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Effects of α-lipoic acid on transforming growth factor β1–p38 mitogen-activated protein kinase–fibronectin pathway in diabetic nephropathy

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Abstract

In diabetic nephropathy, transforming growth factor β1 (TGFβ1) is related to p38 mitogen-activated protein kinase (MAPK) that induces production of fibronectin in mesangial cells. We investigated the effects of α-lipoic acid (ALA), a potent antioxidant, on proteinuria and TGFβ1–p38 MAPK–fibronectin pathway in diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats. After ALA treatment for 5 weeks in OLETF rats at 30 weeks of age, plasma malondialdehyde, urinary protein excretion, renal cortical TGFβ1, and fibronectin protein levels were decreased; and urinary protein excretion was positively correlated with renal cortical TGFβ1 and fibronectin protein levels. Phospho-form but not total-form levels as well as fold activations of each protein consisting of p38 MAPK pathway were also attenuated. These results suggest that ALA ameliorates proteinuria by attenuating expressions of TGFβ1 and fibronectin proteins, and these favorable effects are related to inhibition of phosphorylating activation of p38 MAPK pathway in renal cortex of OLETF rats.

1. Introduction

Diabetes mellitus develops microvascular complications including diabetic nephropathy and has emerged as a leading cause of end-stage renal disease [1]. Diabetic nephropathy is characterized clinically by proteinuria and histologically by glomerular basement membrane thickening and mesangial matrix expansion, resulting in glomerulosclerosis [2]. Transforming growth factor β1 (TGFβ1) is a ubiquitously expressed multifunctional cytokine critical to development and wound healing [3,4]. In fact, TGFβ1 is known as a key mediator of sclerosing process in diseased glomeruli and is related to glomerulosclerosis and interstitial fibrosis in various renal diseases [5-8]. Transforming growth factor β1 is increased in kidney as well as serum of diabetic animals and patients, demonstrating possible contribution of TGFβ1 to diabetic nephropathy [9-12]. Otsuka Long-Evans Tokushima Fatty (OLETF) rat, an animal model of obese type 2 diabetes mellitus established in 1992 by Kawano et al [13], has a renal pathologic feature similar to that of diabetic nephropathy. Yagi et al [10] have reported that TGFβ1 was increased at the stage of non–insulin-dependent diabetes mellitus preceding glomerulosclerosis, indicating responsibility of TGFβ1 for development of glomerulosclerosis in OLETF rats.

Mitogen-activated protein kinase (MAPK) cascades function in a pleiotropic manner to regulate intracellular signal transduction [14-17]. In p38 MAPK pathway, MAPK kinase 3 (MKK3), a specific activator of p38 MAPK, phosphorylates and activates p38 MAPK but not ERK or JNK; and activated p38 MAPK leads to phosphorylation of cyclic adenosine monophosphate response element (CRE) binding protein (CREB) [18,19]. The CRE binding protein plays a critical role in the induction of fibronectin by CRE

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−170 base pairs within fibronectin promoter [20]. In diabetes mellitus, hyperglycemia-induced oxidative stress activates p38 MAPK, which induces phosphorylation of transcriptional factors, altered expression of genes, and production of fibronectin in mesangial cells, resulting in diabetic nephropathy [21,22]. Based on the suggestion that inhibition of p38 MAPK pathway would prevent the development of diabetic nephropathy by blocking formation of extracellular matrix, Wang et al [23] have described that inhibition of phosphorylation of p38 MAPK was involved in the protection of nephropathy by decreasing glomerular fibronectin in diabetic rats.

Transforming growth factor β1 exerts biological activities via MAPK cascades in certain cell lines, especially via p38 MAPK in human mesangial cells, smooth muscle cells, fibroblasts, neutrophils, and rat renal tubular cells [6,24-26]. Although a previous study identified Smad pathway for TGFβ1 signaling in diabetic mice [27], several authors have reported that TGFβ1 was related to p38 MAPK in diabetic kidney, suggesting a role of p38 MAPK as another important mediator of TGFβ1 signal pathway [28,29].

α-Lipoic acid (ALA), a naturally occurring short chain fatty acid with sulfhydryl group, is an essential cofactor of mitochondrial respiratory enzymes and has potent antioxidative capacity [30]. α-Lipoic acid improves insulin sensitivity and stimulates peripheral glucose utilization, resulting in prevention of development of diabetes mellitus in OLETF rats [31-33]. In diabetes mellitus, ALA reduces urinary albumin excretion, fraction clearance of albumin, glomerular volume, glomerular content of TGFβ1, and glomerular mesangial matrix expansion, and inhibits progression of albuminuria [34-37]. It has been reported that ALA reversed increases of fibronectin and collagen in porcine mesangial cells cultured in high glucose concentration [38]. Although ALA has been shown to decrease phosphorylated p38 MAPK but not total p38 MAPK in dystrophin-deficient mice [39], the role of ALA on p38 MAPK pathway in diabetic nephropathy has not been evaluated yet.

