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Effects of eicosapentaenoic acid on the early stage of type 2 diabetic nephropathy in KKA^y/Ta mice: involvement of anti-inflammation and antioxidative stress

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Abstract

Eicosapentaenoic acid (EPA) has been reported to have beneficial effects on the progression of various renal diseases including diabetic nephropathy; however, the precise mechanisms are not completely understood. We examined the effects of EPA on the early stage of type 2 diabetic nephropathy in KKA^y/Ta mice and the possible role of inflammation, oxidative stress, and growth factor in this process. KKA^y/Ta mice were divided into 2 groups. The treatment group was injected with EPA ethyl ester at 1 g/kg per day intraperitoneally from 12 to 20 weeks of age and the control group was injected with saline. Renal morphologic examinations were performed after 8 weeks of treatment. Glomerular macrophage infiltration and expression of monocyte chemoattractant protein 1, malondialdehyde (MDA), nitrotyrosine, transforming growth factor β1 (TGF-β1), and type I collagen were evaluated. Eicosapentaenoic acid decreased the levels of urinary albumin, serum triglyceride and MDA, and improved glucose intolerance in KKA^y/Ta mice. Morphometric analysis showed that accumulation of extracellular matrix and the tubulointerstitial fibrosis area were significantly decreased after treatment. Immunohistochemistry revealed that glomerular macrophage infiltration and the expression of MDA and nitrotyrosine in KKA^y/Ta mice were increased and were inhibited by EPA treatment. Protein and gene expression levels of monocyte chemoattractant protein 1, TGF-β1, and type I collagen, which were evaluated by immunohistochemistry and real-time reverse transcriptase–polymerase chain reaction, were down-regulated in the EPA treatment group. In conclusion, EPA improves type 2 diabetic nephropathy in KKA^y/Ta mice. This beneficial effect might be mediated by attenuation of metabolic abnormalities and inhibition of renal inflammation, oxidative stress, and TGF-β expression.

1. Introduction

Diabetic nephropathy is the leading cause of end-stage renal disease and the most frequent cause of mortality in patients with diabetes [1]. The slow and continuous decline in renal function is characterized by progressive glomerulosclerosis, tubulointerstitial injury, and renal fibrosis [2]. It is essential to identify more methods of treatment that can arrest the disease progression. Several studies have shown that a diet rich in n-3 polyunsaturated fatty acids (PUFA), specifically eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) in fish oil, might influence the progression of renal disease. The effects of dietary PUFA supplementation on renal injury have been reported in the 5/6 renal ablation model [3] and experimental focal glomerulosclerosis model [4]. Moreover, Donadio et al [5] demonstrated that EPA retarded the progression in patients with immunoglobulin A nephropathy. It was also reported that EPA showed beneficial effects in non–insulin-dependent diabetic patients with nephropathy by reducing albuminuria [6].

The mechanism through which PUFA exert their protective effects is still unclear. It appears that the basis is related to their actions on renal inflammation and fibrosis [7]. Many findings from animal and human studies show that PUFA have an anti-inflammatory effect. Polyunsaturated fatty acids

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may have direct effects on cellular production of major cytokine inflammation mediators, on endothelial dysfunction, and on leukocyte chemotaxis. Thus, PUFA treatment has been proposed for various inflammatory diseases such as rheumatoid arthritis and Crohn disease [8]. Diabetic nephropathy is frequently associated with an inflammatory status. Dalla Vestra et al [9] demonstrated that acute-phase markers of inflammation are associated with the severity of renal pathologic changes in diabetic patients with nephropathy. Monocyte chemoattractant protein 1 (MCP-1), which is the strongest known chemokine, may play an important role in the inflammation of renal diseases through recruiting and activating monocytes/macrophages from the circulation to inflammatory sites [10]. The inflammatory status, if not reversed, could accelerate the production of several cellular mediators such as transforming growth factor β (TGF-β) and the subsequent cell activation/proliferation and extracellular matrix (ECM) accumulation [11]. Therefore, the effect of PUFA on the expression of MCP-1 and TGF-β in diabetic nephropathy is worthy to be investigated.

Oxidative stress is another important factor involved in the progression of diabetic nephropathy. However, because PUFA are susceptible to be auto-oxidized and form the lipid peroxidation, there remains a theoretical concern with respect to the potential for increasing the oxidative stress after treatment [12]. However, Mori et al [13] reported that both EPA and DHA reduce oxidative stress in treated hypertensive type 2 diabetic subjects. To date, the data in vivo are inconclusive.

The KKA^y/Ta mice produced by transfection of the yellow obese gene (Ay) into KK/Ta mice are obese diabetic mice showing hyperglycemia, hypertriglyceridemia, hyperinsulinemia, and microalbuminuria. The pathologic changes of KKA^y/Ta mice are consistent with those in the early stage of human diabetic nephropathy, which include glomerular basement membrane thickening and expansion of mesangial matrix. The urinary albumin-creatinine ratio (ACR) in diabetic KKA^y/Ta mice is 250 to 350 mg/g creatinine (Cr) at 8 weeks of age and increases to 550 to 600 mg/g Cr at 16 weeks of age. Therefore, KKA^y/Ta mice are considered as a suitable model for the early stage of type 2 diabetic nephropathy [14].

Based on these findings, we aimed to assess whether EPA treatment could suppress the MCP-1, TGF-β, type I collagen expression, and glomerular macrophage infiltration in diabetic KKA^y/Ta mice. At the same time, the effects of EPA on oxidative stress, measured as serum MDA level and renal MDA and nitrotyrosine expression, were also examined.

