Eicosapentaenoic acid ameliorates diabetic nephropathy of type 2 diabetic KKA\textsuperscript{y}/Ta mice: Involvement of MCP-1 suppression and decreased ERK1/2 and p38 phosphorylation

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Abstract

Background. Previous studies reported that eicosapentaenoic acid (EPA) was effective against any renal diseases including diabetic nephropathy. Monocyte chemoattractant protein-1 (MCP-1) is a regulating macrophage recruitment protein, which is up-regulated in patients with diabetic nephropathy. The objectives of the present study were to evaluate the effects of EPA including renal MCP-1 expression in diabetic KKA\textsuperscript{y}/Ta mice, MCP-1 production and signal transduction in mouse mesangial cells (MMCs).

Methods. KKA\textsuperscript{y}/Ta mice were injected with EPA ethyl ester (1 g/kg/day) intraperitoneally. Immunohistochemical staining of MCP-1, F4/80, phospho-extracellular signal-regulated kinase 1/2 (p-ERK1/2) and phospho-p38 in the renal sections were performed. EPA or specific inhibitors were incorporated in MMCs, and the levels of supernatant MCP-1 were measured. The effect of EPA on ERK1/2, c-jun NH\textsubscript{2}-terminal kinase (JNK), p38 or phosphoinositide 3-kinase (PI3K) activity in MMCs was examined using Western blot.

Results. EPA decreased the levels of serum triglycerides, leptin, urinary albumin and MCP-1, and improved glucose intolerance, mesangial matrix accumulation and tubulointerstitial fibrosis in KKA\textsuperscript{y}/Ta mice. Immunohistochemical staining of MCP-1 and F4/80 in the glomeruli and tubulointerstitial regions was decreased in the EPA-treated group. EPA and specific inhibitors of ERK1/2, JNK and PI3K decreased levels of MCP-1 in MMCs. EPA suppressed phosphorylation of ERK1/2 and p38 in MMCs, and decreased p-ERK positive cells in glomeruli of KKA\textsuperscript{y}/Ta mice.

Conclusions. EPA ameliorates diabetic nephropathy of type 2 diabetic KKA\textsuperscript{y}/Ta mice. We propose that the observed down-regulation of MCP-1 is critically involved in the beneficial effect of EPA, probably in concert with improvement of other clinical parameters.

Keywords: diabetic nephropathy; EPA; ERK1/2; KKA\textsuperscript{y}/Ta mice; MCP-1; PDGF

Introduction

Eicosapentaenoic acid (EPA) is one of the n-3 polyunsaturated fatty acids (PUFA) which are contained in fish oil. It was shown that EPA has many effects such as anti-thrombotic, hypolipidaemic, anti-atherogenic, anti-inflammatory and anti-mitogenic actions. Feeding of fish oil rich in n-3 PUFA reduces \textit{ex vivo} production of interleukin-1 (IL-1), IL-6, tumour necrosis factor (TNF) and IL-2 by peripheral blood mononuclear cells, and reduces the response to endotoxin and to pro-inflammatory cytokines, resulting in increased survival. Such diets were beneficial in some models of bacterial challenge, chronic inflammation and auto-immunity. Moreover, recent studies have shown that dietary supplementation with n-3 PUFA retards disease progression in human and experimental renal diseases. Donadio \textit{et al.} [1] reported that EPA retarded the progression of renal injury in patients with IgA nephropathy. Fish oil containing EPA inhibited mesangial cell activation and proliferation in the anti-Thy1.1 model of mesangial proliferative glomerulonephritis, reduced proteinuria and decreased histological glomerular injuries [2]. There is also a report that EPA improved albuminuria in type 2 diabetic patients [3].
However, the role of EPA in the progression of diabetic nephropathy is not fully understood. Monocyte chemoattractant protein-1 (MCP-1) is the strongest known chemokine, which has the function of recruiting and activating monocytes/macrophages from the circulation to inflammatory sites. Macrophages and macrophage products play an important pathogenic role in tubulointerstitial inflammatory and non-inflammatory conditions and have been implicated as effector cells of tubulointerstitial damage and mesangial matrix accumulation in diabetic nephropathy [4]. Studies using human biopsy and animal model materials showed the presence of macrophage accumulation and MCP-1 expression in diabetic glomeruli, suggesting that MCP-1 may play an important role in the development of diabetic glomerulosclerosis [5]. Recent data suggest that MCP-1 is more than just a chemoattractant. MCP-1 can directly elicit an inflammatory response by inducing cytokines and adhesion molecule expression in the kidney [6]. Platelet-derived growth factor (PDGF) was found to contribute to the pathophysiological process in the development and progression of glomerulosclerosis, characterized by mesangial cell proliferation and accumulation of extracellular matrix. Protein expression of the PDGF B-chain and PDGF beta-receptor was increased in the glomeruli of diabetic rats and inhibition of the PDGF system with trapidil, resulting in the prevention of glomerular hypertrophy, suggesting a significant role for the PDGF system in the development of early glomerular abnormalities in diabetes [7]. PDGF is reported to stimulate the gene expression of MCP-1 through extracellular signal-regulated kinase 1/2 (ERK1/2), c-jun NH2-terminal kinase (JNK) and p38 [8] in cultured human mesangial cells. The phosphoinositide 3-kinase (PI3K) signaling transduction pathway also appears to be involved in MCP-1 expression by PDGF stimulation [9]. Bousserouel et al. observed that IL1-β induced MCP-1 mRNA expression in rat smooth muscle cells was enhanced by arachidonic acid, although EPA reduced MCP-1 mRNA expression to the same level as in control cells [10]. However, little is known about the effects of EPA on the molecular mechanism underlying PDGF and high glucose (HG) induced MCP-1 expression in mesangial cells.

