Research Article

DHA Inhibits Protein Degradation More Efficiently than EPA by Regulating the PPARγ/NFκB Pathway in C2C12 Myotubes

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This study was conducted to evaluate the mechanism by which n-3 PUFA regulated the protein degradation in C2C12 myotubes. Compared with the BSA control, EPA at concentrations from 400 to 600 μM decreased total protein degradation ($P < 0.01$). However, the total protein degradation was decreased when the concentrations of DHA ranged from 300 μM to 700 μM ($P < 0.01$). DHA (400 μM, 24 h) more efficiently decreased the IκBα phosphorylation and increased in the IκBα protein level than 400 μM EPA ($P < 0.01$). Compared with BSA, 400 μM EPA and DHA resulted in a 47% or 68% induction of the NFκB DNA binding activity, respectively ($P < 0.01$). Meanwhile, 400 μM EPA and DHA resulted in a 1.3-fold and 2.0-fold induction of the PPARγ expression, respectively ($P < 0.01$). In C2C12 myotubes for PPARγ knockdown, neither 400 μM EPA nor DHA affected the levels of p-IκBα, total IκBα or NFκB DNA binding activity compared with BSA ($P > 0.05$). Interestingly, EPA and DHA both still decreased the total protein degradation, although PPARγ knockdown attenuated the suppressive effects of EPA and DHA on the total protein degradation ($P < 0.01$). These results revealed that DHA inhibits protein degradation more efficiently than EPA by regulating the PPARγ/NF-κB pathway in C2C12 myotubes.

1. Introduction

Nuclear factor-kappa B (NFκB) is one of the most important signaling pathways linked to the loss of skeletal muscle mass in normal physiological and pathophysiological conditions [1]. NFκB expresses constitutively and exists in the cytosol as part of a heterotrimeric complex [2]. This complex typically comprises the DNA-binding proteins p50 and p65 plus the inhibitory protein IκBα. Activation of NFκB requires phosphorylation of IκBα, followed by ubiquitin conjugation and proteolysis of IκBα by the 26S proteasome [3, 4]. The activated NFκB dimer is then translocated to the cell nucleus, which is a feed-forward signal that leads to the upregulation of pathway components including ubiquitin, E_2/E_3 proteins, and proteasome subunits, subsequently increasing activity of the ubiquitin/proteasome pathway and enhancing skeletal muscle protein degradation [5–7].

There is growing evidence suggesting that long chain eicosapentaenoic acid (EPA, C20:5n-3) can inhibit NFκB activation by preventing the degradation of IκBα in skeletal muscle [8, 9]. Remarkably, most of the previous researches focused on that EPA can decrease muscle protein degradation in cancer cachexia [10–12]. In addition, it was further observed that long chain EPA, but not α-linolenic acid (ALA, C18:3n-3), can inhibit the NFκB activation in C2C12 myotubes by activating transcription factors peroxisome proliferators-activated receptor-γ (PPARγ) [13]. The above results demonstrated that the effect of n-3 polyunsaturated fatty acid (n-3 PUFA) on the IκBα/NFκB pathway may be different because of the variety of fatty acids. Therefore, we hypothesized that longer chain docosahexaenoic acid (C22:6n-3) can more efficiently inhibit the protein degradation by regulating PPARγ/NFκB pathway in C2C12 myotubes than EPA.

In the present study, C2C12 myotubes were treated with EPA and DHA for 24 h, respectively. Meanwhile, knockdown of PPARγ in C2C12 myotubes was achieved by RNA interference (RNAi). C2C12 myotubes for PPARγ knockdown were also treated with EPA or DHA for 24 h, respectively. The actions of EPA or DHA were compared with those of a fatty

acid-free control (containing BSA). The aim of this study was to investigate whether DHA can more efficiently inhibit protein degradation than EPA by regulating $I\kappa B\alpha/NF\kappa B$ pathway in C2C12 myotubes in a $PPAR\gamma$-dependent manner.

