. There seemed to be less tubular atrophy in the cortex in the fluvastatin-treated mice compared with vehicle or pravastatin-treated mice at 10 days after ureteral obstruction.

Effects of fluvastatin on interstitial α -SMA expression in UUO kidneys

Induction of interstitial myofibroblasts was assessed by immunohistochemical detection of α -SMA. Figure 3 shows the representative appearance of α -SMA immunoreactivity in the interstitium of UUO kidneys 10 days after ureteral obstruction. Intense immunostaining of α -SMA was observed in the periglomerular and peritubular interstitium in addition to the vascular smooth muscle of the arterioles in vehicle-treated mouse kidneys 10 days after

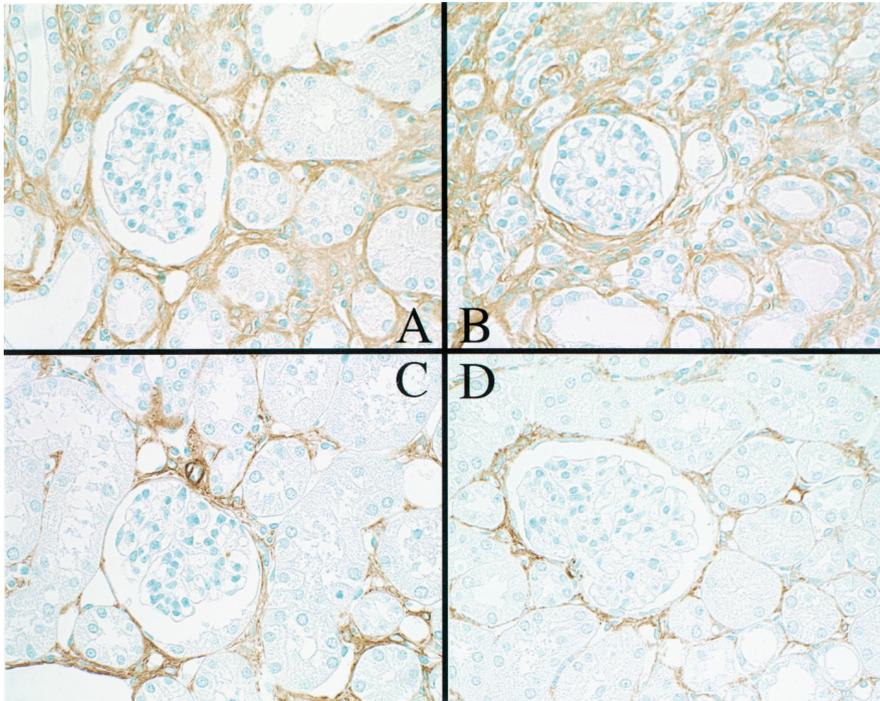


Fig. 3. Immunohistochemical detection of α -smooth muscle actin (α -SMA) in obstructed kidneys. Light field photomicrographs of paraffin sections (A) reacted with mouse monoclonal anti- α -SMA antibody; the cortical region from a 10-day obstructed kidney from a vehicle-treated animal, (B) from a pravastatin-treated (10 mg/kg/day) animal, (C) from a fluvastatin-treated (10 mg/kg/day) animal, (D) and from a fluvastatin-treated (40 mg/kg/day) animal. Sections were counterstained by methyl green. Original magnification was $\times 400$.