The KKA\(\alpha\)/Ta mice produced by transfection of the yellow obese gene (Ay) into KK/Ta mice are obese-diabetic mice showing hyperglycaemia, hypertriglyceridaemia, hyperinsulinaemia and microalbuminuria. Renal lesions in KKA\(\alpha\)/Ta mice closely resemble those in human diabetic nephropathy. The urinary albumin/creatinine ratio (ACR) in diabetic KKA\(\alpha\)/Ta mice is 250–350 mg/g Cr at 8 weeks of age and it increases to 550–600 mg/g Cr at 16 weeks of age. Glomeruli of KKA\(\alpha\)/Ta mice show segmental proliferative glomerular nephritis with expansion of extracellular mesangial matrix (ECM) at 16 weeks of age [11]. Therefore, KKA\(\alpha\)/Ta mice are considered to be a suitable model for the study of type 2 diabetic nephropathy.

It is postulated that one of the effects of EPA on diabetic nephropathy might be suppression of MCP-1 expression and monocyte/macrophage infiltration and activation in the kidney. To examine this hypothesis, we administered EPA ethyl ester to KKA\(\alpha\)/Ta mice, measured the various phenotypes associated with type 2 diabetes, and examined MCP-1 expression and macrophage infiltration in the kidney. Furthermore, we examined the effects of the EPA on MCP-1 expression in mouse mesangial cells (MMCs) stimulated by PDGF under HG conditions in vitro and on mitogen-activated protein kinases (MAPK) activity in MMCs and glomeruli of KKA\(\alpha\)/Ta mice.

Materials and methods

Reagents

EPA ethyl ester was kindly provided by Mochida Pharmaceutical Co., Ltd (Tokyo, Japan). Wortmannin and PD98059 were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). SB203580 and SP600125 were purchased from Alexis Biochemicals Co., Ltd (San Diego, CA, USA). EPA-Na was purchased from Sigma Chemical Co. (St Louis, MO, USA).

In vivo study

Animals and experimental design. Male KKA\(\alpha\)/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; 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 h light/dark cycle and temperature controlled at 24 ± 1 °C. KKA\(\alpha\)/Ta mice were randomly divided into two groups of six mice each. Based on data provided by CLEA Japan (Tokyo, Japan), no proteinuria is observed in KKA\(\alpha\)/Ta mice at 5 weeks of age, but 1+ proteinuria appears in 100% of KKA\(\alpha\)/Ta mice at 10 weeks of age, 2+ proteinuria is observed in 40% and 3+ proteinuria in 60% of KKA\(\alpha\)/Ta mice at 18 weeks of age. 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/day intraperitoneally for 8 weeks. The second group (control group) was injected with saline. Purified EPA ethyl ester stored at −20 °C inside the capsules (containing: EPA943, alpha-tocopherol 2 mg/capsule)) was freshly prepared before injection.

Phenotypic characterization

The ACR, body weight (BW) and fasting blood glucose levels were measured at 12, 16 and 20 weeks of age. Serum triglyceride, total cholesterol, leptin, MCP-1 and urinary MCP-1 levels were measured at 20 weeks of age. Glucose tolerance was estimated by the intraperitoneal glucose tolerance test (IPGTT) at 20 weeks of age. Glucose levels were measured using Glucocard (Kyoto Diamond Kagaku, Kyoto, Japan). Serum immunoreactive insulin (IRI) levels...
were measured by enzyme-linked immunosorbent assay (insulin ELISA Kit, Morinaga & Co., Ltd, Tokyo, Japan). Blood pressure was measured at 12, 16 and 20 weeks of age. The levels of serum fatty acids (EPA, docosahexaenoic acid, arachidonic acid and dihomo-γ-linolenic acid) were also measured at 20 weeks.