2. Materials and Methods

2.1. Materials. Cell culture media and supplements were obtained from Invitrogen (Carlsbad, CA, USA). Reagents for complementary DNA synthesis and the LightCycler system were obtained from Roche Applied Science (Mannheim, Germany). DHA, docosahexaenoic acid (C22:6n-3), EPA (C20:5n-3), essentially fatty acid-free BSA, monoclonal antiphospho-$I\kappa B\alpha$ (Ser32) antibody, and anti-$I\kappa B$a antibody were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies against NF-κB p65 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Finally, $[\gamma\text{-}^{32}P]ATP$ was obtained from Hartmann (Braunschweig, Germany).

2.2. Cell Culture. Mouse C2C12 myoblasts (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (50 units/mL), and streptomycin (50 mg/mL). When cells reached confluence, the medium was transferred to the differentiation medium containing Dulbecco's modified Eagle's medium and 2% horse serum, which was changed every other day. After four additional days, the differentiated C2C12 cells had fused into myotubes.

2.3. Transfection of Stealth RNAi for PPARγ Knockdown in C2C12 Myotubes. Transfection of Stealth RNAi for $PPAR\gamma$ knockdown in C2C12 myotubes was according to Kim et al. [14]. The $PPAR\gamma$ Stealth Select RNAi oligonucleotide (Target Accession nos. NM 138712.1, M015869.2, NM138711.1, and NM 005037.3) was synthesized by Invitrogen. The Stealth RNAi negative control duplex (Invitrogen Corp., Carlsbad, CA, USA) was used as a control oligonucleotide. Transfection efficiency was monitored using a fluorescent oligonucleotide (BLOCK-iT Fluorescent Oligonucleotide; Invitrogen) and estimated to be 40% in C2C12 cells. The Stealth RNAi molecules were transfected into C2C12 myotubes using LipofectAMINE 2000 by following Invitrogen's protocols. The final concentration of 50 nM $PPAR\gamma$ Stealth Select RNAi oligonucleotide was selected for C2C12 myotubes, and the Stealth RNAi oligonucleotides were transfected to the cells 48 h before the treatment of fatty acids. The ability of the Stealth RNAi oligonucleotide to knockdown $PPAR\gamma$ expression was analyzed by western blot and real-time quantitative PCR on whole-cell extract.

2.4. Treatment of Cells. Lipid-containing media was prepared by conjugation of free fatty acids (DHA or EPA) with FFA-free bovine serum albumin, by a method modified from that described by Chavez et al. [15]. Briefly, FFAs were dissolved in ethanol and diluted 1 : 100 in DMEM containing 2% (w/v) fatty-acid-free bovine serum albumin. 3T3-L1 adipocytes and C2C12 myotubes were cocultured in serum free medium overnight and then washed with fresh medium, refed with medium containing the different treatments (BSA or EPA, resp.), and incubated at $37°C$ in 5% CO_2 for 24 h before analysis. Cell viability was measured via trypan blue exclusion. At the end of the incubation, culture supernatant was collected and stored at $-20°C$ until assayed.

2.5. Measurement of Protein Degradation in C2C12 Myotubes. Cells were analyzed for protein degradation using procedures adapted from Desler [16]. C2C12 myotubes were labeled on day 4 for 24 h with $1\,\mu Ci/mL$ of $[^3H]$tyrosine before cells were washed, and the chase medium (DMEM + 2 mM tyrosine) was then added to wells and allowed to incubate for 4 h. Test medium was added and left for 20 h, and medium was sampled and counted. Remaining medium was aspirated off, and 1 mL of 0.5 M NaOH + 0.1% Triton was added. Plates were put in a cold room at $4°C$ overnight to allow the cells to dissolve. The cell suspension was placed in scintillation vials containing 5 mL of scintillation fluid, and wells were washed with 250 mL of 1 N acetic acid. This solution was added to scintillation vials and counted.