In the present study, we hypothesized that ALA has beneficial effects on diabetic nephropathy by inhibiting activation of TGFβ1, p38 MAPK pathway, and fibronectin, and investigated the protective role of ALA in proteinuria and the role of p38 MAPK pathway in this renoprotection by examining expressions of total and phosphorylated MKK3, p38 MAPK, CREB, and fibronectin proteins in renal cortex of OLETF rats.

2. Materials and methods

2.1. Experimental animals

Five-week–old male OLETF rats and Long-Evans Tokushima Otsuka (LETO) rats, a nondiabetic control rat model for OLETF rats, were obtained from the Otsuka Pharmaceutical (Tokushima, Japan). All rats were kept at a specific pathogen-free facility under controlled temperature (23°C ± 2°C) and humidity (60% ± 10%) with a 12-hour light-dark cycle. They were fed standard rat chow (MF; Oriental, Kobo, Japan) and given distilled water ad libitum. All animal procedures were done in accordance with the guidelines of the Institutional Animal Care and Use Committee at the Hallym University Medical Center, ChunCheon, Republic of Korea.

2.2. Experimental protocol

All experiments were performed at 30 weeks of age. At 30 weeks of age, 24 OLETF rats were randomly divided into 2 groups: the untreated OLETF and ALA groups. The untreated OLETF group (n = 12) was fed standard rat chow, and the ALA group (n = 12) was fed standard rat chow with 0.5% (wt/wt, 200 mg per kilogram body weight per day) ALA (Bukwang Pharmaceutical, Seoul, Republic of Korea) for 5 weeks as previously reported [31-33]. The LETO group (n = 12) as a control was fed standard rat chow without ALA. All rats were housed in metabolic chamber individually, and food intake and body weight were monitored weekly from 30 to 35 weeks of age. At 35 weeks of age, all rats were anesthetized with pentobarbital sodium and perfused in situ via aorta with phosphate-buffered saline (pH 7.4) to remove blood. Trunk blood was collected for measurement of biochemical parameters. Kidneys were excised and weighed. Renal cortices were then excised, immediately frozen in liquid nitrogen, and stored at −70°C for analysis.

2.3. Measurement of biochemical parameters

After 12 hours of fasting period, plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose, hemoglobin A_{1c} (HbA_{1c}), insulin, free fatty acid (FFA), cholesterol, triglyceride (TG), malondialdehyde (MDA), and urinary protein and creatinine concentrations of OLETF and LETO rats were measured at 30 and 35 weeks of age. Blood was taken from the tail vein and centrifuged for 15 minutes. Plasma glucose concentration was measured by using a glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH), plasma HbA_{1c} was determined by high-performance liquid chromatography method (Roche Diagnostics, Indianapolis, IN), and plasma insulin concentration was measured by a radioimmunoassay kit (Linco Research, St Charles, MO). Plasma FFA, cholesterol, and TG concentrations were determined by using an automated analyzer. Plasma MDA concentration was measured by thiobarbituric acid method. For measurement of urinary protein and creatinine excretions, urines of all rats in metabolic chambers were collected for 24 hours weekly. Urinary protein concentration was measured by Coomassie quantitative reaction method and corrected by urinary creatinine concentration. Urinary TGFβ concentration was determined by an enzyme-linked immunosorbent assay kit (Promega, Madison, WI). All biochemical parameters were measured twice.