Urinary albumin and creatinine from samples collected for 24 h using metabolic cages (mouse metabolic cage, CLEA Japan, Tokyo, Japan) were measured by immunoassay (DCA 2000 system, Bayer Diagnostics Elkhart, USA). Serum total cholesterol and triglyceride were determined enzymatically by an autoanalyzer (Fuji Dry-Chem 5500, Fujifilm, Tokyo, Japan). Serum leptin levels were measured by enzyme-linked immunosorbent assay (Leptin/Mouse ELISA Kit, Morinaga & Co., Ltd, Tokyo, Japan). Levels of MCP-1 in sera and urinary samples were measured by enzyme-linked immunosorbent assay (Mouse MCP-1 Set, BD Biosciences, San Diego, CA, USA). The IPGTT 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 min after intraperitoneal glucose injection for measurement of the blood glucose and serum insulin levels. Blood pressure was measured at 11:00 a.m. by a non-invasive tail cuff and pulse transducer system (Softron BP-98A, Tokyo, Japan) after the mice were externally prewarmed for 10 min at 38°C. At least three to six recordings were taken for each measurement. Standard deviations of less than 5.0 were defined for the blood pressure levels. Lipids from sera were extracted with chloroform/methanol (1/2 by volume). The phospholipid fraction was isolated from the extracted lipids by thin-layer chromatography in a neutral lipid system (heptane/isopropylether/acetic acid, 60/40/2 by volume) using silica gel plates. Fatty acid methyl esters were formed by the addition of 6% H2SO4 in methanol, and transmethylation was performed at 80°C for 3 h. Following extraction of the dried fatty acid methyl esters using petroleum ether, fatty acid methyl ester derivatives were formed from the isolated phospholipid fraction were analyzed by gas-liquid chromatography (Gas Chromatograph GC14A, Shimadzu Co., Kyoto, Japan) to determine the fatty acid content.

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.

For immunohistochemistry of MCP-1 and F4/80, which recognizes 160 kDa glycoprotein expressed by murine macrophages, 3 μm thick sections were prepared in a cryostat and were fixed in cold acetone at −20°C for 10 min. After blocking with blocking solution, the cryosections were incubated overnight with antibody to MCP-1 (1:100) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and F4/80 (1:50) (Serotec Ltd, Oxford, UK). Immunohistochemistry of phospho-ERK1/2 (p-ERK1/2) and phospho-p38 (p-p38) was performed on 4 μm thick formalin-fixed kidney paraffin sections. Paraffin sections were deparaffinized and rehydrated. After blocking with blocking solution, the sections were incubated overnight with antibody to p-ERK1/2 (1:50) and p-p38 (1:50) (Cell Signaling Technology, Inc., Beverly, MA, USA). The sections were then incubated with horseradish peroxidase (HRP)-labeled antibodies (Dako Co., Carpinteria, CA, USA). Slides were mounted with antifade mounting media and examined on an OLYMPUS BX41 (Olympus Co., Tokyo, Japan) microscope.

Quantification of histopathological damage and immunohistochemistry

To evaluate the extent of glomerular sclerosis, 30 glomeruli were selected randomly from control and EPA treatment groups. PAS 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 tubulo-interstitial fibrosis area were measured automatically using a KS version 3.0 image analysis system (KS400, Carl Zeiss Vision GmbH, Hallbergmoos, Germany), as previously described [12]. Twenty glomeruli were selected randomly for analysis of MCP-1 in each group. The protein production of MCP-1 was measured by immunohistochemical-positive staining using the KS4000 version 3.0 image analysis system. The F4/80 positive cells were counted in 10 randomly chosen fields (×400) within the same section of the renal cortex from an individual mouse. The number of p-ERK1/2 or p-p38 positive cells was counted under a high power microscope (×400) within 20 glomerular cross sections from an individual mouse. The number of the cells in each glomerulus was averaged. Quantification was performed by three independent investigators on blinded slides.