2.6. RNA Isolation. Total RNA was extracted using the TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's specifications. The RNA samples were quantified spectrophotometrically at 260 and 280 nm. The ratio of light absorbance at 260 nm to that at 280 nm was between 1.8 and 2.0, indicating that they were pure and clean. The quality of RNA was also checked by 1.0% agarose gel electrophoresis and staining with $1\,\mu g/mL$ ethidium bromide.

2.7. Reverse Transcription PCR and Real-Time Quantitative PCR Analysis. Reverse transcription $(20\,\mu L)$ of total RNA $(1\,\mu g)$ was performed using avian myeloblastosis virus reverse transcriptase with a first-strand cDNA synthesis kit for reverse transcription PCR. Aliquots $(2\,\mu L)$ of the reverse transcription reactions were then submitted in duplicate to online quantitative PCR with the LightCycler 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany) with SYBR green using the FastStart DNA Master SYBR Green I. Initial real-time amplifications were examined by agarose gel electrophoresis, followed by ethidium bromide staining to verify that the primer pairs amplified a single product of the predicted size. Subsequent aliquots of the PCR reaction were checked by melting curve analysis as provided by the LightCycler system. Primer sequences and optimal PCR annealing temperatures (ta) are listed in Table 1. The PCR was performed in a volume of $20\,\mu L$: $2\,\mu L$ of FastStart DNA Master SYBR Green I, 3 mM $MgCl_2$ and primers according to a primer concentration of $1\,\mu M$. The instrument settings were denaturing at $95°C$ for 10 min, $45 \times$ denaturing at $95°C$ for 30 s, annealing at $59°C$ for 30 s, and elongation at $72°C$ for 8 min for ($PPAR\gamma$ and β-actin). Quantification was performed by online monitoring for identification of the exact time point at which the logarithmic linear phase was distinguishable from the background. Serially diluted samples obtained by PCR with the above-mentioned primers

TABLE 1: Oligonucleotide polymerase chain reaction primers.

Gene[1]	Accession number	Source	Primer sequences ($5' \rightarrow 3'$)	Product size (bp)	ta[2] (°C)	Amplification Efficiency (%)
PPARγ	NM011146	Mus	ATGGAGCCTAAGTTTGAGTT CAGCAGGTTGTCTTGGATGT	153	58	98
β-actin	NM007393	Mus	CAGTGCGTGCTAAAGGGAGA CGCTCGTTGCCAATAGTGAT	148	59	97

[1]PPARγ: Peroxisome proliferator-activated receptor γ.
[2]ta, optimal PCR annealing temperature.

from human myotubes were used as external standards in each run. The cycle numbers of the logarithmic linear phase were plotted against the logarithm of the concentration of the template DNA, and the concentration of cDNA in the different samples was calculated with the LightCycler software (version 5.32).

2.8. Isolation of Nuclear Extracts.
Nuclear extracts were isolated according to Andrews and Faller [17]. Cells were scraped into 1.5 mL of cold phosphate-buffered saline, pelleted for 10 seconds, and resuspended in 400 μL of cold BufferA (10 mM HEPES-KOH pH 7.9 at 4°C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 5 μg/mL aprotinin, and 2 μg/mL leupeptin) by flicking the tube. Cells were allowed to swell on ice for 10 minutes and then vortexed for 10 seconds. Then, samples were centrifuged for 10 seconds and the supernatant fraction was discarded. Pellets were resuspended in 50 μL of cold BufferC (20 mM HEPES-KOH pH 7.9 at 4°C, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 5 μg/mL aprotinin, and 2 μg/mL leupeptin) and incubated on ice for 20 minutes for high-salt extraction. Cellular debris was removed by centrifugation for 2 minutes at 4°C and the supernatant fraction (containing DNA binding proteins) was stored at −80°C. Nuclear extract concentration was determined by using the Bradford method.