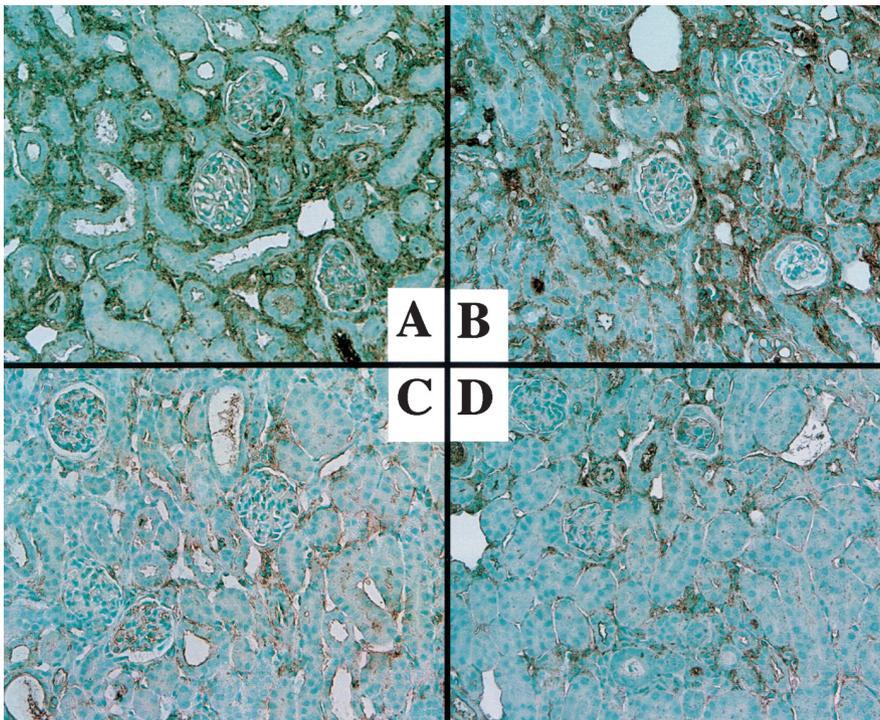


Fig. 6. Horseradish peroxidase immunostaining of advanced glycation end products (AGE) in ureteral obstructed kidneys. Light field photomicrographs of paraffin sections (A) reacted with mouse monoclonal anti-AGE antibody; the cortical region from a 10-day obstructed kidney from a vehicle-treated animal, (B) from a pravastatin-treated (10 mg/kg/day) animal, (C) from a fluvastatin-treated (10 mg/kg/day) animal, and (D) from a fluvastatin-treated (40 mg/kg/day) animal. Sections were counterstained by methyl green. Original magnification was $\times 300$.

ureteral ligation (Fig. 3A). No interstitial immunostaining of α -SMA was detected in the contralateral unobstructed or sham-operated kidneys (data not shown). Fluvastatin but not pravastatin treatment reduced the appearance of α -SMA-positive myofibroblasts in the interstitium (Fig. 3B–D). Quantitative analysis of α -SMA immunostaining is shown in Figure 4. Fluvastatin at both doses

(10 and 40 mg/kg/day) significantly reduced the α -SMA immunostaining at 10 days after ureteral obstruction.

Expression of heme oxygenase-1 in the obstructed kidneys

In our previous study, there was an induction of HO-1 mRNA expression as early as 12 hours after the onset of

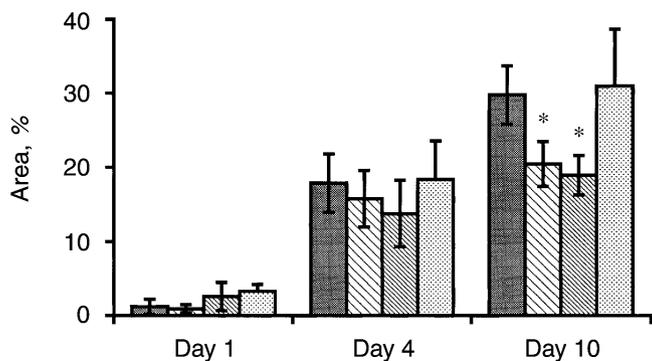


Fig. 4. Quantitative analysis of α -SMA immunostaining in kidneys with ureteral obstruction treated with fluvastatin or pravastatin. The immunostaining positive area was quantitated with a computer-aided image manipulator. Eight animals were analyzed for each point, and five randomly selected high-power fields were quantitated and averaged for the value of each animal. Symbols are: (□) vehicle; (▨) fluvastatin 10 mg/kg/day; (▩) fluvastatin 40 mg/kg/day; (▤) pravastatin 10 mg/kg/day; * $P < 0.05$ vs. vehicle-treated animals.

ureteral obstruction. The early induction of HO-1 is likely indicative of early onset of oxidative stress in the interstitium of obstructed kidneys. To investigate whether fluvastatin blunts the early increase of oxidative stress in the obstructed kidneys following ureteral obstruction, the induction of HO-1 mRNA one day after ureteral obstruction was examined by Northern blot analysis. As shown in Figure 5, there was a significant reduction in HO-1 mRNA levels in the high-dose fluvastatin-treated group.