2. Materials and methods

2.1. Animals and experimental design

Male KKA^y/Ta mice (7 weeks of age) were purchased from CLEA Japan (Tokyo, Japan). The mice were individually housed in plastic cages with free access to food (rodent pellet diet NMF; 1456 kJ [348 kcal]/100 g, containing 5.5% crude fat) and water throughout the experimental period. All mice were maintained in the same room under conventional conditions with a regular 12-hour light/dark cycle and temperature controlled at 24°C ± 1°C. KKA^y/Ta mice were randomly divided into 2 groups of 8 mice each. Administration of EPA was started at 12 weeks of age, which is considered as the early stage of diabetic nephropathy. The first group (treatment group) was injected with EPA ethyl ester at 1 g/kg per day intraperitoneally for 8 weeks. The second group (control group) was injected with saline [15]. Purified EPA ethyl ester was kindly provided by Mochida Pharmaceutical (Tokyo Japan). It was stored at −20°C inside the capsules (containing EPA943 mg and α-tocopherol 2 mg per capsule) and was freshly prepared before injection. KKA^y/Ta mice were killed at 20 weeks of age. Kidneys were removed for light microscopy, immunohistochemical examination, and RNA extraction.

2.2. Phenotypic characterizations

The body weight, blood pressure, fasting blood glucose levels, and ACR were measured at 12, 16, and 20 weeks of age. Serum triglyceride, total cholesterol, glucose tolerance, immunoreactive insulin (IRI), and MDA levels were measured at 20 weeks of age.

Blood pressure was measured at 11:00 AM by a noninvasive tail cuff and pulse transducer system (Softron BP-98A, Tokyo, Japan) after the mice were externally prewarmed for 10 minutes at 38°C. At least 3 to 6 recordings were taken for each measurement. Standard deviations of less than 5.0 were defined for the blood pressure levels. Urinary albumin and creatinine from samples collected for 24 hours using metabolic cages (mouse metabolic cage, CLEA Japan) were measured by immunoassay (DCA 2000 system, Bayer Diagnostics, Elkhart, IN). Serum total cholesterol and triglyceride were determined enzymatically by an autoanalyzer (Fuji Dry-Chem 5500, Fujifilm, Tokyo, Japan). Glucose tolerance was estimated by the intraperitoneal glucose tolerance test (IPGTT). It was performed by injection of glucose (2 g/kg in 20% solution) in overnight-fasted mice. Blood was obtained from the retro-orbital sinus at 0 (fasting blood glucose level) and 120 minutes after intraperitoneal glucose injection for measurement of the blood glucose and IRI levels. Glucose levels were measured using Glucocard (Kyoto Daiichi Kagaku, Kyoto, Japan). Immunoreactive insulin levels were measured by enzyme-linked immunosorbent assay (insulin enzyme-linked immunosorbent assay kit, Morinaga & Co, Tokyo, Japan).

Serum MDA was determined colorimetrically by a commercial kit (lipid peroxidation assay kit, Calbiochem, San Diego, CA) according to the manufacturer's instructions. Briefly, 0.65 mL of reagent 1 (7.7 mmol/L *N*-methyl-2-phenylindole in 75% acetonitrile and 25% methanol) was added to 0.2 mL of serum. After vortexing for 3 to 4 seconds and adding 0.15 mL of 12 N hydrochloric acid, samples were mixed and closed with a tight stopper and incubated at

45°C for 60 minutes. The samples were then cooled on ice and the absorbance was measured spectrophotometrically at 586 nm. A calibration curve of an accurately prepared standard MDA solution (from 0 to 20 μmol/L) was run at the same time for quantification.

2.3. Light microscopy and immunohistochemical staining

For light microscopy, 3-μm sections were prepared and stained with periodic acid–Schiff (PAS) and Azan reagent after paraffin embedding.

Immunohistochemical studies were performed with the following antibodies: anti–MCP-1 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-F4/80 (a 160-kd glycoprotein expressed by murine macrophages) rat monoclonal antibody (Serotec, Oxford, UK), anti–TGF-β1 rabbit polyclonal antibody (Santa Cruz Biotechnology), anti–type I collagen goat polyclonal antibody (Southern Biotechnology Associates, Birmingham, AL), anti-MDA rabbit polyclonal antibody (Alpha Diagnostic International, San Antonio, TX), and anti-nitrotyrosine rabbit polyclonal antibody (Upstate Biotechnologies, Lake Placid, NY). Frozen cryostat sections of 3 μm thick were fixed in acetone for 10 minutes and air-dried. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide/methanol for 15 minutes. The sections were then blocked by blocking solution (2% fetal bovine serum, 0.2% fish gelatin, and 10% normal serum in phosphate-buffered saline). After incubation with primary antibodies at 4°C overnight, the sections were incubated with antirabbit Envision+ polymer reagents (DAKO, Carpinteria, CA), antigoat polymer reagents (Histofine, Tokyo, Japan), or antirat polymer reagents (Histofine) at room temperature for 90 minutes. Bound antibody was visualized by light microscopy with diaminobenzidine. The omission of primary antibodies served as negative controls for each antibody in this study. Slides were mounted with anti-fade mounting media and examined on an OLYMPUS BX41 (Olympus, Tokyo, Japan) microscope.