2.9. Electrophoretic Mobility Shift Assay.
The transcription factor consensus oligonucleotides for the NFκB responsive element ($5'$-AGT TGA GGG GAC TTT CCC AGG C-$3'$) and the AP1-responsive element ($5'$-CGC TTG ATG AGT CAG CCG GAA-$3'$) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The probes were labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (Boehringer, Mannheim, UK) and purified on Sephadex G-25 (Pharmacia, UK) spin chromatography columns. For the binding reaction, nuclear extract (1 μg of protein) was incubated in a 20 μL volume with EMSA binding buffer (20 mM HEPES, pH 7.5; 50 mM NaCl; 1 mM EDTA; 1 mM dithiothreitol; 0.05% NP-40; 10% glycerol), 0.25 ng [γ-^{32}P]-labelled probe, 1 mg/mL bovine serum albumin (Boehringer, Mannheim, UK), and 100 ng/mL poly d(I-C) for 20 min at room temperature. The specificity of the binding reaction was determined by coincubating duplicate samples with either 100-fold molar excess of unlabeled oligonucleotide probe or an anti-NFκB antibody (anti-p65; Santa Cruz Biotechnology). Protein-nucleic acid complexes were resolved using a nondenaturing polyacrylamide gel consisting of 6% acrylamide gel run in 5 mM Tris, pH 8.3, and 38 mM glycine for

2 h at 200 V. Gels were transferred to Whatman 3M paper (Whatman, Inc., Clifton, NJ, USA), dried under a vacuum at 80°C for 1 h, and exposed to photographic film at −70°C with an intensifying screen.

2.10. Immunoblotting.
In order to obtain total proteins C2C12, myotubes were homogenized in cold lysis buffer (5 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 5.4 μg/mL aprotinin). The homogenate was centrifuged at 10,000 ×g for 30 min at 4°C. For obtaining total membranes from C2C12 myotubes, cells were collected into 10 mL of ice cold HES buffer (250 mmol/L sucrose, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μmol/L pepstatin, 1 μmol/L aprotinin, 1 μmol/L leupeptin, and 20 mmol/L HEPES, pH 7.4) and subsequently homogenized at 4°C. After centrifugation at 1,000 ×g for 3 minutes at 4°C to remove large cell debris and unbroken cells, the supernatant was then centrifuged at 245,000 ×g for 90 min at 4°C to yield a pellet of total cellular membranes. Protein concentration was measured by the Bradford method. Proteins (30 μg) were separated by SDS-PAGE on 10% separation gels and transferred to Immobilon Polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Western blot analysis was performed using antibodies against phospho IκBα (Ser32), IκBα, and PPARγ (Santa Cruz Biotechnology, Inc.). Detection was achieved using the EZ-ECL chemiluminescence detection kit (Biological Industries, Beit Haemek Ltd., Israel). Equal loading of proteins was assessed by red phenol staining. Size of detected proteins was estimated using protein molecular-mass standards (Invitrogen, Barcelona, Spain).

2.11. Statistical Analysis.
Data were presented as means ± S.E. Differences between group means were determined by a one-way ANOVA using the computer program GraphPad Instat (version 2.03; GraphPad Software Inc., San Diego, CA, USA). When significant variations were found, the Tukey-Kramer multiple comparisons test was performed. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effect of Increasing Concentrations of n-3PUFA on Total Protein Degradation in C2C12 Myotubes.
In the present study, two long chain n-3 PUFA were chosen for study: eicosapentaenoic acid (EPA), a C20:5n-3 n-3 polyunsaturated
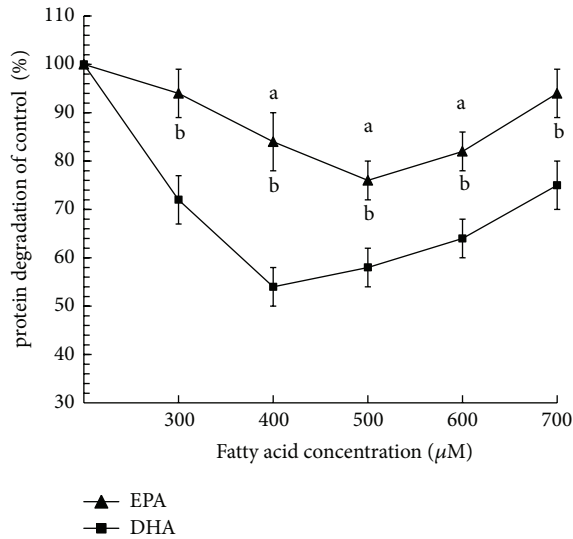
FIGURE 1: Effect of n-3 PUFA on total protein degradation in C2C12 myotubes. C2C12 myotubes were incubated (24 hours) with increasing concentrations of EPA (▲) or DHA (■), respectively. The radioactivity associated with cellular proteins is shown in the presence of 300 μM, 400 μM, 500 μM or 600 μM EPA, or DHA, respectively. Each treatment mean ($n = 8$) reported as a percentage of a FFA-free control; bars, SE. a, differences from controls in the presence of EPA ($P < 0.01$) and b, differences from the presence of DHA ($P < 0.01$). EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid.

