Original Article

DOI: 10.5582/bst.8.120

EPA and DHA increased PPARγ expression and deceased integrin-linked kinase and integrin β1 expression in rat glomerular mesangial cells treated with lipopolysaccharide

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Summary **Fish oil containing n-3 polyunsaturated fatty acids (n-3 PUFAs) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is known to prevent the progression of nephropathy and retard the progression of kidney disease. This study sought to investigate the underlying mechanisms of EPA and DHA in terms of peroxisome proliferator-activated receptor γ (PPARγ), integrin-linked kinase (ILK), and integrin β1 expression in glomerular mesangial cells (GMCs) because of their critical roles in the development and progression of nephropathy. Lipopolysaccharide (LPS) significantly reduced the expression of PPARγ and increased the expression of ILK at the mRNA level and at the protein level in GMCs as indicated by real-time PCR and Western blotting. In addition, LPS increased integrin β1 expression in GMCs at the mRNA level. Treatment with EPA and DHA significantly increased the expression of PPARγ and decreased the expression of ILK and integrin β1 in GMCs. These data suggest that the renoprotective effects of EPA and DHA may be related to their potential to increase the expression of PPARγ and decrease the expression of ILK and integrin β1.**

Keywords: Eicosapentaenoic acid, docosahexaenoic acid, mesangial cells, PPARγ, integrin-linked kinase, integrin

1. Introduction

Glomerular diseases are a leading cause of chronic and end-stage kidney failure worldwide, and an array of glomerular diseases is distinguished by glomerular mesangial cell (GMC) injury, including membranoproliferative glomerulonephritis, IgA nephropathy, and diabetic nephropathy. In a pathophysiological state, GMCs typically lead to expansion of the mesangial matrix and they undergo cell proliferation and hypertrophy and apoptosis. The phenomena are closely correlated with deterioration of renal function, so GMCs have long been considered an important factor in progressive renal failure (*1*).

Integrin-linked kinase (ILK) is known to be a widely expressed serine/threonine protein kinase localized to focal adhesion plaques and centrosomes (*2,3*). ILK plays a fundamental role in the regulation of cell survival, proliferation, and migration by connecting the cytoplasmic domains of β-integrins to the actin cytoskeleton, mediating integrin signaling in diverse cell types (*3*). An increase in the ILK level in the mesangium is associated with diffuse mesangial expansion. ILK is a downstream mediator of integrin β1 activity, and integrin/ILK signal pathways are involved in the regulation of cell adhesion, changes in cell morphology, and extracellular matrix (ECM) deposition (*4*). Overexpression of ILK can result in ECM remodeling and cell proliferation by GMCs (*5*).

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor family consisting of three subtypes (α, δ, and γ) with distinct and overlapping expression patterns (*6*). These lipid-

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sensitive receptors regulate many important physiological processes including glucose and lipid metabolism, energy homeostasis, cell proliferation, inflammation, immunity and reproduction (*7*). PPARγ is expressed in several types of tissue, including kidney tissue. In addition to regulating glucose and lipid metabolism, PPARγ has antiinflammatory and anti-fibrotic action in kidney diseases (*8*).

Eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), which belong to the n-3 polyunsaturated fatty acid (PUFA) family, are the main components of fish oil from deep sea fish. Fish oil, a type of PPARγ natural ligand, has been reported to slow the progress of kidney disease. A diet rich in n-3 PUFA, like EPA and DHA, has been associated with reduced triacylglycerol (TAG) levels, anti-inflammatory action, pulmonary disease (*9*), and a lowered risk of cardiovascular disease and mortality (*10-13*). A higher dietary intake of PUFA may protect against progression of chronic kidney disease (*14*). Clinical trials have provided conflicting results with regard to the efficacy of n-3 PUFA in treating IgA nephropathy, although *in vitro* and *in vivo* experimental studies have indicated that n-3 PUFA act on inflammatory pathways involved in the progression of kidney disease (*15*).

Despite increasing knowledge of the beneficial effects of fish oil in treating nephropathy, the exact mechanisms underlying its renoprotective effects are not fully understood. Previous studies by the current authors showed that EPA and DHA inhibited the proliferation of GMCs induced by lipopolysaccharide (LPS) (*16*). Furthermore, the protective effects of EPA and DHA on the kidney were found to be related to their action to suppress TGF-β1 and MCP-1 expression in GMCs (*17*). In addition, EPA and DHA may protect GMCs by regulating the imbalance of MMP and TIMP expression (*18*). The present study sought to examine the effects of EPA and DHA on the expression of PPARγ, ILK, and integrin β1 in rat GMSs in order to further investigate the renoprotective action of fish oil.