2.4. Western blot analysis and periodic acid Schiff reagent immunostaining

Samples for immunoblot analysis were prepared from renal cortices. Total cellular extracts were obtained by lysis of tissue homogenate, washed with ice-cold phosphate-buffered saline, and lysed in 500 μL of lysis buffer containing 20 mmol/L HEPES (pH 7.2), 1% Triton X-100, 10% glycerol, 20 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μ/mL leupeptin at 4°C. After incubation for 5 minutes, lysates were sonicated for 1 minute and then centrifuged for 15 minutes at 15 000 rpm at 4°C to pellet insoluble portion. Protein concentrations in supernatants were determined using bicinchoninic acid assay (Pierce, Rockford, IL). An equal volume of sodium dodecyl sulfate (SDS) loading buffer consisting of 0.125 mmol/L Tris-HCl (pH 7.4), 4% SDS, and 20% glycerol was added; and protein samples were boiled for 5 minutes at 95°C. The samples (100 μg of protein) were electrophoresed on a 12% SDS–polyacrylamide gel electrophoresis and then transferred onto Immobilon-P membrane (Immobilon; Millipore, Bedford, MA). Nonspecific binding sites were blocked in Tris-buffered saline buffer containing 10 mmol/L Tris-HCl (pH 7.4), 0.15 mol/L NaCl, and 5% skim milk overnight at 4°C. Blot was washed in Tris-buffered saline buffer containing 0.05% Tween 20 and probed with the specific antibodies for TGFβl (Santa Cruz Biotechnology, Santa Cruz, CA), total p38 MAPK, phospho–p38 MAPK, total MKK3/6, phospho-MKK3/6, total CREB, phospho-CREB (New England Biolabs, Beverly, MA), and fibronectin (Gibco-BRL, Grand Island, NY) (1:1000 dilution) overnight at 4°C and incubated with horseradish peroxidase–conjugated secondary antibodies appropriate for the species of primary antibodies used for 2 hours at room temperature with gentle shaking. Total-form protein antibodies detect the total (phosphorylation state independent) proteins, whereas phospho-form protein antibodies detect specifically the phosphorylated proteins. Bands were detected using electrochemiluminescence Western blotting system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), scanned, and quantitatively analyzed by an image densitometer. Results are expressed as percentage of control signals in each blot to correct for variations between blots. Immunoblottings for β-actin and for the sample without each antibody were carried out as positive and negative controls, respectively. All experiments were done in triplicate.

For periodic acid Schiff reagent (PAS) immunostaining, renal cortices were fixed, embedded in paraffin section at 3 μm, and stained with PAS.

2.5. Statistical analysis

Data are presented as means ± SEM. Statistical analysis was performed by unpaired Student *t* test or by analysis of variance. A *P* value less than .05 was considered to be statistically significant. All analyses were performed using Statistical Package for Social Science, version 9.0 (SPSS, Chicago, IL).

3. Results

3.1. Effects of ALA on the food intake and body weight in OLETF rats

Basic characteristics of the LETO, ALA, and untreated OLETF groups at 30 and 35 weeks of age are summarized in Table 1. At 30 weeks of age, the food intake and body weight of OLETF rats were significantly higher than those of LETO rats ($P < .05$); but there was no difference in the food intake and body weight between the ALA and untreated OLETF groups because of random allocation of OLETF rats to the ALA and untreated OLETF groups.

The food intake was significantly decreased in the ALA group at 35 weeks of age compared with the untreated

Table 1
The food intake, body weight, and biochemical parameters of the LETO, ALA, and untreated OLETF groups at 30 and 35 weeks of age

	LETO group		ALA group		Untreated OLETF group	
	30 wk	35 wk	30 wk	35 wk	30 wk	35 wk
Food intake (g/d)	23.10 ± 1.54	23.30 ± 1.65	26.40 ± 1.57*	22.30 ± 1.67†,‡	26.20 ± 1.48*	28.60 ± 1.39
Body weight (g)	512.60 ± 11.19	518.70 ± 10.22	660.10 ± 11.54*	602.30 ± 9.85†,‡	658.20 ± 10.84*	698.90 ± 10.73
AST (IU/L)	52.4 ± 2.3	58.7 ± 2.5	65.3 ± 3.1*	79.2 ± 3.8‡	64.7 ± 3.4*	83.1 ± 4.2†
ALT (IU/L)	49.6 ± 1.4	51.1 ± 1.9	58.1 ± 1.6*	72.7 ± 2.1‡	56.9 ± 1.6*	75.3 ± 2.3†
Plasma glucose (mmol/L)	6.3 ± 1.1	6.3 ± 1.2	9.3 ± 1.8*	9.2 ± 1.5	9.2 ± 1.7*	9.8 ± 1.8
HbA_{1c} (%)	5.30 ± 0.33	5.79 ± 0.41	8.31 ± 0.41*	8.74 ± 0.42	8.29 ± 0.26*	8.98 ± 0.30
Plasma insulin (pmol/L)	254 ± 72	257 ± 79	851 ± 304*	782 ± 264†,‡	843 ± 291*	886 ± 273
Plasma FFA (mmol/L)	0.66 ± 0.08	0.69 ± 0.11	0.76 ± 0.11*	0.81 ± 0.12	0.74 ± 0.09*	0.92 ± 0.10
Plasma cholesterol (mmol/L)	2.18 ± 0.15	2.21 ± 0.27	3.19 ± 0.74*	3.36 ± 0.41	3.16 ± 0.52*	3.57 ± 0.44
Plasma TG (mmol/L)	0.23 ± 0.04	0.51 ± 0.12	2.12 ± 0.63*	1.95 ± 0.53†	2.07 ± 0.59*	2.49 ± 0.62
Plasma MDA (μmol/L)	1.8 ± 0.5	1.9 ± 0.7	3.8 ± 1.1*	2.9 ± 0.5†,‡	3.7 ± 0.8*	4.1 ± 0.7
Urinary protein/Cr (mg/mg·Cr)	0.341 ± 0.192	0.396 ± 0.254	3.591 ± 0.386*	2.634 ± 0.279†,‡	3.483 ± 0.317*	5.112 ± 0.311

Data are presented as mean ± SEM. Cr indicates creatinine.

