

Effect of Pitavastatin on Urinary Liver-Type Fatty Acid-Binding Protein Levels in Patients With Early Diabetic Nephropathy

TSUKASA NAKAMURA, MD¹
TAKESHI SUGAYA, PHD²
YASUHIRO KAWAGOE, MD¹

YOSHIHIKO UEDA, MD³
SHIWORI OSADA, MD⁴
HIKARU KOIDE, MD⁴

OBJECTIVE — Liver-type fatty acid-binding protein (L-FABP) is expressed in renal proximal tubules and is reported to be a useful marker for progression of chronic glomerulonephritis. The aim of this study was to determine whether urinary L-FABP levels are altered at various stages of diabetic nephropathy and whether pitavastatin affects urinary L-FABP levels in early diabetic nephropathy.

RESEARCH DESIGN AND METHODS — Fifty-eight patients with type 2 diabetes (34 men and 24 women, median age 52 years) and 20 healthy, age-matched subjects (group E) were recruited for the study. The diabetic patients included 12 patients without nephropathy (group A), 20 patients with microalbuminuria (group B), 14 patients with macroalbuminuria and normal renal function (group C), and 12 patients with chronic renal failure but not undergoing hemodialysis (blood creatinine >1.2 mg/dl; mean 2.5 mg/dl, group D). Twenty group B patients were randomly assigned to receive 1 mg/day pitavastatin (10 patients, group B1) or placebo (10 patients, group B2). Treatment was continued for 12 months. Urinary L-FABP levels were measured by enzyme-linked immunosorbent assay. Urinary 8-hydroxydeoxyguanosine and serum free fatty acids (FFAs) were also measured in group B.

RESULTS — Urinary L-FABP levels in groups A-D were 6.2 ± 4.6 $\mu\text{g/g}$ creatinine, 19.6 ± 13.5 $\mu\text{g/g}$ creatinine, 26.8 ± 20.4 $\mu\text{g/g}$ creatinine, and 52.4 ± 46.8 $\mu\text{g/g}$ creatinine, respectively. Urinary L-FABP levels in groups B-D were significantly higher than those in healthy subjects (group E, 5.8 ± 4.0 $\mu\text{g/g}$ creatinine) (group B, $P < 0.05$; group C, $P < 0.01$; group D, $P < 0.01$). In group B1, urinary albumin excretion (UAE) and urinary L-FABP levels were decreased after pitavastatin treatment (UAE before, 110 ± 74 $\mu\text{g/min}$; 6 months, 88 ± 60 $\mu\text{g/min}$, $P < 0.05$; 12 months, 58 ± 32 $\mu\text{g/min}$, $P < 0.01$; L-FABP before, 18.6 ± 12.5 $\mu\text{g/g}$ creatinine; 6 months, 12.2 ± 8.8 $\mu\text{g/g}$ creatinine, $P < 0.05$; 12 months, 8.8 ± 6.4 $\mu\text{g/g}$ creatinine, $P < 0.01$). In group B2, UAE and L-FABP levels showed little change during the experimental period. In group B1, urinary 8-hydroxydeoxyguanosine was decreased 12 months after pitavastatin treatment (before 32.5 ± 19.5 ng/mg creatinine, after 18.8 ± 14.5 ng/mg creatinine, $P < 0.01$), but in group B2, these showed little difference during the experimental period. In both groups B1 and B2, serum FFAs showed little difference during the experimental period.

CONCLUSIONS — Urinary L-FABP levels appear to be associated with the progression of diabetic nephropathy, and pitavastatin may be effective in ameliorating tubulointerstitial damage in early diabetic nephropathy.

Diabetes Care 28:2728–2732, 2005

From the ¹Department of Medicine, Shimatsudo Central General Hospital, Chiba, Japan; the ²Research Unit for Organ Regeneration, Riken Kobe Institute, Hyogo, Japan; the ³Department of Pathology, Koshigaya Hospital, Dokkyo University School of Medicine, Saitama, Japan; and the ⁴Department of Medicine, Koto Hospital, Tokyo, Japan.

Address correspondence and reprint requests to Hikaru Koide, MD, Department of Medicine, Koto Hospital, 6-8-5 Ojima, Koto-ku, Tokyo 136-0072, Japan. E-mail: hkoide@koto-hospital.or.jp.

Received for publication 3 February 2005 and accepted in revised form 26 July 2005.