Effects of fluvastatin on the immunostaining of AGE in the interstitium of UUO kidneys

Figure 6 shows the representative appearance of the immunostaining of AGE in the UUO kidneys after 10 days of ureteral obstruction from four experimental groups. At 10 days following ureteral obstruction, AGE immunoreactivity was observed in the periglomerular and peritubular interstitium of the obstructed kidneys from vehicle-treated mouse (Fig. 6A). AGE immunostaining was reduced in fluvastatin-treated animals but not in pravastatin-treated animals (Fig. 6B) at both low (10 mg/kg/day; Fig. 6C) and high (40 mg/kg/day; Fig. 6D) doses. Quantitative analysis of AGE immunostaining in interstitium is shown (Fig. 7). A significant reduction in the AGE-positive area was observed in fluvastatin-treated mouse kidneys.

Effects of fluvastatin and pravastatin on serum lipids levels in UUO mice

Serum cholesterol or triglyceride levels were not affected by fluvastatin or pravastatin treatment at 10 days after ureteral obstruction (Table 1). This observation is consistent with the previous recognition that HMG-CoA reductase inhibitors have little lipid-lowering effect in mice [22].

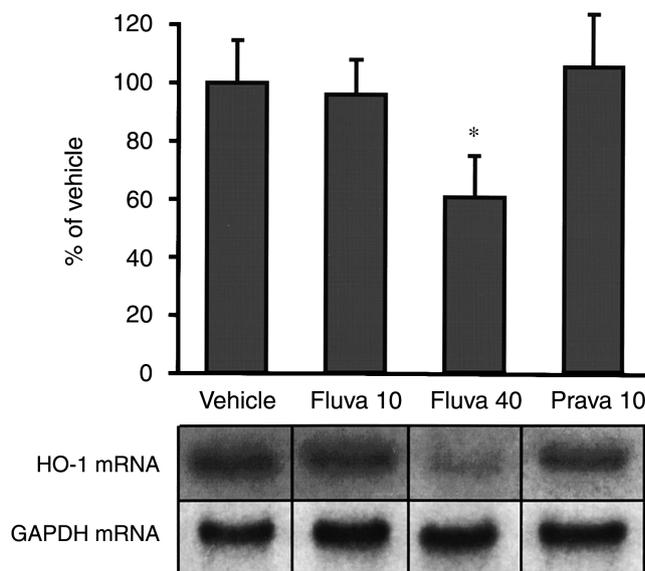


Fig. 5. Heme oxygenase-1 (HO-1) mRNA in mouse kidneys one day after the onset of ureteral obstruction. The relative induction of HO-1 mRNA by densitometry of the Northern blotting autoradiograms is shown. The densitometric values of vehicle treated mice were taken as 1. Results are mean \pm SD of four independent animals. * $P < 0.05$ vs. vehicle-treated animals. Representative samples are shown in this autoradiogram. Each lane represents RNA (20 μ g) from obstructed kidneys from four experimental groups. The top panel shows the HO-1 mRNA, and the bottom panel shows the GAPDH mRNA. Abbreviations are: Fluva 10, fluvastatin (10 mg/kg/day); Fluva 40, fluvastatin (40 mg/kg/day); Prava 10, pravastatin (10 mg/kg/day).

Table 1. Serum lipids levels in unilateral ureteral obstructed (UUO) mice 10 days after ureteral obstruction

	Vehicle	Fluva 10	Fluva 40	Prava 10
Total cholesterol mg/dL	105.8 \pm 8.6	115.4 \pm 9.7	118.7 \pm 11.7	120.6 \pm 7.9
Triglyceride mg/dL	58.6 \pm 10.8	61.0 \pm 7.8	50.8 \pm 2.6	56.2 \pm 11.4

Data are mean \pm SD; $N = 5-8$. Abbreviations are: Fluva 10, fluvastatin (10 mg/kg/day)-treated group; Fluva 40, fluvastatin (40 mg/kg/day)-treated group; Prava 10; pravastatin (10 mg/kg/day)-treated group.