2.4. Morphometric analysis of histopathologic damage and immunohistochemistry

Thirty glomeruli were selected randomly from the control and EPA treatment groups to evaluate the extent of glomerular sclerosis. Periodic acid–Schiff–positive materials in the glomerular mesangial areas were determined as the extent of ECM. The extent of tubulointerstitial fibrosis in Azan-stained sections of the renal cortex was estimated by the blue-stained area in the interstitium of 10 randomly selected fields (×200). The whole glomerular area (WGA), ECM area (ECMA), and tubulointerstitial fibrosis area were measured automatically using a KS version 3.0 image analysis system (KS400, Carl Zeiss Vision, Hallbergmoos, Germany) [15]. Twenty glomeruli were selected randomly in each group to quantify the expression of MCP-1, MDA, nitrotyrosine, TGF-β1, and type I collagen. The protein production was measured by a ratio of immunohistochemical positive staining area to WGA using the KS400 version 3.0 image analysis system [15]. Values for WGA from the 2 study groups were compared statistically for any differences that might influence the calculated results. The number of F4/80-positive cells in the glomerulus was counted under a high-power microscope (×400) within 20 glomerular cross sections from an individual mouse. The number of cells in each glomerulus was averaged.

2.5. Real-time reverse transcriptase–polymerase chain reaction

Total RNA was extracted from the renal cortex with Trizol (Life Technologies, Carlsbad, CA) and the purity was checked by spectrophotometry. The samples were treated with DNase in all experiments to avoid contamination of genomic DNA. Two micrograms of RNA isolated was reverse-transcribed using random decamer primer (Ambion, Austin, TX) and M-MLV Reverse Transcriptase (Life Technologies). Quantitative polymerase chain reaction (PCR) was performed using the SYBR Green PCR Master Mix (Perkin-Elmer Applied Biosystems, Foster City, CA) and analyzed on an ABI PRISM 7500 Sequence Detector System (Perkin-Elmer Applied Biosystems). The forward and reverse primers used for each molecule were as follows: 5′-AACTTTGGCATTGTGGAAGG-3′ and 5′-GGATGCAGGGATGATGTTCT-3′ for mouse GAPDH, 5′-GCCCCACTCACCTGCTGCTACT-3′ and 5′-CCTGCTGCTGGTGATCCTCTTGT-3′ for mouse MCP-1, 5′-GCAACATGTGGAACTCTACCAGA-3′ and 5′-GACGTCAAAA GACAGCCACTCA-3′ for mouse

Table 1
Phenotypic values of KKA^{y}/Ta mice treated with EPA

	Age (wk)	EPA treatment (n = 8)	Controls (n = 8)	*P*
Body weight (g)	12	44.9 ± 0.7	43.3 ± 0.4	NS
	16	45.7 ± 0.7	45.8 ± 0.8	NS
	20	47.9 ± 1.4	48.1 ± 0.6	NS
Systolic blood pressure (mm Hg)	12	106.4 ± 3.0	113.6 ± 3.4	NS
	16	110.7 ± 2.4	116.3 ± 5.0	NS
	20	113.1 ± 2.1	116.1 ± 3.9	NS
Fasting blood glucose levels (mg/dL)	12	112.1 ± 7.7	98.9 ± 5.3	NS
	16	93.8 ± 5.0	84.6 ± 5.9	NS
	20	93.6 ± 5.0	96.0 ± 7.7	NS
ACR (mg/g Cr)	12	321.9 ± 51.2	386.0 ± 36.9	NS
	16	161.3 ± 34.4	587.4 ± 31.6	.0002
	20	188.3 ± 34.6	610.4 ± 79.8	.0019
Serum triglyceride (mg/dL)	20	114.1 ± 8.4	203.4 ± 21.4	.0006
Serum total cholesterol (mg/dL)	20	126.0 ± 8.3	141.9 ± 6.9	NS
Blood glucose levels (mg/dL) after IPGTT	20	196.1 ± 52.6	386.0 ± 79.8	.0451
Serum IRI levels (ng/mL) after IPGTT	20	3.2 ± 1.0	15.2 ± 1.7	.0003
Serum MDA levels (μmol/L)	20	8.8 ± 1.0	13.4 ± 0.5	.0198

Data are expressed as means ± SEM. Glucose levels were measured at 0 and 120 minutes after intraperitoneal glucose injection. Immunoreactive insulin levels were measured at 120 minutes after glucose administration. NS indicates not significant.

TGF-β1, and 5′-CCCCGGGACTCCTGGACTT-3′ and 5′-GCTCCGACACGCCCTCTCTC-3′ for mouse type I collagen. The thermal cycling comprised a denaturation step at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 40 cycles at 60°C for 1 minute. Amplification of specific transcripts was confirmed by melting curve profiles (cooling the sample to 60°C and heating slowly to 95°C with measurement of fluorescence) at the end of each PCR. Each data point was repeated 4 times. The relative messenger RNA (mRNA) level in the sample was normalized to GAPDH content. The data presented are expressed as the fold decrease in mRNA.

2.6. Statistical analysis

All results are expressed as mean ± SEM. Mann-Whitney *U* test was used in the comparison of phenotypic values between the EPA treatment group and the control group. Student *t* test was used in the other studies. $P < .05$ was considered as statistically significant.