Abbreviations: 8-OHdG, 8-hydroxydeoxyguanosine; FFA, free fatty acid; L-FABP, liver-type fatty acid-binding protein; NAG, N-acetyl- β -D-glucosaminidase; UAE, urinary albumin excretion.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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Diabetes-associated nephropathy is the leading cause of end-stage renal disease. The pathogenesis of diabetic nephropathy is multifactorial, and the precise mechanisms are unclear. It is now widely accepted that the rate of functional deterioration correlates with the degree of renal tubulointerstitial fibrosis (1). Epithelial cells of the proximal tubules play a major role in orchestrating events in the renal interstitium in diabetic nephropathy (2). Morcos et al. (3) reported activation of tubular epithelial cells in diabetic nephropathy. In addition, oxidative stress may contribute to the progression of tubulointerstitial injury in patients with diabetic nephropathy (4).

Free fatty acids (FFAs) are bound to albumin, filtered through the glomeruli, and reabsorbed into the proximal tubules (5). FFAs in the proximal tubules are then bound to cytoplasmic fatty acid-binding protein (FABP) and transported to mitochondria or peroxisomes (6). In humans, liver-type (L)-FABP of 14 kDa molecular weight is expressed in proximal tubules (7). The physiologic role of L-FABP has not been completely elucidated, but it may be a key regulator of fatty acid homeostasis in the cytoplasm (8). Kamijo et al. (9) reported that urinary L-FABP levels reflect the extent of tubulointerstitial damage and that, of the factors tested, only urinary L-FABP correlates with the progression of chronic kidney disease. However, little is known about the regulation of urinary L-FABP levels in patients with diabetic nephropathy.

Previous studies have shown that statins decrease proliferation, increase apoptosis, and enhance fibrinolytic activity of renal tubular cells (10). Ota et al. (11) reported that cerivastatin decreases albuminuria independently of its cholesterol-lowering effect in rats. We reported that cerivastatin decreases microalbuminuria and plasma and urinary endothelin-1 levels in patients with early diabetic nephropathy, and we speculated that this may represent amelioration of renal injury (12). In this study, we sought to clarify the clinical relevance of urinary L-FABP levels at various stages of diabetic nephropathy. In addition, we studied the

Table 1—Subject characteristics by study group

	A	B	C	D	E
n	12	20	14	12	20
Sex (M/F)	7/5	12/8	8/6	7/5	12/8
Age (years)	48 (36–62)	50 (42–60)	54 (46–64)	57 (50–66)	56 (42–66)
Diabetes duration (years)	8 (6–12)	13 (9–15)	16 (10–18)	20 (12–24)	—
A1C (%)	7.6 ± 1.2	7.5 ± 1.3	8.1 ± 1.4	8.0 ± 1.3	4.6 ± 0.8
SBP (mmHg)	122 ± 14	129 ± 15	134 ± 16	140 ± 16	112 ± 8
DBP (mmHg)	70 ± 8	75 ± 8	82 ± 10	84 ± 12	70 ± 4
Hypertension drugs	25%	25%	29%	42%	0%
Insulin	0%	0%	14%	25%	0%
Total cholesterol (mg/dl)	160 ± 22	182 ± 31	190 ± 32	210 ± 28	146 ± 10
Serum creatinine (mg/dl)	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	2.5 ± 1.0	0.8 ± 0.1
UAE (μg/min)	8 ± 4	107 ± 71	340 ± 220	880 ± 640	6 ± 2
Urinary NAG (IU/g creatinine)	4.4 ± 2.5	4.7 ± 3.1	5.8 ± 4.2	9.8 ± 6.8	4.0 ± 2.2
Urinary-α1 microglobulin (mg/g creatinine)	4.5 ± 3.8	5.0 ± 4.0	5.4 ± 4.2	9.5 ± 8.0	4.0 ± 2.8

Data are means ± SD or median (range). DBP, diastolic blood pressure; SBP, systolic blood pressure.

effect of pitavastatin on L-FABP levels in early-stage diabetic nephropathy.