DISCUSSION

The possible involvement of oxidative stress in the pathogenesis of obstructive nephropathy has recently begun to attract attention. We have recently revealed increased oxidative stress in the interstitium of obstructed kidneys by the increased immunostaining of CML at 10 days after ureteral obstruction and an early up-regulation of HO-1 mRNA expression [12]. The present study tested the therapeutic value of fluvastatin, an HMG CoA reductase inhibitor, as a potential antioxidant agent targeting increased oxidative stress and fibrosis in the tubulointerstitium of UUO kidneys.

The major finding in this study is the reduction of oxidative stress in the interstitium of obstructed kidneys from mice treated by fluvastatin. Of note, the effect was

observed in the absence of the lipid-lowering effect of the compound. This is the first demonstration of *in vivo* antioxidant activity of fluvastatin toward kidney. Two different doses were tested, and both high and low doses were revealed to be effective in reducing the accumulation of AGE in the interstitium of the obstructed kidneys. Fluvastatin was shown to have *in vitro* antioxidant activity against oxidative modification of low-density lipoprotein [16]. Yamamoto, Hoshi, and Ichihara investigated the effect of fluvastatin and pravastatin on the lipid peroxidation *in vitro* and found that the order of magnitude of inhibition was butylated hydroxytoluene > probucol \geq fluvastatin > pravastatin, with 0.3 $\mu\text{mol/L}$, 8.2 $\mu\text{mol/L}$, 11.8 $\mu\text{mol/L}$, and 2.3 mmol/L of IC_{50} , respectively [15]. They also found that fluvastatin but not pravastatin has scavenging activity of superoxide anion *in vitro*. The glycoxidation reaction only proceeds in the presence of oxygen mediated by a free radical mechanism involving superoxide radicals and hydrogen peroxide [23]. This may explain the different effects between fluvastatin and pravastatin on the accumulation of AGE in the obstructed kidneys.

We found that fluvastatin but not pravastatin has a suppressive effect on the interstitial expansion and interstitial expression of $\alpha\text{-SMA}$ in the obstructed kidneys. The therapeutic efficacy of HMG-CoA reductase inhibitors in renal diseases has been shown in experimental settings. Yoshimura et al found in rat anti-Thy 1.1 glomerulonephritis that simvastatin suppressed glomerular expression of $\alpha\text{-SMA}$ and glomerular hypercellularity [24]. More recently, Kim, Han, and Lee demonstrated that lovastatin administration in streptozotocin-induced diabetic rats significantly suppressed the increase in urine albumin excretion, kidney weight, glomerular volume, and TGF- β mRNA expression [25]. They also found in cultured mesangial cells that lovastatin suppressed the high glucose-induced up-regulation of TGF- β and fibronectin mRNA. These studies were focused on the glomerular lesion. Our study is the first demonstration, to our knowledge, of the therapeutic efficacy of HMG-CoA reductase inhibitor toward tubulointerstitial lesion. In contrast to earlier studies, we did not find significant suppression of the tubulointerstitial lesion in pravastatin-treated animals. Pravastatin did not affect the appearance of myofibroblast assessed by $\alpha\text{-SMA}$ immunostaining or increased oxidative stress in the interstitium revealed by HO-1 expression or AGE immunostaining. It was previously reported that an *in vivo* inhibitory effect of pravastatin and fluvastatin on *de novo* cholesterol synthesis is comparable in rat kidneys when administered orally at a dose of 12.5 mg/kg [26]. Our study suggests that the differential effect on interstitial fibrosis of fluvastatin and pravastatin cannot be attributed to the bioavailability of these drugs in the kidney *in vivo*.