3. Results

3.1. Phenotypic characterizations

The phenotypic characterizations of KKA^y/Ta mice treated with EPA ethyl ester or saline at different ages are shown in Table 1. There was no significant difference in the levels of body weight, systolic blood pressure, and fasting blood glucose between the EPA treatment group and the control group at all ages. For reference, fasting blood glucose levels in nondiabetic BALB/cA mice (CLEA Japan, n = 8), which were housed in the same conditions as KKA^y/Ta mice without any treatment, were 68.5 ± 3.0, 69.7 ± 3.6, and 76.0 ± 0.8 mg/dL at 12, 16, and 20 weeks of age, respectively, in our laboratory. The mean level of ACR at 16 and 20 weeks of age in the EPA group was significantly lower than that in the control group ($P = .0002$ and $P = .0019$ respectively). There was a significant change in the levels of serum triglyceride ($P = .0006$), but not in the levels of total cholesterol between EPA treatment and control groups, at 20 weeks of age. Impaired glucose tolerance evaluated by IPGTT in the EPA treatment group was significantly improved compared with that in the control group at 20 weeks of age ($P = .0451$). The serum IRI levels after glucose administration in the EPA treatment group were significantly decreased compared with those in the control group ($P = .0003$). The mean level of serum MDA in the EPA treatment group was significantly lower than that in the control group at 20 weeks of age ($P = .0198$).

3.2. Light microscopy

In light microscopy, diffuse mesangial matrix expansion was observed in control mice. Furthermore, segmental sclerosis was present in some glomeruli of the control

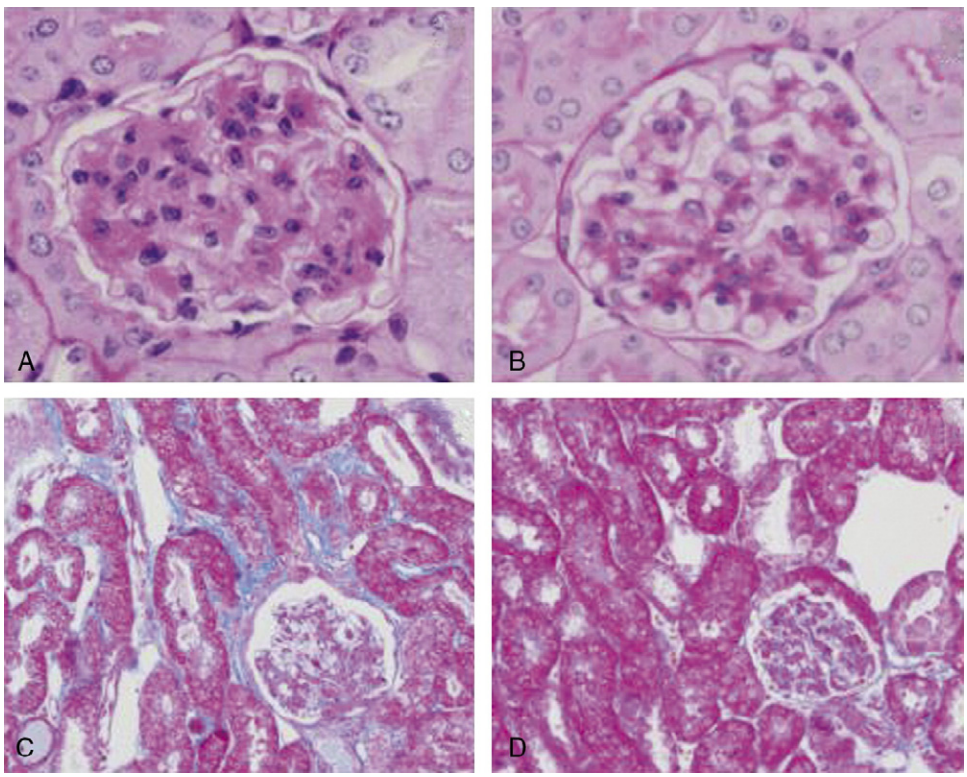

Fig. 1. Representative histopathologic changes of light microscopy in KKA^y/Ta mice at 20 weeks of age. A, Diffuse mesangial matrix expansion and segmental sclerosis in the glomeruli were observed in the control group (PAS, original magnification ×400). B, Matrix expansion and segmental sclerosis were decreased by EPA treatment (PAS, original magnification ×400). C, Interstitial fibrosis was observed in control mice (Azan, original magnification ×200). D, Interstitial fibrosis was significantly decreased in EPA-treated mice (Azan, original magnification ×200).

Table 2
Morphometric analysis of renal pathologic changes in KKA^y/Ta mice treated with EPA

	EPA treatment (n = 8)	Controls (n = 8)	*P*
ECMA/WGA (%)	27.0 ± 1.8	48.1 ± 2.6	<.0001
Tubulointerstitial fibrosis area (%)	2.3 ± 0.4	6.6 ± 0.9	.0006
MCP-1–stained area/WGA (%)	35.3 ± 5.2	58.8 ± 4.6	.0017
F4/80-positive cells per glomerulus	0.20 ± 0.09	0.85 ± 0.18	.0028
MDA-stained area/WGA (%)	37.2 ± 3.5	55.7 ± 4.4	.0022
Nitrotyrosine-stained area/WGA (%)	28.3 ± 2.6	49.4 ± 5.1	.0007
TGF-β1–stained area/WGA (%)	8.4 ± 1.1	20.4 ± 3.4	.0019
Type I collagen-stained area/WGA (%)	27.2 ± 2.4	44.5 ± 5.2	.0043

Data are expressed as means ± SEM. Whole glomerular area values between the 2 groups showed no significant statistical differences for all measured markers (data not shown).

group at 20 weeks of age (Fig. 1A), whereas matrix expansion and segmental sclerosis decreased in EPA-treated mice (Fig. 1B). The extent of interstitial fibrosis was more prominent in the control group than in the EPA-treated mice (Fig. 1C and D). In morphometric analysis, the mean ECMA/WGA ratio in the EPA treatment group was significantly lower than that in the control group (P < .0001, Table 2). The tubulointerstitial fibrosis area was also significantly decreased in the EPA-treated group compared with the control group (P = .0006, Table 2).