RESEARCH DESIGN AND METHODS

Fifty-eight patients with type 2 diabetes (34 men and 24 women, median age 52 years, range 36–66 years) were recruited from consecutive attendees. Twenty healthy, age-matched subjects (12 men and 8 women, median age 56 years, range 42–66 years, group E) were also included for the present study. The diabetic patients included 12 patients without nephropathy (group A, 7 men and 5 women; median age 48 years, range 36–62 years), 20 patients with microalbuminuria (20–200 μg/min) (group B, 12 men and 8 women; median age 50 years, range 42–60 years), 14 patients with macroalbuminuria (>200 μg/min) and normal renal function (blood creatinine <1.2 mg/dl) (group C, 8 men and 6 women; median age 54 years, range 46–64 years), and 12 patients with chronic renal failure but not undergoing hemodialysis (group D, 7 men and 5 women; median age 57 years, range 50–66 years). Type 2 diabetes was diagnosed according to the World Health Organization criteria. Patient characteristics in groups A–D are shown in Table 1. The status of diabetic nephropathy of each patient was based on urinary albumin excretion (UAE) levels as measured by immunoturbidimetry of urine samples obtained over 24 h. Urinary N-acetyl-β-D-glucosaminidase (NAG), urinary α1-microglobulin, serum creatinine, serum cholesterol, and HbA_{1c} (A1C) were also measured for all participants. This study was carried out in accordance with the

principles of the Declaration of Helsinki. Informed consent was obtained from each patient and healthy subject. Since we selected the patients who agreed with our projects, the sample size was rather small.

Determination of FABP levels

BALB/C mice were injected subcutaneously with 50 μg purified recombinant human L-FABP in Freund's complete adjuvant, and the same dose was injected again 2 weeks later. We prepared recombinant human L-FABP with a fusion plasmid system (pMAL-cRI). Spleen cells from immunized mice were fused with murine myeloma P3/X63-AG8.6.5.3 cells. Hybridomas were selected in a hypoxanthine-aminopterin-thymidine medium and screened for antibody production by enzyme-linked immunosorbent assay with purified recombinant L-FABP-coated plates. We obtained 18 positive clones by limiting dilution. Expanded cultures from two hybridomas, FABP-2 and FABP-L, were injected into the peritoneal cavities of pristine primed mice, after which ascites fluid was collected, and IgG was isolated by protein A column chromatography. Monoclonal antibody (mAb) FABP-2 was conjugated to horseradish peroxidase with the use of succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate according to the manufacturer's instructions (Pierce Chemical, Rockford, IL) (9). Ninety-six-well microtiter plates were coated with 10 mg/l FABP-L mAb overnight. Unreacted sites were blocked overnight with PBS containing 10 g/l BSA. The plates were washed three times with PBS containing 0.5 g/l Tween-20 and 1 g/l BSA and then

dried. One hundred microliters of properly diluted standards or samples were incubated in the wells of each plate at room temperature for 1 h. The wells were then washed four times with PBS containing 0.5 g/l Tween-20 and allowed to react with 100 μl horseradish peroxidase-conjugated FABP-2 for 1 h. After four more washes, 100 μl enzyme substrate solution was added and incubated at room temperature for 30 min, after which the reaction was terminated by the addition of 100 μl 2 mol/l sulfuric acid. Absorbance was measured at 492 nm on a microplate reader (10). We prepared standards for the assay by measuring the protein concentration of purified recombinant L-FABP according to Lowry's method and adjusting the concentration with PBS plus 10 g/l BSA to create a series ranging from 0 to 400 ng/ml. There are no effects of sex in urinary L-FABP levels (T.N., T.S., H.K., unpublished data).

Statin treatment

Twenty group B patients were randomly assigned (sealed envelope method) to one of two groups: group B1, comprising 10 patients treated with 1 mg/day pitavastatin (Kowa, Nagoya, Japan) or group B2, comprising 10 patients who received placebo. Characteristics of these two patient groups are shown in Table 2. Treatment was continued for 12 months. Urinary L-FABP and UAE levels were measured before treatment, 6, and 12 months after treatment in each group. In addition, serum FFAs and urinary 8-hydroxydeoxyguanosine (8-OHdG) were measured before and 12 months after treatment in each group. Urinary 8-OHdG was mea-

Table 2—Basic clinical data of diabetic patients with microalbuminuria (group B) with or without pitavastatin

	Pitavastatin treatment	No pitavastatin treatment
n	10	10
Sex (M/F)	6/4	6/4
Age (years)	51 (42–60)	49 (44–58)
Diabetes duration (years)	13 (9–15)	12 (9–14)
A1C (%)	7.4 ± 1.4	7.5 ± 1.2
Serum creatinine (mg/dl)	0.9 ± 0.2	0.8 ± 0.1
Blood urea nitrogen (mg/dl)	16 ± 2	17 ± 3
Total cholesterol (mg/dl)	180 ± 28	185 ± 34
UAE (μg/min)	110 ± 74	104 ± 68
SBP (mmHg)	128 ± 14	130 ± 16
DBP (mmHg)	76 ± 8	74 ± 8
NAG (IU/g creatinine)	4.5 ± 3.0	4.8 ± 3.2

Data are means ± SD or median (range). DBP, diastolic blood pressure; SBP, systolic blood pressure.

sured by an enzyme-linked immunosorbent assay kit using the highly sensitive mAb described previously (8-OHdG Check; Nikken Foods, Fukuroi, Shizuoka, Japan [13,14]). Serum FFA concentrations were determined by enzymatic method with 3-octenoic acid (15).