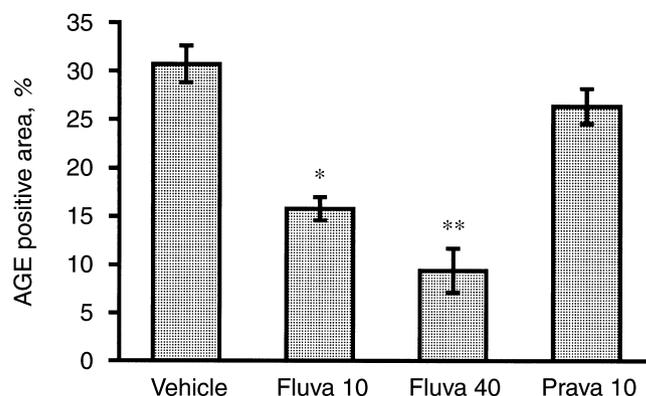


Fig. 7. Quantitative analysis of AGE immunostaining in obstructed kidneys 10 days after ureteral obstruction. An immunostaining positive area was quantitated with a computer-aided image manipulator. Six to eight animals were analyzed for each point, and five randomly selected high-power fields were quantitated and averaged for the value of each animal. * $P < 0.05$ vs. vehicle-treated animals. ** $P < 0.01$ vs. vehicle-treated animals. Abbreviations are: Fluva 10, fluvastatin (10 mg/kg/day); Fluva 40, fluvastatin (40 mg/kg/day); Prava 10, pravastatin (10 mg/kg/day).

Tubulointerstitial injury is an invariant finding in the chronically diseased kidney, irrespective of the type of disease or the compartment in which the disease originates. Recent investigations revealed a close association between the interstitial damage and the prognosis of renal dysfunction [27, 28]. The mechanisms of the development of tubulointerstitial injury and interstitial fibrosis in UUO kidney are a complex pathophysiological sequel of cellular and molecular events. The appearance of $\alpha\text{-SMA}$ -positive activated interstitial cells is one of the early changes leading to a consequent fibrosis in tubulointerstitium. Recent findings demonstrated that the enhanced expression of $\alpha\text{-SMA}$ is a marker of interstitial phenotypic change in various forms of renal disease, and these activated interstitial cells are called “myofibroblasts” [29]. The appearance of myofibroblasts has also been considered to play a key role in the progression of renal fibrosis in both glomerular and interstitial lesions, and to predict a progressive decline in renal function in patients with several glomerulonephritis [30–33]. Myofibroblasts are also described in experimental renal damages [11, 34]. A better understanding of the molecular mechanism of myofibroblast formation would give a new therapeutic option targeting glomerular and tubulointerstitial fibrosis. To date, little is known about the molecular mechanism of oxidative stress in the formation of myofibroblasts and consequent fibrotic lesion in the kidney. Our previous and present studies showed the possible involvement of oxidative stress in the pathogenesis of interstitial fibrosis in UUO [12]. Modification of proteins and lipids by oxidative stress is believed to play a central role in a variety of biological activities caused by increased oxidative stress such as cell proliferation [35],

apoptosis [36–38], and extracellular matrix expansion [39, 40]. It is tempting to establish the molecular link between oxidative stress and myofibroblast formation, especially from the viewpoint of transcription factors. Our new findings regarding the regulatory mechanism of α -SMA gene induction in myofibroblasts and involvement of oxidative stress described in the present study may help to uncover the molecular link between oxidative stress and formation of myofibroblast and consequent fibrotic lesion in the kidney.

In summary, our present study shows the antioxidant activity of fluvastatin in mouse kidneys with UUO. The significant improvement of interstitial fibrosis along with decreased AGE immunostaining strongly suggests a causal relationship between oxidative stress and the development of interstitial fibrosis in UUO. The appearance of α -SMA-positive myofibroblasts was also significantly suppressed by fluvastatin treatment. These antioxidant and antifibrotic actions were independent of the cholesterol-lowering effect of the compound. Novel application of fluvastatin treatment targeting increased oxidative stress is suggested in the clinical setting.

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APPENDIX

Abbreviations used in this article are: AGE, advanced glycation end products; CML, N^ε-(carboxymethyl) lysine; GADPH, glyceraldehyde-3-phosphate-dehydrogenase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HO-1, heme oxygenase-1; HSP47, heat shock protein 47; pRHO-1, rat heme oxygenase-1 cDNA; ROS, reactive oxygen species; α -SMA, α -smooth muscle actin; UUO, unilateral ureteral obstruction.

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