3.3. Effect of EPA on renal MCP-1 expression and macrophage infiltration

Increased immunostaining of MCP-1 was observed in the glomeruli of control mice at 20 weeks of age and expressed within both the capillary and mesangial areas, whereas staining of MCP-1 in the glomeruli of EPA-treated mice was much less intense (Fig. 2A and B). The mean MCP-1–stained area/WGA ratio evaluated by morphometric analysis was significantly decreased in the EPA treatment group compared with that in the control group (P = .0017,

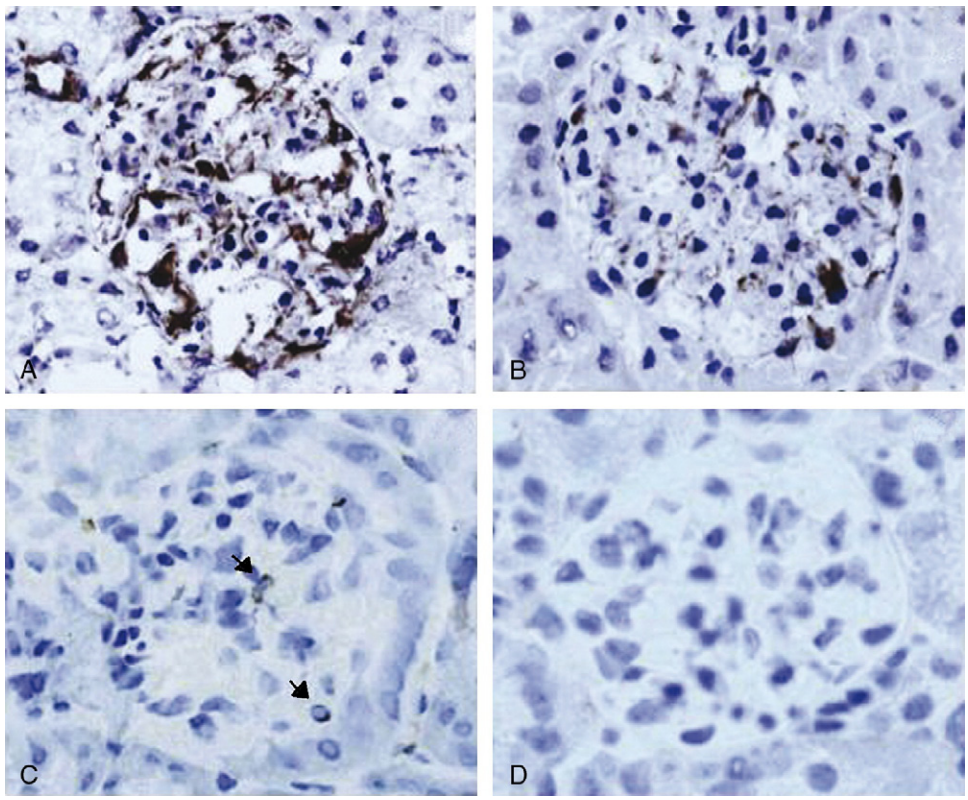

Fig. 2. Representative immunohistochemical staining of MCP-1 and F4/80 in KKA^y/Ta mice at 20 weeks of age. A, Monocyte chemoattractant protein 1 expression was found within the capillary and mesangial areas of the glomeruli in the control group. B, Monocyte chemoattractant protein 1 immunostaining was much less intense in the EPA treatment group. C, Expression of F4/80 was observed in the glomeruli (arrows) of the control group. D, F4/80 expression was significantly suppressed in the EPA treatment group (original magnification ×400).

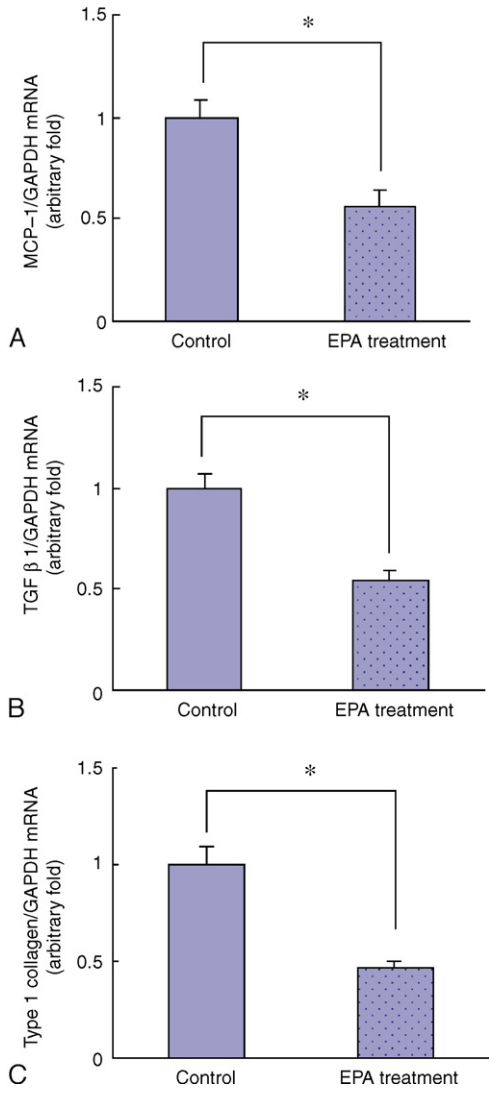

Fig. 3. Effect of EPA on MCP-1 (A), TGF-β1 (B), and type I collagen (C) mRNA expression estimated by the real-time RT-PCR. Monocyte chemoattractant protein 1, TGF-β1, and type I collagen mRNA expression was normalized to GAPDH mRNA content. Bar graph shows mean ± SEM from 8 mice in each group, expressed as fold-over control. *P < .05 vs control.