Statistical analysis

Data are shown as means ± SD. Patient age and duration of diabetes are shown as the median and range. Linear regression analysis was used to evaluate correlation between the two variables. Differences in variables among the three groups were analyzed with Scheffle's test. To analyze differences in clinical variables between patient and healthy subject groups, the Mann-Whitney *U* test was used for unpaired data, and the Wilcoxon's rank-sum test was used for paired data. *P* values <0.05 were considered statistically significant.

RESULTS — Urinary L-FABP levels for each group are shown in Table 3. Urinary L-FABP levels showed little difference between diabetic patients without nephrop-

athy (group A) and healthy subjects (group E). Urinary L-FABP levels increased with the progression of diabetic nephropathy. Table 4 shows the results of univariate regression analysis of urinary L-FABP levels with serum creatinine, serum total cholesterol, A1C, UAE, urinary NAG, urinary α1-microglobulin, and systolic and diastolic blood pressure. Urinary L-FABP levels did not correlate with any of these variables. Urinary 8-OHdG levels were significantly higher in group B (30.5 ± 18.5 ng/mg creatinine) than in group E (10.5 ± 8.5 ng/mg creatinine) (*P* < 0.01). However, serum FFA levels in group B (0.50 ± 0.36 mEq/l) were not significantly different from those in group E (0.42 ± 0.32 mEq/l). In addition, urinary L-FABP levels did not correlate with UAE levels, NAG levels, or serum fatty acid levels in group B. However, urinary L-FABP levels were significantly correlated with urinary 8-OHdG levels (*P* < 0.01). In group B1, UAE and urinary L-FABP levels decreased after pitavastatin treatment (6 months, UAE *P* < 0.05, L-FABP *P* < 0.05; 12 months, UAE *P* < 0.01, L-FABP *P* < 0.01) (Table 5). Furthermore, urinary 8-OHdG levels de-

Table 3—Urinary L-FABP levels

Group	L-FABP (μg/g creatinine)
A	6.2 ± 4.6
B	19.6 ± 13.5
C	26.8 ± 20.4
D	52.4 ± 46.8
E	5.8 ± 4.0

Data are means ± SD. A vs. B, *P* < 0.05; A vs. C, *P* < 0.01; A vs. D, *P* < 0.001; A vs. E not significant; B vs. C, *P* < 0.05; B vs. D, *P* < 0.01; B vs. E, *P* < 0.05; C vs. D, *P* < 0.01; C vs. E, *P* < 0.01; D vs. E, *P* < 0.001.

Table 4—Stepwise regression analysis for urinary L-FABP levels

Independent variables	<i>F</i> ratio
Serum creatinine	2.0
Serum total cholesterol	0.01
A1C	0.01
UAE	2.0
Urinary NAG	0.2
Urinary α1-macroglobulin	1.5

All data are not significant.

creased from 32.5 ± 19.5 ng/mg creatinine to 18.8 ± 14.5 ng/mg creatinine after 12 months (*P* < 0.01) in group B1. However, in group B2, UAE, L-FABP, and urinary 8-OHdG levels showed little changes during the experimental period (Table 5). Serum FFAs showed little changes in both groups B1 and B2 during the experimental period.