2. Materials and Methods

2.1. *Chemicals and antibodies*

EPA, DHA, and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 and fetal bovine serum (FBS) were purchased from Life Technologies (Carlsbad, CA, USA). Rabbit anti-rat ILK and rabbit anti-rat PPARγ were from Santa Cruz Biotechnology (Dallas, TX, USA). Monoclonal antibody against β-actin was purchased from Beijing Jing Mei Biotechnology (Beijing, China). All primers were synthesized by BioSune (Shanghai, China). Green real-time PCR master mix was purchased from Toyobo Biotech (Shanghai, China).

2.2. *Cell culture*

Rat GMCs (HBZY-1) were purchased from the Chinese Center for Typical Culture Collection (Wuhan, Hubei, China). GMCs were routinely cultured in RPMI-1640 media supplemented with 10% FBS. For experiments, GMCs were divided into six groups: *i*) Control group: cells were cultured in RPMI-1640 during the entire study; *ii*) LPS group: cells were treated with 10 μg/mL LPS; *iii*) Low-dose EPA group: cells were treated with 10 μg/mL LPS and 10 μmol/L EPA; *iv*) High-dose EPA group: cells were treated with 10 μg/mL LPS and 100 μmol/L EPA; *v*) Low-dose DHA group: cells were treated with 10 μg/mL LPS and 10 μmol/L DHA; *vi*) High-dose DHA group: cells were treated with 10 μg/mL LPS and 100 μmol/L DHA. Each experiment was repeated three times.

2.3. *RNA isolation and quantitative real-time polymerase chain reaction (PCR)*

After incubation for 24 h or 48 h, GMCs were harvested and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Complement DNA (cDNA) was synthesized using a Super Script III first strand cDNA synthesis Kit (Invitrogen). Real-time PCR was performed with SYBR Green PCR Master Mix kits (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Gene-specific primer sequences are listed in Table 1.

β-Actin was used as an internal control to quantify mRNA expression. The relative expression of the target gene = intensity of fluorescence of target gene fragments/ intensity of fluorescence of the β-actin gene. Cycling conditions were 94ºC, 3 min, followed by 40 cycles of 94ºC, 30 sec; 60ºC, 30 sec; and 72ºC, 1 min.

2.4. *Western blotting*

After incubation for 24 h or 48 h, cells were harvested and lysed. The protein concentration was determined using the Coomassie Brilliant Blue method. Equal amounts of lysate proteins from whole-cell lysates were loaded onto

Table 1. Primer pairs used in RT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')
PPARγ	AACCGGAACAAATGCCAGTA	TGGCAGCAGTGGAAGAATCG
ILK	CTTCTGTGGGAACTGGTGAC	CACATGGGGGGAAATACCTG
Intergrin β1	AGGAGGAGTAAAGTAAGTAG	AACCACCATAACAAAATGGG
β-Actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

10% gels and separated using SDS-PAGE. Then gels were transferred to a polyvinylidene difluoride membrane. After blocking for 2 h at room temperature in blocking buffer, the membrane was incubated overnight with rabbit anti-rat PPARγ or rabbit anti-rat ILK. Blots were washed and then incubated with a secondary goat antibody raised against rabbit IgG conjugated to horseradish peroxidase. After further washing, the bound antibodies were visualized using an ECL kit, and band densities were measured using TINA imaging software (Raytest, Straubenhardt, Germany). The level of β-actin protein served as a protein loading control.

2.5. *Statistical analysis*

Data are expressed as mean ± SD. One-way ANOVA followed by Dunnett's test was performed using SPSS/Win11.0 software (SPSS, Inc., Chicago, IL, USA). A significant difference was defined as $p < 0.05$.

3. Results

3.1. *EPA and DHA increased PPARγ expression in GMCs*

The effects of EPA and DHA on PPARγ expression were examined at the mRNA level and at the protein level in GMCs treated with LPS.