Table 2). The renal cortex expression of MCP-1 mRNA was estimated by quantitative real-time reverse transcriptase (RT)-PCR. The results showed that MCP-1 expression was down-regulated to 56% after EPA treatment (P = .0176, Fig. 3A).

Immunostaining of F4/80 was observed in the glomeruli of the control group, but it was significantly decreased in the EPA treatment group (Fig. 2C and D). The number of F4/80-positive cells per glomerular cross section in the treatment group was significantly decreased when compared with the control group (P = .0028, Table 2).

3.4. Effect of EPA on renal MDA and nitrotyrosine expression

Malondialdehyde expression was observed in the glomeruli of KKA^y/Ta mice at 20 weeks of age, especially within the mesangial area. This expression was significantly suppressed in the EPA treatment group (Fig. 4A and B). The mean MDA-stained area/WGA ratio in the EPA-treated mice was significantly lower than that in the control mice in morphometric analysis (P = .0022, Table 2).

Accumulation of nitrotyrosine was found in the glomeruli and expressed within both the capillary and mesangial areas of KKA^y/Ta mice. Eicosapentaenoic acid treatment significantly decreased the accumulation of nitrotyrosine (Fig. 4C and D). The glomerular nitrotyrosine-stained area/WGA ratio in the EPA treatment group was significantly lower than that in the control group (P = .0007, Table 2).

3.5. Effect of EPA on renal TGF-β1 and type I collagen expression

Transforming growth factor β1 protein expression was determined immunohistochemically within the mesangial area of glomeruli, as well as in the tubulointerstitium. Transforming growth factor β1 immunostaining was much stronger in the control group than in the EPA treatment group (Fig. 5A and B). Morphometric analysis showed that the mean TGF-β1–stained area/WGA ratio was significantly decreased after treatment (P = .0019, Table 2). Eicosapentaenoic acid inhibited the increase of TGF-β1 mRNA expression to 54% in KKA^y/Ta mice in real-time RT-PCR analysis (P = .0336, Fig. 3B).

A similar pattern was observed in the glomeruli and tubulointerstitium of KKA^y/Ta mice, with increased type I collagen expression in the control group and attenuated expression in the EPA treatment group (Fig. 5C and D). There was a significant difference in the levels of the mean type I collagen-stained area/WGA ratio between these 2 groups (P = .0043, Table 2). The renal cortex mRNA expression of type I collagen was suppressed to 46% in the treatment group compared with that in the control mice (P = .0072, Fig. 3C).

4. Discussion

In the present study, our data demonstrated that KKA^y/Ta mice, an animal model for the early stage of type 2 diabetic nephropathy, showed decreased levels of urinary ACR, serum triglyceride and MDA, and improved glucose intolerance after EPA treatment, whereas systemic blood pressure and fasting blood glucose levels remained unchanged. Eicosapentaenoic acid also attenuated mesangial matrix accumulation and tubulointerstitial fibrosis in KKA^y/Ta mice. Before we have shown that EPA ethyl ester injected at 1 g/kg per day intraperitoneally significantly increased EPA levels among serum phospholipids, confirming that EPA was adequately absorbed and 1 g/kg per day intraperitoneal injection was a suitable dosage. Measurement of EPA in serum phospholipids appears to be a useful biological indicator for EPA intake and nutritional status [15].

Decreasing serum triglyceride and glucose intolerance by EPA might contribute to the improvement of diabetic nephropathy. It is well established that n-3 PUFA can lower