* *P* less than .05 vs the LETO group at 30 weeks of age.

† *P* less than .05 vs the untreated OLETF group at 35 weeks of age.

‡ *P* less than .05 vs the ALA group at 30 weeks of age.

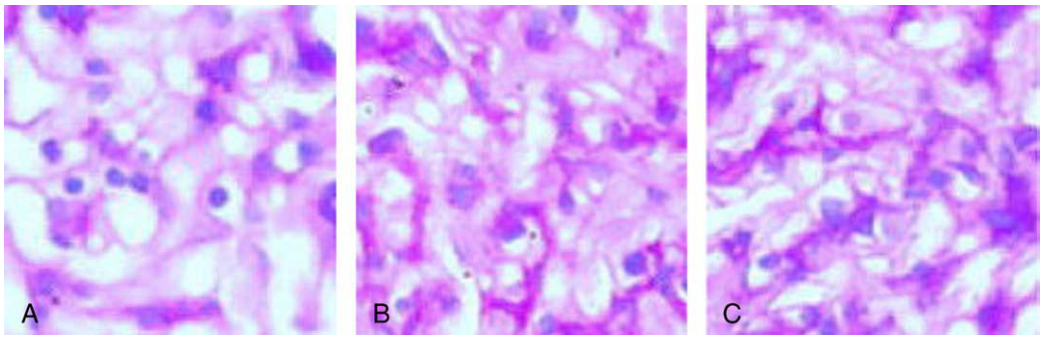

Fig. 1. Periodic acid Schiff immunostaining of glomeruli in LETO and OLETF rats at 35 weeks of age. The glomerular hypertrophy and mesangial matrix expansion were shown in the ALA (B) and the untreated OLETF (C) groups except the control group (A), and were decreased in the ALA group compared with the untreated OLETF group (hematoxylin and eosin stain, ×400).

OLETF group at 35 weeks of age (22.30 ± 1.67 g/d vs 28.60 ± 1.39 g/d, $P < .05$) and the ALA group at 30 weeks of age (22.30 ± 1.67 g/d vs 26.40 ± 1.57 g/d, $P < .05$). At 35 weeks of age, the ALA group weighed significantly lower than the untreated OLETF group (602.30 ± 9.85 g vs 698.90 ± 10.73 g, $P < .05$) and the ALA group at 30 weeks of age (602.30 ± 9.85 g vs 660.10 ± 11.54 g, $P < .05$).

3.2. Effects of ALA on the plasma metabolic parameters and MDA concentrations in OLETF rats

The fasting plasma AST, ALT, glucose, HbA_{1c}, insulin, FFA, cholesterol, TG, and MDA concentrations of OLETF rats were significantly higher than those of LETO rats at 30 weeks of age ($P < .05$) (Table 1). As with the food intake and body weight, these parameters were not different between the ALA and untreated OLETF groups at 30 weeks of age.

The fasting plasma glucose concentrations were decreased in the ALA group compared with the untreated OLETF group at 35 weeks of age, but the differences were not statistically significant. In the ALA group, the fasting plasma glucose concentrations and HbA_{1c} levels before and after ALA treatment did not differ. The increases of the plasma insulin concentrations in the ALA group at 35 weeks of age were significantly attenuated by 16.5% (vs the untreated OLETF group at 35 weeks of age, $P < .05$) and 11.6% (vs the ALA group at 30 weeks of age, $P < .05$), respectively. At 35 weeks of age, the plasma FFA and cholesterol concentrations were not different between the ALA and untreated OLETF groups; but the plasma TG concentrations were significantly lower in the ALA group than in the untreated OLETF group ($P < .05$). The plasma MDA concentrations were significantly reduced in the ALA group at 35 weeks of age compared with the untreated OLETF group at 35 weeks of age ($P < .05$) and the ALA group at 30 weeks of age ($P < .05$).