PPARγ protein expressed by GMCs was evaluated using Western blotting. Exposure of cultured GMCs to 10 μg/mL LPS induced a significant decrease in PPARγ expression in comparison to that in the control group ($p < 0.01$ at 24 h and 48 h, Figure 1). Incubation GMCs cultured with 10 μg/mL LPS and treated with EPA (100 μmol/L) or DHA (100 μmol/L) for 24 h resulted in a significant increase in PPARγ protein expression ($p < 0.01$, *vs*. LPS group, Figure 1A). In addition, incubation of GMCs treated with 100 μmol/L of EPA or 10 μmol/L or 100 μmol/L of DHA for 48 h markedly increased the expression of PPARγ ($p < 0.05$ or $p < 0.01$, *vs*. LPS group, Figure 1B).

Levels of PPARγ mRNA in GMCs were determined using real-time PCR. As shown in Figure 2, the ratio of the level of PPARγ mRNA to the level of β-actin mRNA was determined to be 1.0 for the control group, and this value decreased dramatically to 0.68 (24 h, $p < 0.05$) and 0.31 (48 h, $p < 0.01$) for the LPS group. Incubation of GMCs treated with EPA (10 μmol/L and 100 μmol/L) or DHA (10 μmol/L and 100 μmol/L) for 24 h (Figure 2A) and 48 h (Figure 2B) resulted in a significant increase in PPARγ mRNA expression in comparison to that in the LPS group ($p < 0.05$ or $p < 0.01$).

3.2. *EPA and DHA reduced ILK expression in GMCs*

The rise in the ILK level in the mesangium is associated with diffuse mesangial expansion. Whether EPA or DHA is able to diminish ILK expression in GMCs was ascertained at the mRNA level and at the protein level.

ILK protein expressed by GMCs was evaluated using Western blotting. Exposure of cultured GMCs to 10 μg/mL LPS induced a significant increase in ILK expression in comparison to that in the control group ($p < 0.01$ at 24 and 48 h, Figure 3). Incubation of GMCs treated with EPA

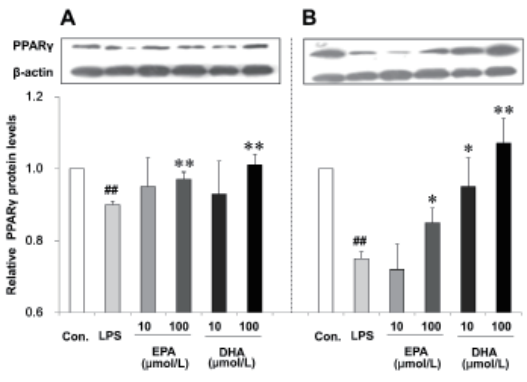

Figure 1. Effects of EPA and DHA on PPARγ protein expression in GMCs. Expression of PPARγ was detected using Western blotting at 24 h **(A)** and 48 h **(B)**. $^{\#\#}$ $p < 0.01$, *vs*. control group. * $p < 0.05$, ** $p < 0.01$, *vs*. LPS group.

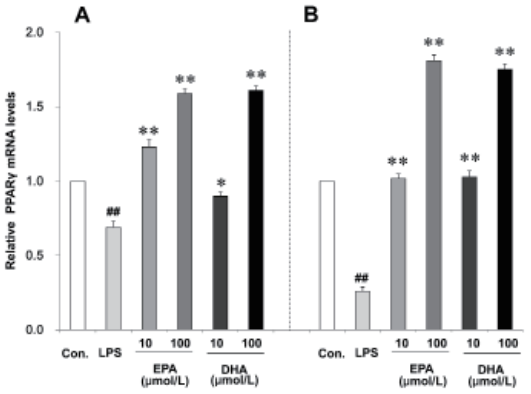

Figure 2. Effects of EPA and DHA on PPARγ mRNA expression in GMCs. PPARγ mRNA expression was determined with real-time PCR at 24 h **(A)** and 48 h **(B)**. The level of mRNA expression was normalized to that of β-actin. $^{\#\#}$ $p < 0.01$, *vs*. control group. * $p < 0.05$, ** $p < 0.01$, *vs*. LPS group.