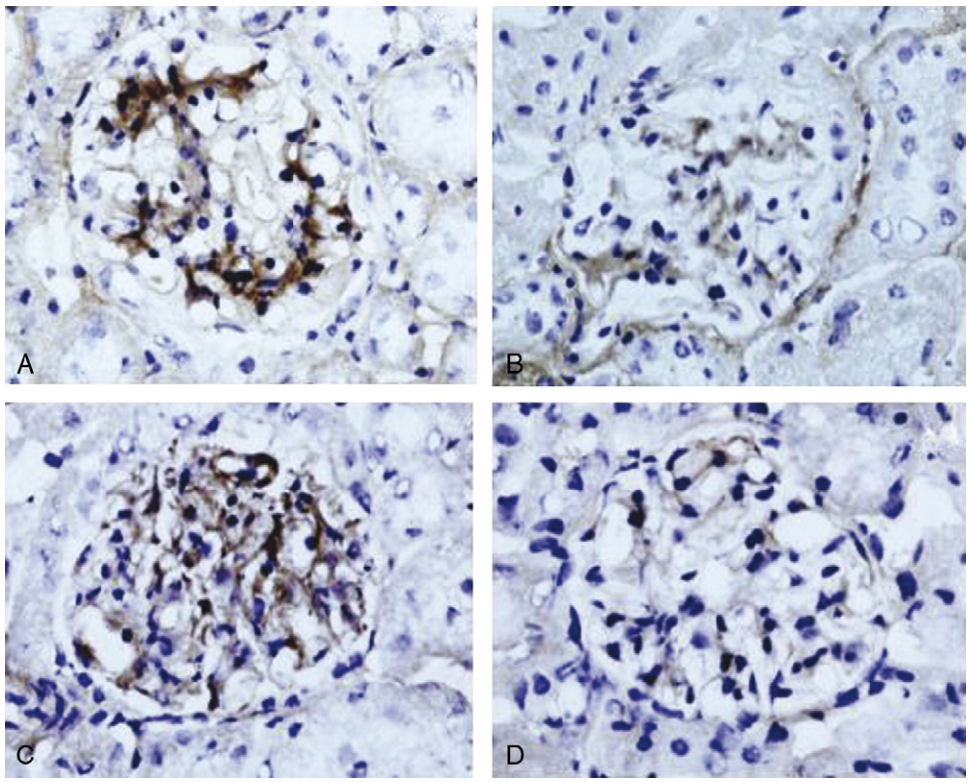

Fig. 4. Representative immunohistochemical staining of MDA and nitrotyrosine in KKA^y/Ta mice at 20 weeks of age. A, Expression of MDA was observed in the glomeruli and especially within the mesangial area in the control group. B, Malondialdehyde expression was significantly suppressed in the EPA treatment group. C, Accumulation of nitrotyrosine was found in the glomeruli and expressed within both the capillary and mesangial areas in the control group. D, Eicosapentaenoic acid treatment significantly decreased the accumulation of nitrotyrosine (original magnification ×400).

triglyceride levels in subjects with type 2 diabetes mellitus. Lipid-lowering treatment has been shown to attenuate renal fibrosis in obese Zucker rats [16]. Although why EPA treatment could improve glucose intolerance was still unknown, some studies suggested that it might be explained by its effect on insulin resistance. Both the amount and the type of fatty acids ingested alter insulin sensitivity in target tissues of rats (ie, muscle, adipose tissue, and liver) associated with glucose intolerance and obesity [17]. n-3 PUFA also have a protective effect against high-fat diet–induced insulin resistance in rodents [18]. On the other hand, n-3 PUFA are natural ligands of the peroxisome proliferator-activated receptor family. It was demonstrated that peroxisome proliferator-activated receptor γ agonists improved glucose intolerance and enhanced insulin sensitivity in patients with type 2 diabetes mellitus [19]. Therefore, EPA may show beneficial effects in KKA^y/Ta mice indirectly through attenuation of metabolic abnormalities.

However, Shimizu et al [6] reported that EPA administration improved urinary ACR in diabetic patients without affecting blood pressure levels, glucose control, and lipid metabolism. It was shown that dietary supplementation with n-3 PUFA retarded the disease progression in nondiabetic renal diseases such as immunoglobulin A nephropathy [5]. These facts indicate that EPA may also exert a direct effect on diabetic nephropathy, besides the effects on hypertriglyceridemia and glucose intolerance.

As reported previously, increased MCP-1 expression was observed in the kidneys of diabetic models and human biopsies [20]. Monocyte chemoattractant protein 1 induces monocyte immigration and differentiation to macrophages. Recent data suggest that MCP-1 is more than just a chemoattractant. Monocyte chemoattractant protein 1 can directly elicit an inflammatory response by inducing cytokines and adhesion molecule expression in the kidney [21]. Thus, diabetic nephropathy could be considered as an inflammatory process that is characterized by up-regulation of MCP-1 and infiltration of macrophages. In this study, EPA suppressed MCP-1 expression in the kidney of diabetic KKA^y/Ta mice and inhibited glomerular macrophage infiltration, indicating that EPA might possess anti-inflammatory properties in diabetic nephropathy. In vitro study, it was also demonstrated that high glucose level stimulated MCP-1 expression in mouse mesangial cells and administration of EPA could inhibit MCP-1 production in high glucose medium [15].

Our results showed that EPA treatment ameliorated the histopathologic damage of diabetic mice including both ECM accumulation and tubulointerstitial fibrosis. Transforming growth factor β is a well-known fibrogenic factor that has been reported to be up-regulated in the kidney of diabetic animals and patients in many studies [22]. We showed here that decreased TGF-β1 and type I collagen expression in KKA^y/Ta mice was found after EPA