3.3. Effects of ALA on the urinary protein excretion, kidney weight, and expressions of renal cortical TGFβ1 and fibronectin proteins in OLETF rats

The urinary protein excretion of OLETF rats was significantly higher than that of LETO rats at 30 weeks of age (Table 1). The urinary protein excretion between the ALA and untreated OLETF groups at 30 weeks of age did not differ.

The urinary protein excretion in the ALA group at 35 weeks of age compared with the untreated OLETF group at 35 weeks of age and the ALA group at 30 weeks of age was significantly decreased by 52.5% ($P < .05$) and 30.0% ($P < .05$), respectively. At 35 weeks of age, the kidney weight per body weight was lower in the ALA group than in the untreated OLETF group; but the difference was not statistically significant (the LETO vs the ALA vs the untreated OLETF groups, 3.11 ± 0.14 vs 3.16 ± 0.18 vs 3.24 ± 0.23 mg/g; P = not significant [NS]).

The PAS immunostaining of glomeruli was observed in the ALA and untreated groups and was decreased in the ALA group compared with the untreated OLETF group (Fig. 1).

At 35 weeks of age, the renal cortical TGFβ1 protein levels were significantly increased in the untreated OLETF group by 14-fold (vs the LETO group, $P < .01$); and the

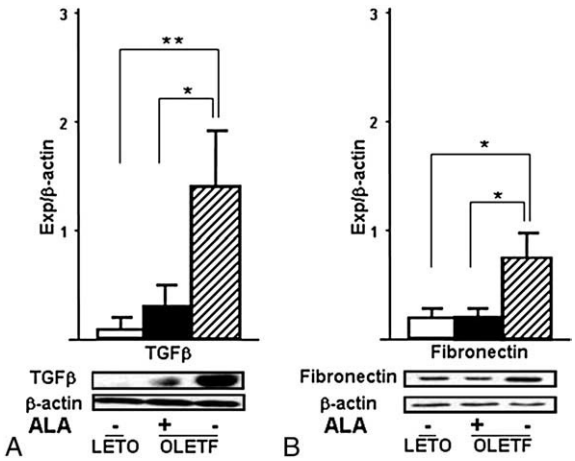

Fig. 2. Effects of ALA on the expression of the renal cortical TGFβ1 and fibronectin proteins in OLETF rats at 35 weeks of age. The levels of renal cortical TGFβ1 (A) and fibronectin (B) proteins were significantly increased in the untreated OLETF group compared with the LETO group; and the increments were significantly attenuated in the ALA group. Data are presented as mean ± SEM. *$P < .05$, **$P < .01$.