In vitro study

Mesangial cell culture. MMCs were obtained from isolated glomeruli of male C57BL/6 mice (Japan SLC, Inc., Shizuoka, Japan) at 7 weeks of age by conventional sieving techniques as described previously [2]. The mesangial cells were identified by immunostaining of desmin, von Willebrand factor and cytokeratin and by morphological observation [2]. MMCs were cultured in RPMI 1640 supplemented with 20% FCS, 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 mM t-glutamine, 5 μg/ml insulin and 5 μg/ml transferrin and then incubated in humidified 5% CO2 atmosphere at 37°C. For measurement of supernatant MCP-1 levels under various conditions, near-confluent mouse mesangial cells in the 2–3 passage were transferred to 6-well plates and were left undisturbed in serum-free medium for 24 h. Thereafter, the cells were cultured in media containing normal glucose (5 mM), HG (30 mM) or mannitol (30 mM) for 24 h. The cells were treated or not treated (control) with PDGF (50 ng/ml) in the presence or absence of EPA (1, 10, 20, 30, 40, 50 and 100 μM), PD98059 (25 μM), SP600125 (1 μM) or wortmannin (100 nM). The dosages of PDGF and inhibitors for MAPK or PI3K were obtained from previous reports [9,10]. The cells were pretreated with EPA for 1 h, and PD98059, SB203580, SP600125 or wortmannin for 30 min. The products of PI3K activity were required for consequent activation of PKB. Therefore, the activity of PKB for readout of activation of the PI3K pathway was examined. For the assessment of ERK1/2, p-p38, JNK and PI3K activation, the quiescent cells were cultured in the HG (30 mM) media for 24 h and then treated or not treated (control)
with PDGF (50 ng/ml) for 10 min in the presence or absence of EPA (30 μM).

**Cytotoxic activity**

To exclude cell damage by EPA, the level of lactate dehydrogenase (LDH) in the cultured medium was examined using a LDH-cytotoxic test (Wako, Osaka, Japan). The maximum release was determined by incubation in 0.2% Tween 20. The control was incubated in EPA-free medium. The absorbance at 560 nm was measured.

**Measurements of MCP-1 by ELISA**

To quantify the levels of MCP-1 under various experimental conditions, the level of supernatant MCP-1 was measured using a solid phase quantitative sandwich enzyme-linked immunosorbent assay (Mouse MCP-1 Set, BD Biosciences, San Diego, CA, USA), which is specific for mouse MCP-1.

**Western blot analysis**

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and Western blot analyses were carried out according to standard protocols and visualized using enhanced chemiluminescence immunoblot detection kits (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). The first antibodies used in this study were as follows: mouse anti-mouse p-ERK1/2 (1:1000), rabbit anti-mouse ERK1/2 (1:1000), rabbit anti-mouse p-p38 (1:1000), rabbit anti-mouse phospho-JNK (p-JNK) (1:1000), rabbit anti-mouse phospho-protein kinase B (p-PKB) (1:1000) (Cell Signaling Technology, Inc., Beverly, MA, USA) and goat anti-human actin (1:1000) (Santa Cruz, Biotechnology, Inc., Santa Cruz, CA, USA) antibodies. HRP-conjugated second antibodies (Jackson-Immunoresearch Laboratories, Inc., West Grove, PA, USA) were used in this study.

**Statistical analyses**

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’s unpaired t-test was used in the comparison of quantitative evaluation of histopathological damage or immunohistochemical staining between the EPA-treatment group and the control group. ANOVA was used to assess the differences among multiple groups. P < 0.05 was considered as statistically significant.

**Results**

**Phenotypic characterizations of KKA^Y/Ta mice treated with EPA**

The serum fatty acid compositions of phospholipids from KKA^Y/Ta mice treated with EPA ethyl ester or saline at 20 weeks of age are shown in Table 1. Serum EPA levels in KKA^Y/Ta mice treated with EPA were significantly higher than those in the control group (0.049). As shown in Table 2, 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 non-diabetic BALB/cA mice (CLEA Japan, Tokyo, Japan (n = 8)), which were housed in the same conditions as KKA^Y/Ta mice and without any treatment, were 68.5 ± 3.0, 69.7 ± 3.6, 76.0 ± 0.8 at 12, 16 and 20 weeks of age each 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 = 0.0005 and P = 0.0017, respectively). As shown in Table 3, there was a significant change in the levels of serum triglyceride (P = 0.002) but not in the levels of total cholesterol between EPA-treatment and control groups at 20 weeks of age. Serum leptin levels in the EPA-treatment group were significantly decreased compared with those in the control group (P = 0.0033). Impaired glucose tolerance (IGT) evaluated by IPGTT in the EPA-treatment group was significantly improved compared with that in the control group at 20 weeks of age (P = 0.0201). The serum IRI levels after glucose administration in the EPA-treatment group were significantly decreased compared with those in the control group (P = 0.0003). The mean level of urinary