CONCLUSIONS — We found that urinary L-FABP levels increased with the progression of diabetic nephropathy and that pitavastatin was effective in decreasing urinary L-FABP and UAE levels in patients with early diabetic nephropathy. FFAs are overloaded in the proximal tubules, not only in massive proteinuria but also in response to various kinds of stress to the proximal tubules, including ischemic or toxic insults, both of which have been implicated in the progression of renal disease (9,16). In the human kidney, two types of FABP have been localized (7). L-FABP is expressed in proximal tubules, and a heart-type FABP is expressed in distal tubules. L-FABP plays a key role in fatty acid metabolism in proximal tubules, and its expression is induced by fatty acids (9). Tubulointerstitial inflammation induced by lipid toxicity may be provoked not only by proteinuria but also by other stressors. Kamiyo et al. (9) hypothesized that various stressors to proximal tubules overload fatty acids in the cytoplasm and thereby damage the tubules by releasing inflammatory factors. In this way, tubulointerstitial inflammation deteriorates renal function over time. Previous studies have shown that renal function in type 2 diabetes correlates better with tubular changes than with glomerular pathology (2,3). Further studies of tubulointerstitial disease in addition to glomerular injury in diabetes may provide additional insight into the pathogenesis of diabetic nephropathy and lead to the identification of targets for therapeutic intervention (17). Increased oxidative stress plays an important role in the progression of diabetic nephropathy (4). Urinary 8-OHdG has been reported as a sensitive biomarker of oxidative DNA damage, and its excretion is significantly correlated with severity of tubulointerstitial lesions (4). In the present study, we showed that urinary 8-OHdG levels in diabetic patients with microalbuminuria (group B) were significantly higher than those in healthy control subjects (group E) and that urinary L-FABP levels were significantly correlated with urinary

Table 5—UAE, urinary L-FABP levels, and total cholesterol before and after treatment in diabetic patients with microalbuminuria (group B)

	Before treatment	6 months	12 months
UAE ($\mu\text{g}/\text{min}$)			
Pitavastatin	110 \pm 74	88 \pm 60*†	58 \pm 32*§
Nonpitavastatin	104 \pm 68	110 \pm 72	118 \pm 74
L-FABP ($\mu\text{g}/\text{g}$ creatinine)			
Pitavastatin	18.6 \pm 12.5	12.2 \pm 8.8*†	8.8 \pm 6.4*§
Nonpitavastatin	20.6 \pm 14.5	22.0 \pm 16.0	24.0 \pm 18.0
Total cholesterol (mg/dl)			
Pitavastatin	180 \pm 28	172 \pm 24	170 \pm 26
Nonpitavastatin	185 \pm 34	180 \pm 28	184 \pm 30

Data are means \pm SD. Versus before treatment: * $P < 0.05$ and † $P < 0.01$. Versus nonpitavastatin treatment: ‡ $P < 0.05$ and § $P < 0.01$.

8-OHdG levels. Our data suggest that increased urinary L-FABP levels may occur, in part, in response to oxidative stress. Kamijo et al. (9) detected a significant correlation of urinary L-FABP levels with urinary protein levels in chronic glomerular diseases, excluding diabetic nephropathy. However, our data did not show any correlation between urinary L-FABP levels and UAE levels in diabetic nephropathy. Therefore, regulation of L-FABP levels may differ between diabetic nephropathy and other chronic glomerular diseases. In addition, we did not find any correlation between urinary L-FABP levels and urinary $\alpha 1$ -microglobulin or NAG levels. Urinary NAG levels reflect structural damage to tubular cells. We presume that the clinical significance of L-FABP levels differs from that of NAG levels.

Although a recent meta-analysis study showed that statins can decrease proteinuria and preserve glomerular filtration in patients with chronic renal disease (18), little is known about the precise mechanisms involved. Ota et al. (11) reported that cerivastatin decreases albuminuria by suppressing glomerular hyperfiltration, mesangial expansion, and loss of the charge barrier independently of its cholesterol-lowering effect in rats. Usui et al. (19) reported that the beneficial effect of statins in early diabetic nephropathy is mediated by pleiotropic effects, including anti-inflammatory reduction of oxidative stress. The antioxidant effects of statins likely contribute to their clinical efficacy in treating cardiovascular diseases as well as other chronic conditions associated with increased oxidative stress in humans (20). Pitavastatin is a new, chemically synthesized, potent statin (21). The cytochrome P450 system only

slightly modifies pitavastatin, suggesting a clinical advantage of this agent, because the potential for clinically significant interactions with commonly used drugs is considered extremely low (21). Recently, Hayashi et al. (22) have shown that statins, including pitavastatin, reduced ischemic brain injury through decreasing oxidative stress. However, little is known about the effect of pitavastatin on renal disease. We are the first to report that pitavastatin reduces microalbuminuria, urinary 8-OHdG, and L-FABP levels in patients with early diabetic nephropathy independently of its cholesterol-lowering effect, in part due to reducing oxidative stress; however, the precise mechanisms remain unclear.

In summary, urinary L-FABP may be a useful clinical marker for diabetic nephropathy, and pitavastatin may ameliorate the progression of tubulointerstitial lesions in diabetic nephropathy.

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