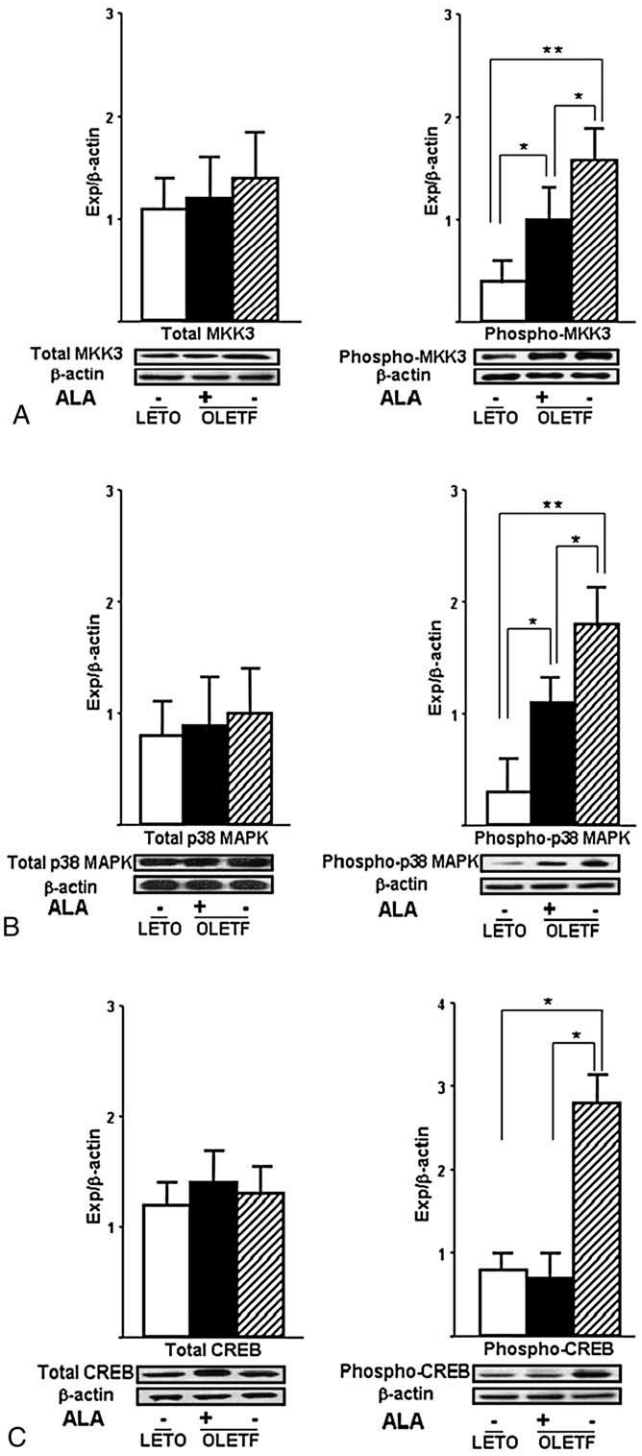

Fig. 3. Effects of ALA on the expressions of the renal cortical MKK3, p38 MAPK, and CREB proteins in OLETF rats at 35 weeks of age. In the untreated OLETF group compared with the LETO group, the expressions of the total MKK3 (A), p38 MAPK (B), and CREB (C) proteins were activated; but the differences were not statistically significant. In contrast, the expressions of the phospho-MKK3 (A), phospho–p38 MAPK (B), and phospho-CREB (C) proteins were significantly activated. After ALA treatment in OLETF rats, the phospho-form levels but not total-form levels of each protein were significantly reduced. Data are presented as mean ± SEM. *$P < .05$, **$P < .01$.

increments were significantly attenuated in the ALA group by 84.6% ($P < .05$) (Fig. 2). The renal cortical TGFβ1 protein levels were positively correlated with the urinary protein excretion in the ALA ($r = 0.681$, $P < .05$) and untreated OLETF ($r = 0.752$, $P < .05$) groups.

The renal cortical fibronectin protein levels were also significantly increased by 3.5-fold in the untreated OLETF group compared with the LETO group at 35 weeks of age ($P < .05$), and the increments were significantly attenuated by 100% in the ALA group at 35 weeks of age ($P < .05$). There were significant positive correlations between the renal cortical fibronectin protein levels and the urinary protein excretion in the ALA ($r = 0.418$, $P < .05$) and untreated OLETF ($r = 0.597$, $P < .05$) groups.

3.4. Effects of ALA on the expressions of the renal cortical MKK3–p38 MAPK–CREB proteins in OLETF rats

In the untreated OLETF group compared with the LETO group, the expression of the total MKK3 protein was activated by 1.3-fold, which was not statistically significant; and the expression of the phospho-MKK3 protein was significantly activated by 4.3-fold ($P < .01$) (Fig. 3A). After ALA treatment in OLETF rats, the phospho-MKK3 but not total MKK3 protein levels were significantly decreased by 50.0% ($P < .05$). Similarly, compared with the LETO group, the total and phospho–p38 MAPK protein levels were increased in the untreated OLETF group by 1.3-fold (P = NS) and 6.0-fold ($P < .01$), respectively (Fig. 3B). In the ALA group compared with the untreated OLETF group, the phospho–p38 MAPK but not total p38 MAPK protein levels were significantly reduced by 46.7% ($P < .05$). The total and phospho-CREB protein levels were higher in the untreated OLETF group than the LETO group by 1.1-fold (P = NS)

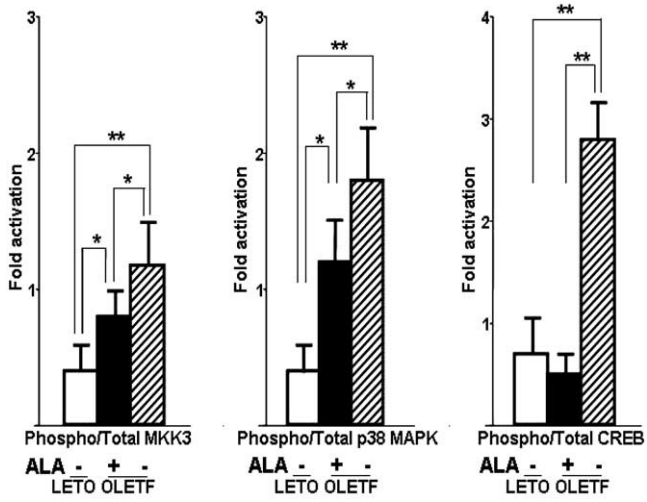

Fig. 4. The fold activations, the ratios of phospho to total forms, of the MKK3, p38 MAPK, and CREB protein levels in the untreated OLETF group compared with the LETO group were 1.2, 1.8, and 2.8, respectively; and these were significantly decreased in the ALA group. Data are presented as mean ± SEM. *$P < .05$, **$P < .01$.

and 3.5-fold ($P < .05$), respectively (Fig. 3C). After ALA treatment in the OLETF rats, the increments of the phospho-CREB protein levels were significantly attenuated by 100% ($P < .05$). The fold activations, the ratios of phospho to total forms, of the MKK3, p38 MAPK, and CREB protein levels in the untreated OLETF group compared with the LETO group were 1.2, 1.8, and 2.8; and these were significantly decreased in the ALA group by 50.0% ($P < .05$), 42.9% ($P < .05$), and 100% ($P < .01$), respectively (Fig. 4).