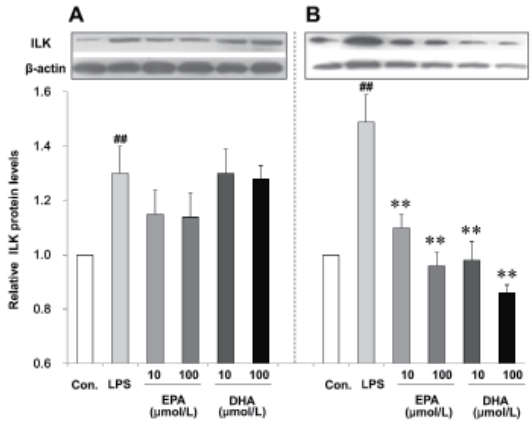

Figure 3. Effects of EPA and DHA on ILK protein expression in GMCs. Expression of ILK was detected using Western blotting at 24 h **(A)** and 48 h **(B)**. $^{\#\#}$ $p < 0.01$, *vs*. control group. ** $p < 0.01$, *vs*. LPS group.

(10 μmol/L and 100 μmol/L) or DHA (10 μmol/L and 100 μmol/L) for 24 h had no significant effect on ILK protein expression ($p > 0.05$, *vs.* LPS group, Figure 3A). However, incubation of GMCs treated with 10 μmol/L or 100 μmol/L of EPA or 10 μmol/L or 100 μmol/L of DHA for 48 h significantly decreased the expression of ILK protein ($p < 0.01$, Figure 3B).

Levels of ILK mRNA in GMCs were determined using real-time PCR. As shown in Figure 4, the levels of ILK mRNA dramatically increased in the LPS group ($p < 0.01$ at 24 h and 48 h). Incubation of GMCs treated with EPA (10 μmol/L and 100 μmol/L) or DHA (10 μmol/L and 100 μmol/L) for 24 h resulted in a significant decrease in ILK mRNA expression in comparison to that in the LPS group ($p < 0.01$, Figure 4A). Incubation of GMCs treated with 100 μmol/L of EPA or 10 μmol/L or 100 μmol/L of DHA for 48 h significantly decreased the expression of ILK mRNA ($p < 0.05$ or $p < 0.01$ *vs.* LPS group, Figure 4B).

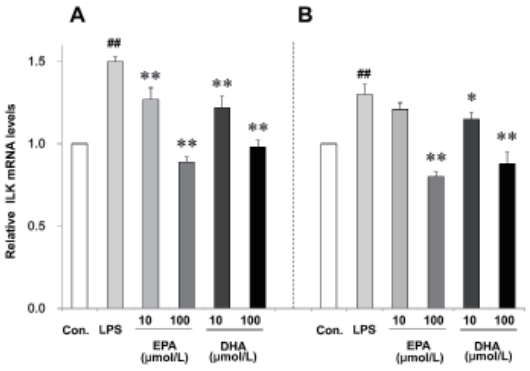

Figure 4. Effects of EPA and DHA on mRNA expression of ILK in GMCs. ILK mRNA expression was determined with real-time PCR at 24 h **(A)** and 48 h **(B)**. The level of mRNA expression was normalized to that of β-actin. $^{\#\#}$ $p < 0.01$, *vs*. control group. * $p < 0.05$, ** $p < 0.01$, *vs*. LPS group.

3.3. *EPA and DHA reduced integrin β1 expression in GMCs*

ILK is a downstream mediator of integrin β1 activity, and integrin/ILK signal pathways are involved in the regulation of cell adhesion, changes in cell morphology, and ECM deposition. Whether EPA or DHA was able to diminish integrin β1 mRNA expression in GMCs was ascertained.

Levels of integrin β1 mRNA in GMCs were determined using real-time PCR. As shown in Figure 5, the levels of integrin β1 mRNA dramatically increased in LPS-treated cells ($p < 0.01$ at 24 h and 48 h, *vs.* control group). Incubation of GMCs treated with EPA (100 μmol/L) for 24 h resulted in a significant decrease in integrin β1 mRNA expression in comparison to that in the LPS group ($p < 0.05$; Figure 5A). Incubation of GMCs treated with 10 μmol/L or 100 μmol/L of EPA or 10 μmol/L or 100 μmol/L of DHA for 48 h significantly decreased the expression of integrin β1 mRNA in comparison to that in the LPS group ($p < 0.05$ or $p < 0.01$, Figure 5B).