**Table 1.** Serum fatty acid composition of KKA^Y/Ta mice treated with EPA at 20 weeks of age

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>EPA treatment (n = 6)</th>
<th>Controls (n = 6)</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dihomo-γ-linolenic acid (C20:3o6) (μg/ml)</td>
<td>32.5 ± 5.3</td>
<td>26.5 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>Arachidonic acid (C20:4o6) (μg/ml)</td>
<td>195.6 ± 24.4</td>
<td>185.9 ± 14.9</td>
</tr>
<tr>
<td></td>
<td>Eicosapentaenoic acid (C20:5o3) (μg/ml)</td>
<td>125.8 ± 15.5</td>
<td>69.2 ± 7.8</td>
</tr>
<tr>
<td></td>
<td>Docosahexaenoic acid (C22:6o3) (μg/ml)</td>
<td>215.1 ± 21.7</td>
<td>174.8 ± 12.1</td>
</tr>
</tbody>
</table>

NS, not significant. Data expressed as means±SE.

**Table 2.** Phenotypic values of KKA^Y/Ta mice treated with EPA

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>EPA treatment (n = 6)</th>
<th>Controls (n = 6)</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body weight (kg)</td>
<td>44.5 ± 0.8</td>
<td>43.6 ± 0.5</td>
</tr>
<tr>
<td>12</td>
<td>Systolic blood pressure (mmHg)</td>
<td>104.3 ± 2.7</td>
<td>112.3 ± 2.4</td>
</tr>
<tr>
<td>16</td>
<td>Fasting blood glucose (mg/dl)</td>
<td>102.2 ± 5.9</td>
<td>96.8 ± 5.9</td>
</tr>
<tr>
<td>20</td>
<td>Levels (mg/ml)</td>
<td>93.8 ± 6.1</td>
<td>97.7 ± 3.0</td>
</tr>
<tr>
<td>12</td>
<td>ACR (mg/g Cr)</td>
<td>357.1 ± 61.3</td>
<td>425.7 ± 43.0</td>
</tr>
<tr>
<td>16</td>
<td>162.3 ± 42.6</td>
<td>566.3 ± 46.3</td>
<td>0.0005</td>
</tr>
<tr>
<td>20</td>
<td>216.1 ± 38.2</td>
<td>660.7 ± 58.6</td>
<td>0.0017</td>
</tr>
</tbody>
</table>

NS, not significant. Data expressed as means±SE.
MCP-1 in the EPA-treatment group was significantly lower than that in the control group at 20 weeks of age ($P = 0.0039$). However, there were no significant changes in the serum mean value of MCP-1 between the EPA-treatment and control groups. There was a significant correlation between the urinary levels of albumin and those of MCP-1 in the EPA-treatment and control groups ($r = 0.98$, $P < 0.001$; Figure 1).

**Light microscopy and immunohistochemical staining of MCP-1 and F4/80**

In light microscopy, diffuse mesangial matrix expansion was observed in control mice. Furthermore, segmental sclerosis was present in some glomeruli of the control group at 20 weeks of age (Figure 2a), whereas matrix expansion and segmental sclerosis decreased in EPA treated mice (Figure 2b). The extent of interstitial fibrosis was more prominent in the control group than in the EPA-treated mice (Figure 2). In morphometric analysis, the mean ECMA/WGA ratio and tubulointerstitial area in the EPA-treatment group were significantly lower than those in the control group ($P < 0.0001$ and $P = 0.0018$, Table 4).

Increased immunostaining of MCP-1 was observed in glomeruli of control mice from 20 weeks of age, whereas the staining of MCP-1 in the glomeruli of EPA-treated mice was much less intense. Immunoreactivity to MCP-1 was also identified by weak staining in tubulointerstitial regions of control mice. Similarly, tubulointerstitial immunostaining for MCP-1 was diminished in EPA-treated mice (Figure 3). The quantification of glomerular MCP-1 expression in the EPA-treatment group was significantly lower than that in the control group ($P = 0.0017$, Table 4). Staining of F4/80 was significantly suppressed in the EPA-treatment group compared with the control group. Increased mononuclear cell infiltration was observed in the interstitium and glomeruli in the control group.
group. However, it was decreased in the EPA-treatment group (Figure 3). The number of F4/80 positive cells in the EPA-treatment group was significantly decreased as compared with the control group (\(P = 0.0008\), Table 4).