4. Discussion

The food intake and body weight of OLETF rats begin to increase from 24 weeks of age, and diabetes mellitus eventually develops [10,13]. Recently, Kim et al [33] have reported that ALA exerted antiobesity effect by reducing food intake and enhancing energy expenditure with suppression of hypothalamic cyclic adenosine monophosphate–activated protein kinase activity in OLETF rats. Our results that ALA decreased the food intake and body weight in OLETF rats are in good agreement with a previous study [33]; but it is not clear, at least in our study, whether gain of body weight is inhibited by reduction of food intake or other mechanisms. In the present study, after ALA treatment in OLETF rats, the plasma AST and ALT levels were decreased, although the changes were not significant. It means that ALA at our dose does not have any toxic effect as assessed by the plasma AST and ALT levels and that the effects of ALA on the food intake and body weight are not related to toxicity of ALA.

Otsuka Long-Evans Tokushima Fatty rats display insulin resistance, hyperinsulinemia, impaired pancreatic β-cell function, and glucose intolerance from 24 weeks of age [13,40]. In OLETF rats, the plasma cholesterol levels at 30 weeks of age and the plasma TG levels at 6 to approximately 8 weeks of age are 1.5 and 5 times higher than those of age-matched LETO rats, respectively [40]. Previous studies have shown that ALA improved insulin sensitivity and hyperglycemia by its antioxidative ability [31,32]. In this study, after ALA treatment in OLETF rats, the fasting plasma glucose and HbA_{1c} levels decreased compared with those in the untreated OLETF group at 35 weeks of age and those in the ALA group at 30 weeks of age; but the improvements were not significant, in contrast with previous reports [31,32]. At 35 weeks of age, the plasma insulin and TG but not FFA and cholesterol levels were significantly lower in the ALA group than the untreated OLETF groups. In the present study, the plasma MDA, an oxidative stress marker, levels of OLETF rats at 30 weeks of age were significantly higher than those of LETO rats, suggesting increased oxidative stress in hyperglycemic condition. As in a previous study [38], the plasma MDA levels were significantly reduced in the ALA group at 35 weeks of age compared with the untreated OLETF group at 35 weeks of age as well as the ALA group at 30 weeks of age. These results demonstrate that ALA may have favorable effects on the plasma glucose and lipid parameters related to decreasing oxidative stress in OLETF rats. Whether the plasma MDA levels were reduced directly by ALA or indirectly by decreasing food intake, blood glucose, and lipid levels or both is not clear. Therefore, the mechanism of reduction of MDA by ALA should be further clarified.

The urinary protein excretion in OLETF rats is increased from 22 weeks of age and reaches a range of overt proteinuria from 30 weeks of age [10]. Melhem et al [34,35] have described that ALA prevented elevation of urinary albumin excretion, fraction clearance of albumin, glomerular volume, glomerular content of TGFβ, and glomerular mesangial matrix expansion in diabetes mellitus. The data reported by Morcos et al [36] have shown that ALA inhibited progression of albuminuria in diabetic patients. In our study, the urinary protein excretion was significantly decreased in the ALA group at 35 weeks of age compared with the untreated OLETF group at 35 weeks of age and the ALA group at 30 weeks of age. As the kidney weight at 35 weeks of age was measured, the kidney weight corrected by body weight was higher in OLETF rats than LETO rats and was lower in the ALA group than the untreated OLETF group, although the kidney weights before and after ALA treatment in OLETF rats were not different. Taken together, our data indicate that ALA may attenuate the increments of the urinary protein excretion and kidney weight at a less extent, suggesting a significant role of ALA as a renoprotective agent in diabetic nephropathy.