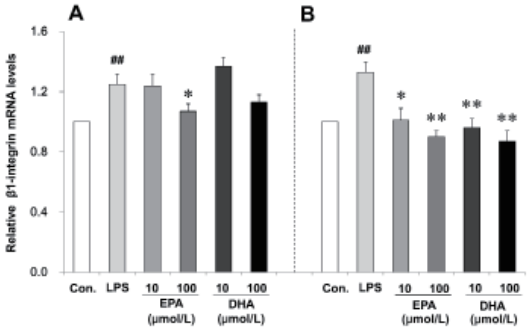

Figure 5. Effects of EPA and DHA on integrin β1 mRNA expression in GMCs. Integrin β1 mRNA expression was determined with real-time PCR at 24 h **(A)** and 48 h **(B)**. The level of mRNA expression was normalized to that of β-actin. $^{\#\#}$ $p < 0.01$, *vs*. control group. * $p < 0.05$, ** $p < 0.01$, *vs*. LPS group.

4. Discussion

This study investigated the mechanisms underlying the renoprotective effect of EPA and DHA in GMCs *in vitro*. Results indicated that EPA and DHA significantly increased the expression of PPARγ and reduced the expression of ILK and integrin β1 in GMCs, and these levels of expression changed dramatically when cells were cultured with LPS. These results suggest that the renoprotective effects of EPA and DHA are possibly related to their effects on the expression of PPARγ, ILK, and integrin β1 in GMCs.

PPARγ plays an important role in mesangial cells responding to inflammatory stress. PPARγ protein expression increased dramatically in human mesangial cells stimulated with interleukin-1β (IL-1β), and the levels of interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) increased significantly in comparison to those in untreated cells (*19*). A key finding is that PPARγ agonists, including troglitazone, rosiglitazone, and prostaglandin J2, significantly decrease the increased expression of TNFα and IL-6 (*19*). Rosiglitazone inhibits mesangial cell proliferation by blocking reactive oxygen species (ROS)-dependent epidermal growth factor receptor (EGFR) intracellular signaling, and telmisartan has powerful anti-inflammatory action *via* PPARγ activation in mesangial cells (*20*). PPARγ may prove to be a pharmacological target for treatment of glomerulonephritis (*19*). Fish oil reduced an LPS-induced inflammatory response in HK-2 cells *via* a PPARγ dependent pathway (*21*). The current results indicated that both EPA and DHA effectively up-regulated PPARγ mRNA and protein expression. Thus, fish oil may play a role by activating PPARγ. PPARγ agonists are effective in delaying and even preventing the progression of many renal diseases (*7*). These data suggest that PPARγ plays an important role in the response of mesangial cells to inflammatory stress. PPARγ may represent a potential target for the treatment of renal diseases (*7*).

ILK is a focal adhesion adaptor and a serine/threonine

protein kinase that regulates cell proliferation, survival, epithelial-mesenchymal transition (EMT), and kidney development (*22*,*23*). It acts as a central component of a heterotrimer (the PINCH-ILK-parvin complex) at ECM adhesions, mediating interactions with a large number of proteins via multiple sites including its pseudoactive site. ILK links integrins to the actin cytoskeleton and catalytic proteins and thereby regulates focal adhesion assembly, cytoskeleton organization, and signaling (*24*). Increased activity of ILK and integrin β1 in GMCs may contribute to the development of sustained mesangial cell proliferation and lead to glomerular scarring (*5*). In aging kidneys, integrin β1 and ILK may be involved in the process of fibrosis and related senescence (*25*). ILK also plays an important role in the pathogenesis of nephropathy in GMCs and also in podocytes of the kidney (*26,27*). In the present study, EPA and DHA decreased the expression of ILK and integrin β1 in GMCs stimulated with LPS. This finding confirms the assertion that ILK and integrin β1 play a role in lipid-induced kidney injury.

Interestingly, there may be a relationship between PPARγ and ILK. A study found that fish oil inhibited the proliferation of non-small cell lung carcinoma by suppressing ILK expression via activation of PPARγ (*28*).

In summary, the present study suggests that EPA and DHA are capable of increasing PPARγ expression and decreasing ILK and integrin β1 expression in GMCs treated with LPS. This study provides novel insights into the mechanisms of the renoprotective effects of fish oil.

Acknowledgement

This work was supported by a grant from the Shandong Provincial Natural Science Foundation (ZR2013HM099), China.

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(Received March 10, 2014; Revised April 19, 2014; Accepted April 23, 2014)