**Cytotoxicity of EPA and effective dose of EPA in suppression of MCP-1 production in MMCs**

As shown in Figure 4a, more than 50 \(\mu M\) of EPA have cytotoxic effects, but the levels of LDH release in concentrations of less than 40 \(\mu M\) EPA were the same as in the control, suggesting no cytotoxic effect of EPA at less than 40 \(\mu M\). The suppression of MCP-1 production in MMCs was examined at different doses of EPA (1, 10, 20, 30 and 40 \(\mu M\)). EPA at 10–40 \(\mu M\) significantly suppressed PDGF-induced MCP-1 production dose dependently (\(P < 0.005\), Figure 4b) at 24 h. In the following in vitro studies, 30 \(\mu M\) EPA was employed to avoid the cytotoxic effect of EPA.

**Effects of EPA on MCP-1 production induced by PDGF and/or HG in MMCs**

PDGF induced MCP-1 production in a time-dependent manner. PDGF increased MCP-1 production by about 120% in control cells (\(P < 0.05\)) and EPA inhibited PDGF-induced MCP-1 production by about 60% (\(P < 0.05\)) at 24 h (Figure 5).

As shown in Figure 6, PDGF-and/or HG-induced MCP-1 production was examined in MMCs. MCP-1 production from MMCs cultured in PDGF or HG medium was markedly increased by about 80% or 34%, respectively, compared with controls at 12 h (\(P < 0.0005\)). MCP-1 production induced by PDGF with HG in MMCs was increased by about 102% (\(P < 0.0005\)). Mannitol as osmotic control did not significantly increase MCP-1 production.
significantly decreased MCP-1 production induced by PDGF, HG or PDGF with HG at 12 h (about 30, 23 or 35%, respectively, \( P < 0.0005 \)).

**Effects of ERK, JNK and PI3K inhibitors on PDGF-induced MCP-1 production in MMCs under HG conditions**

Figure 7 shows the effect of p38 inhibitor (SB203580), ERK inhibitor (PD98059), JNK inhibitor (SP600125) and PI3K inhibitor (wortmannin) on MCP-1 production in MMCs stimulated by PDGF (50 ng/ml) under HG conditions (30 mM). MCP-1 production from MMCs cultured with PDGF and HG was markedly increased by about 150% compared with controls at 12 h (\( P < 0.0005 \)). EPA, PD98059, SP600125 and wortmannin inhibited PDGF with HG-induced MCP-1 expression by 60, 50, 45 and 52%, respectively, at 12 h (\( P < 0.0005 \)). However, SB203580 did not inhibit MCP-1 expression. These results suggested that MCP-1 expression is ERK1/2, JNK and PI3K dependent, but p38 independent in MMCs.

**Effects of EPA on MAPK and PI3K activation in MMCs**

As shown in Figure 8, ERK1/2, p38 JNK and PKB were phosphorylated after incubation with PDGF (50 ng/ml) for 10 min. Incubation with EPA had decreased the phosphorylation of ERK1/2 and p38 (\( P < 0.05 \)), but had no effect on phosphorylation of JNK and PKB induced by PDGF.
MAPK activity in the glomeruli of KKA\(^{y}\)/Ta mice

To examine the effect of EPA on MAPK activity in glomeruli of KKA\(^{y}\)/Ta mice, an immunohistochemical study was performed using antibodies of p-ERK and p-p38 (Figure 9), because these two MAPK signalings are suppressed by EPA in vitro. The numbers of p-ERK and p-p38 positive cells at 12 weeks in pre-treated KKA\(^{y}\)/Ta mice were higher than those at 20 weeks in control mice and declined with the progression of diabetes \((P < 0.005)\). The number of p-ERK positive cells in the glomeruli of EPA-treated mice at 20 weeks of age was significantly lower than that of control mice \((P < 0.05)\). However, the number of p-p38 positive cells in the glomeruli of EPA-treated mice at 20 weeks of age was not significantly lower than that of control mice. It seems that the effect of EPA on MAPK activation is more powerful in ERK activation than in p38 activation in KKA\(^{y}\)/Ta mice.

Discussion

The present study demonstrated that EPA, one of the n-3 PUFA, ameliorates urinary ACR and MCP-1 levels, and attenuates mesangial matrix accumulation and tubulointerstitial fibrosis in KKA\(^{y}\)/Ta mice without changing systemic blood pressure and fasting blood glucose levels. Moreover, EPA ameliorates IGT and hypertriglyceridemia, and lowers leptin levels in KKA\(^{y}\)/Ta mice. EPA, as EPA ethyl ester injected at 1 g/kg/day intraperitoneally, significantly increased EPA levels among serum phospholipids, confirming that EPA was adequately absorbed. Measurement of EPA in serum phospholipids appears to serve as a useful biological indicator for EPA intake and nutritional status.