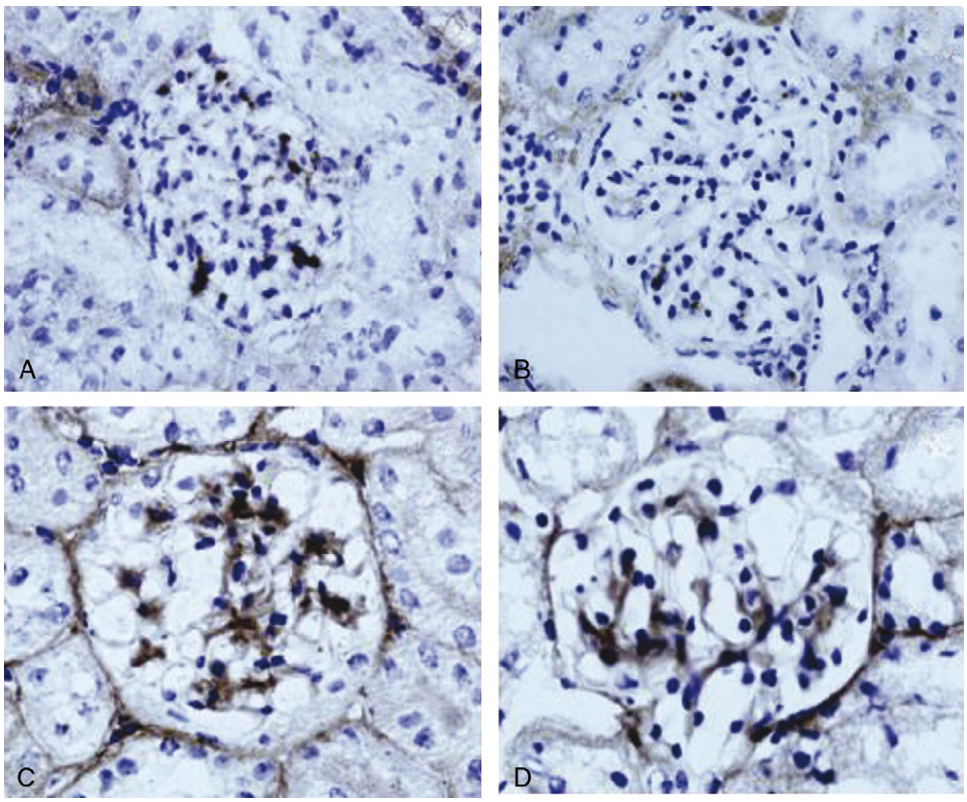

Fig. 5. Representative immunohistochemical staining of TGF-β1 and type I collagen in KKA^y/Ta mice at 20 weeks of age. A, Transforming growth factor β1 expression was localized within the mesangial area of the glomeruli in the control group. B, Transforming growth factor β1 immunostaining was decreased in the EPA treatment group. C, Increased expression of type I collagen was found within the mesangial area of the glomeruli in the control group. D, Immunostaining of type I collagen was decreased in the EPA treatment group (original magnification ×400).

treatment. These data confirmed that EPA inhibited renal fibrosis by controlling overexpression of TGF-β. Because TGF-β can be synthesized by activated macrophages, EPA treatment might decrease the TGF-β expression by inhibiting macrophage accumulation in the kidney [23]. Moreover, MCP-1 has been reported to have fibrogenic effects through stimulation of TGF-β in experimental glomerulonephritis; so increased expression of MCP-1 and TGF-β might be a common pathway involved in the development of diabetic nephropathy [24]. The inhibitory effects of EPA on both MCP-1 and TGF-β expression might play an additive role in preventing mesangial expansion and renal fibrosis.

Eicosapentaenoic acid might also have a direct role in suppression of TGF-β. It has been found that EPA may influence the diacylglycerol–protein kinase C–extracellular signal-regulated kinase pathway by replacement of arachidonic acid in plasma membrane phospholipids. An increase in de novo synthesis of diacylglycerol followed by activation of the protein kinase C–extracellular signal-regulated kinase pathway leads to enhanced TGF-β and ECM expression [25]. We previously demonstrated that EPA suppressed phosphorylation of ERK1/2 and p38 induced by platelet-derived growth factor under high glucose conditions in mouse mesangial cells and decreased p-ERK positive cells in glomeruli of KKA^y/Ta mice [15]. Therefore, EPA might improve the diabetic nephropathy by interference in cellular events involved in renal fibrosis.

Oxidative stress is known to play an important role in the progression of diabetic nephropathy. Reactive oxygen species mimic the stimulatory effects of high glucose level and up-regulate TGF-β and ECM expression [26]. The hypothesis that PUFA intake may lead to increased oxidative stress is not supported in our study. We demonstrated that in KKA^y/Ta mice, EPA decreased the serum MDA levels and attenuated the renal accumulation of MDA and nitrotyrosine. Our results are in accordance with the study reported by Mori et al [13], which showed that EPA could reduce in vivo oxidative stress rather than increasing the susceptibility. Another randomized controlled trial of type 2 diabetic patients also revealed significantly beneficial effects of low-dose n-3 PUFA on oxidative stress parameters [27]. Thus, we speculate that EPA might attenuate renal fibrosis through inhibition of oxidative stress.

The detailed mechanisms associated with decreased oxidative stress after EPA treatment remain unclear. The α-tocopherol fraction in the EPA capsules may be partly involved in the prevention of oxidative stress. Because the dosage of α-tocopherol in the capsule is extremely low compared with that of EPA, it seems unlikely that the small amount of α-tocopherol present significantly interferes with EPA. n-3 PUFA may assemble in membrane lipids and

lipoproteins, making the double bonds less available for free radical attack [28]. Eicosapentaenoic acid can inhibit the production of phospholipase A_2, which is an important prooxidant enzyme [29]. Diep et al [30] showed that DHA decreased the oxidative stress in the vascular wall of angiotensin II–infused rats by decreasing nicotinamide adenine dinucleotide phosphate oxidase activity. In addition, the degree of oxidative stress in vivo may depend on an imbalance between prooxidative and antioxidative status. It has been found that n-3 PUFA up-regulates the gene expression of antioxidative enzymes and down-regulates genes associated with production of reactive oxygen species [31]. However, further studies are required to explore potential mechanisms for the association of oxidative stress and n-3 PUFA in diabetic nephropathy.

In conclusion, our study showed that EPA improved type 2 diabetic nephropathy in KKA^y/Ta mice by decreasing hypertriglyceridemia, glucose intolerance, and albuminuria. Diabetes-induced up-regulation of MCP-1 and TGF-β expression was inhibited by EPA, along with the reduction of glomerular macrophage infiltration and oxidative stress. This study raises the possibility that EPA might be one of the therapeutic methods for diabetic patients with nephropathy, especially from the early stage, to slow and prevent disease progression.

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