Recently, the evidence of relationship between TGFβ1 and glomerulosclerosis has markedly increased; and the pathogenic importance of TGFβ1 in various glomerular diseases including glomerulosclerosis is supported by many clinical and experimental reports [5-7]. Several authors have described that elevated TGFβ1 production was induced by exposure to high glucose and was completely or partially reversed by neutralizing anti-TGFβ1 antibody in diabetic kidney [41,42]. It has been revealed that expression of TGFβ1 was increased in renal cortex of OLETF rats at 30 weeks of age and that ALA attenuated the increased expression of glomerular TGFβ1, resulting in improvement of glomerular injury in cultured mesangial cells and streptozocin-induced diabetic rats [10,37,43]. In this study, at 35 weeks of age, the renal cortical TGFβ1 protein levels were significantly increased in the untreated OLETF group; and the increments were significantly attenuated in the ALA group. The renal cortical TGFβ1 protein levels were positively correlated with the urinary protein excretion in the ALA and untreated OLETF groups. These data suggest that the renal cortical TGFβ1 protein levels may be related to a degree of proteinuria and that ALA may decrease proteinuria by reducing expression of the renal cortical TGFβ1 protein in OLETF rats.

Fibronectin accumulates in glomeruli of OLETF rats from 30 weeks of age [44]. McKay et al [45] have reported that TGFβ1 caused extracellular matrix accumulation by enhancing production of fibronectin in mesangial cell. In addition, the results described by Isono et al [27] have shown that

hyperglycemia activated intrarenal TGFβ1-Smad signaling pathway, resulting in promotion of mesangial fibronectin expression in streptozocin-induced diabetic mice. In the present study, the renal cortical fibronectin protein levels were significantly increased in OLETF rats compared with LETO rats at 35 weeks of age and were fully attenuated after ALA treatment, similar to the results of a previous study [38]. There were significant positive correlations between the renal cortical fibronectin protein levels and the urinary protein excretion in the ALA and untreated OLETF groups. Our results demonstrate association of the renal cortical fibronectin as well as TGFβ1 proteins with proteinuria, and the favorable effects of ALA on proteinuria probably by reducing expression of the renal cortical TGFβ1 and fibronectin proteins in OLETF rats.

Mitogen-activated protein kinase cascades linked to reactions of p38 MAPK have been shown to modulate signal transduction of biological actions such as regulation of cellular growth, differentiation, and apoptosis either alone or in concert with other pathways [15,24]. Previous studies have revealed that p38 MAPK was activated in aortic smooth muscle cells and mesangial cells exposed to high glucose and that phosphorylated p38 MAPK was increased in rat glomeruli, suggesting involvement of p38 MAPK in diabetic vascular complications including diabetic nephropathy [46,47]. In regard to relationship between TGFβ1 and p38 MAPK, it has been shown that TGFβ1 activated p38 MAPK in human mesangial cells, smooth muscle cells, fibroblasts, neutrophils, and rat renal tubular cells [6,24-26]. Despite the report that ALA decreased phosphorylated p38 MAPK but not total p38 MAPK in dystrophin-deficient mice [39], the effects of ALA on p38 MAPK pathway in diabetic nephropathy have not been elucidated yet; and therefore, we investigated the activation of p38 MAPK pathway and the effects of ALA on p38 MAPK pathway in kidney of OLETF rats. In our study with the untreated OLETF group compared with the LETO group at 35 weeks of age, expressions of the total MKK3, p38 MAPK, and CREB proteins were activated; but the differences were not significant. In contrast, expressions of the phospho-MKK3, phospho–p38 MAPK, and phospho-CREB proteins were significantly activated. After ALA treatment in OLETF rats, the phospho-form levels but not total-form levels of each protein were significantly reduced. The fold activations of the MKK3, p38 MAPK, and CREB protein levels in the untreated OLETF group compared with the LETO group were decreased in the ALA group. These results first display that phosphorylating activation rather than increased biosynthesis of components of p38 MAPK pathway may be a predominant phenomenon and that ALA may attenuate activation of p38 MAPK pathway as well as expression of TGFβ1 in renal cortex of OLETF rats. The inhibitory mechanism of ALA on phosphorylation of p38 MAPK pathway is unknown. Moreover, it is not clear whether effects of ALA on activation of p38 MAPK pathway is caused by direct inhibition of phosphorylation of p38 MAPK pathway or indirect suppression of expression of TGFβ1. Therefore, further study is required for elucidation of the inhibitory effects of ALA on p38 MAPK pathway.

In conclusion, these results confirm that ALA decreases the food intake and body weight, and improves the plasma glucose and lipid parameters related to reducing oxidative stress in OLETF rats. Furthermore, our results suggest that ALA ameliorates proteinuria by decreasing expressions of the TGFβ1 and fibronectin proteins; and these favorable effects are related to inhibition of phosphorylating activation of p38 MAPK pathway in renal cortex of OLETF rats. This study would provide new insights into the therapeutic roles of ALA related to modulation of p38 MAPK pathway in patients with diabetic nephropathy.

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