We cannot deny a possibility that the correction of metabolic abnormalities by EPA may contribute...
to the improvement of diabetic nephropathy in the present study. However, Shimizu et al. reported that EPA administration improved urinary ACR without affecting blood pressure levels, glycaemic control and lipid metabolism [3]. Recent studies have shown that dietary supplementation with n-3 PUFA retards disease progression in non-diabetic renal diseases including IgA nephropathy [1]. Therefore, besides the effects of EPA on IGT or hypertriglyceridemia, we assume that EPA has a direct renal effect on diabetic nephropathy. In vitro studies may support the assumption of direct renal effects of EPA.

Because MCP-1 induces monocyte immigration and differentiation to macrophages, which augment extracellular matrix production and tubulointerstitial fibrosis, the beneficial effects of EPA in diabetic nephropathy may also be exerted by down-regulation of renal MCP-1. There may be other factors involved in the improvement of diabetic nephropathy by EPA treatment, since EPA has many effects such as anti-thrombotic, hypolipidaemic, anti-atherogenic, anti-inflammatory and anti-mitogenic actions. The effect of EPA on anti-mitogenic action is particularly well documented in renal disease of experimental animals and in in vitro studies [2,13]. In the present study, we focused on its effects against inflammation and macrophage monocyte activation because mesangial cell proliferation is not a characteristic feature of diabetic nephropathy, and mesangial matrix accumulation and tubulointerstitial fibrosis are more common. There were also reports that EPA can inhibit monocyte/macrophage differentiation [14]. We observed decreases in MCP-1 expression and macrophage infiltration, matrix expansion, nodular lesions and tubulointerstitial fibrosis in renal histopathology of EPA-treated mice. There was a significant correlation between the urinary level of albumin and MCP-1, but EPA treatment did not affect the serum MCP-1 levels. These results suggested that urinary MCP-1 did not simply reflect the glomerular filtration of circulating MCP-1, but reflected the degree of progression of diabetic nephropathy. There is a possibility that at least part of the beneficial effect of EPA treatment in the interstitial compartment could be mediated indirectly by decreasing the albuminuria, since proteinuria is known to induce tubular interstitial inflammation through tubular epithelial cell activation by producing chemokines such as MCP-1.

MCP-1 is a member of the C–C chemokine family regulating macrophage recruitment and is up-regulated in many renal diseases including diabetic nephropathy [5]. Glycated albumin up-regulated MCP-1 expression in human mesangial cells and urinary MCP-1 concentration showed a significant correlation with the extent of albuminuria in diabetic nephropathy [5]. Moreover, Viedt et al. found that urinary MCP-1 directly activates tubular epithelial cells, leading to an increase in (IL-6) and intracellular adhesion molecule-1 production, contributing to tubulointerstitial inflammation [6]. MCP-1 exhibited direct effects on not only tubular epithelial cells, but also on vascular smooth muscle cells. Stimulation of vascular smooth muscle with MCP-1 induced proliferation and resulted in release of IL-6. The effects of MCP-1 on vascular smooth muscle cells may not only be important in the progression of cardiovascular damage in general, but also in the progression of vascular lesions in the kidney, which could contribute further to the progression of renal damage [6]. Thus, reduced MCP-1 expression by EPA is considered effective for the suppression of macrophage activation, tubulointerstitial inflammation and vascular damage in the diabetic kidney. In experimental crescentic glomerulonephritis, the administration of antibodies to MCP-1 decreased the urinary protein excretion, reduced glomerulosclerosis, and improved renal dysfunction [15]. However, the roles of MCP-1 in diabetic kidneys are not fully clarified and long-term studies using specific pharmacological suppression or inhibition of MCP-1 in diabetic nephropathy are needed.

We performed in vitro studies using MMCs to examine the molecular mechanism of suppression of MCP-1 expression induced by EPA. MCP-1 expression seems to be ERK1/2, JNK and PI3K dependent, but p38 independent in MMCs, although a previous study reported that MCP-1 was expressed via ERK, JNK and p38 in human mesangial cells [8]. The difference may be due to inter-species differences between human mesangial cells and MMCs. EPA suppressed ERK1/2 and p38 activation induced by PDGF under HG conditions in MMCs. These findings indicated that EPA decreased MCP-1 expression at least via suppression of ERK1/2 activity.

Hida et al. reported that 2 μg/ml EPA inhibited PDGF-induced mitogenesis and cyclin D1 expression via transforming growth factor (TGF)-β without altering ERK in mesangial cells [13]. In contrast, we showed that 30 μM EPA suppressed ERK activation of MMCs. The difference may be due to the different EPA concentrations used. Denys et al. reported that EPA-modulated phorbor 12-myristate 13-acetate induced ERK activation at 20, 40 and 60 μM, but not at 5 and 10 μM in human T cells [16]. These findings indicate that the ability of EPA to influence ERK activation may be dose dependent.

EPA may influence the diacylglycerol (DAG) – protein kinase C (PKC)–ERK pathway by replacement of arachidonic acid in plasma membrane phospholipids. An increase in de novo synthesis of DAG followed by activation of the PKC–ERK pathway leading to enhanced extracellular matrix and TGF has been proposed to explain the pathogenesis of diabetic nephropathy. ERK is an important kinase in the intracellular signal transduction system leading to cell proliferation and extracellular matrix protein synthesis. Activation of the PKC–MAPK pathway in glomeruli seems to be responsible for the high expression of TGF-β1 [18]. Suppression of the PKC–MAPK pathway by EPA treatment may affect not only MCP-1 expression but also TGF-β1 expression, leading to improvement of histological damage of diabetic nephropathy. In a study of MAPK activity in the
glomeruli of KKA\textsuperscript{+}/Ta mice, p-ERK and p-p38 were markedly increased in the early stage of diabetic nephropathy, suggesting that MAPK activation may play an important role in the progression of the early stage of diabetic nephropathy. Therefore, the suppression of ERK activation by EPA treatment seems more effective in the early stages of diabetic nephropathy to retard the progression of nephropathy. Further studies of the effects of EPA on MAPK activation in diabetic nephropathy are warranted.

The results of the improvement of IGT and decreased leptin levels in the KKA\textsuperscript{+}/Ta mouse given EPA may reflect the size of body fat deposition. Hun et al. also reported that KKA\textsuperscript{+}/Ta mice given fish oil had increased uncoupling protein 2 mRNA in white adipose tissue, and decreased leptin, visceral fat, blood glucose and cholesterol without significant changes in energy intake and body weight [18]. Leptin is a hormone secreted by adipocytes that regulates the energy balance. The loss of white adipose tissue leads to a decrease in leptin, which in turn increases neuropeptide Y production and stimulates both food intake and energy conservation. White adipose tissue accumulation leads to an increase in leptin levels and enhances energy expenditure via the melanocortin receptor [19]. EPA has recently been found to induce expression of mitochondrial uncoupling protein-2 and -3 in skeletal muscles, and to increase the less efficient peroxisomal fatty acid oxidation, pathway found in liver and skeletal muscle. EPA not only induces the genes of fatty acid oxidation, but also reduces body fat deposition in animals and humans by altering gene expression involved in thermogenesis, thereby increasing total body heat production. EPA down-regulates lipogenic gene expression by reducing the hepatic content of both precursor and matures sterol regulatory element-binding proteins, whereas EPA up-regulates genes of fatty acid oxidation and thermogenesis by functioning as a ligand activator for peroxisome proliferator-activated receptor alpha [19].

Decreasing serum triglycerides by EPA might also contribute to improvement of diabetic nephropathy. Experiments on the obese Zucker rat, which represents a model of dyslipidaemia with peripheral insulin resistance, showed that hyperlipidaemia may induce glomerular injury, especially focal glomerular sclerosis, independently of glomerular haemodynamics. Furthermore, lipid-lowering treatment has been shown to attenuate renal lesions in Zucker rats emphasizing a possible pivotal role for lipids in the pathogenesis of progressive renal disease [20].

In conclusion, EPA reduces albuminuria and renal MCP-1 expression, attenuates glomerular sclerosis, mesangial matrix accumulation and tubulointerstitial inflammation, and suppresses glomerular ERK activation in KKA\textsuperscript{+}/Ta mice. EPA also ameliorates IGT and hypertriglyceridaemia, and lowers leptin levels in KKA\textsuperscript{+}/Ta mice. Because MCP-1 induces monocyte immigration and differentiation to macrophages, which augment extracellular matrix production and tubulointerstitial fibrosis, and MCP-1 directly induces tubulointerstitial inflammation and vascular damage in the kidney, we propose that the observed down-regulation of MCP-1 is critically involved in the beneficial effect of EPA, probably in concert with improvement of other clinical parameters. The potential of EPA in the treatment of diabetic nephropathy might be of particular relevance to patients with comorbidities such as dyslipidaemia and obesity.

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