fatty acid, and docosahexaenoic acid (DHA), a C22:6n-3 n-3 polyunsaturated fatty acid. BSA was used as fatty acid-free control. C2C12 myotubes were incubated (24 hours) with 300 μM, 400 μM, 500 μM, 600 μM, or 700 μM EPA and DHA, respectively. The effect of EPA or DHA on total protein degradation was dependent on the concentration added in the cell medium, as shown in Figure 1. Compared with the BSA control, EPA decreased total protein degradation when added at final concentrations of 400, 500, or 600 μM for 24 hours ($P < 0.01$). The maximal effect was observed with 500 μM of EPA. Compared with the BSA control, a 24 h incubation of C2C12 myotubes with 300 μM, 400 μM, 500 μM, 600 μM, or 700 μM DHA, respectively, significantly decreased the total protein degradation ($P < 0.01$). Remarkably, the maximal effect was observed with 400 μM of DHA.

3.2. Effect of 24 h Treatment with 400 μM n-3PUFA on IκBα/NF-κB Pathway in C2C12 Myotubes.
After being treated with 400 μM EPA, and DHA for 24 h in C2C12 myotubes, respectively, the levels of p-IκBα and total IκBα were measured by western blot method. The effect of 400 μM EPA and DHA on the IκBα protein level in C2C12 myotubes was present in Figure 2(a). As expected, compared with the BSA control, EPA (400 μM, 24 h) decreased the IκBα phosphorylation and caused approximately the 72% increase in the IκBα protein level ($P < 0.01$). In addition, a 24 h incubation of C2C12 myotubes with 400 μM DHA also decreased the IκBα phosphorylation and caused approximately the 89% increase in the IκBα protein level ($P < 0.01$). Taken together, these

data suggested that 400 μM DHA more effectively prevented the degradation of IκBα by decreasing the phosphorylation of IκBα and increased the IκBα protein level in C2C12 myotubes than 400 μM EPA.

To test whether incubation of C2C12 cells with 400 μM EPA or DHA for 24 h affected NFκB activity, we performed EMSA studies (Figure 2(b)). Compared with BSA, incubation of C2C12 myotubes with 400 μM EPA for 24 h decreased the IκBα phosphorylation and resulted in a 47% induction of the NFκB DNA binding activity ($P < 0.01$). Furthermore, C2C12 myotubes incubated in the presence of 400 μM DHA for 24 h decreased the IκBα phosphorylation and caused a 68% reduction in the levels of the NFκB DNA binding activity ($P < 0.01$). These results demonstrated that the inhibitory effect of 400 μM DHA on the NFκB DNA binding activity in C2C12 myotubes was greater than that of 400 μM EPA.

3.3. Effect of 24 h Treatment with 400 μM n-3PUFA on the Gene Expression of PPARγ in C2C12 Myotubes.
PPARγ gene expression data were presented in Figure 3. C2C12 myotubes incubated in the presence of 400 μM EPA for 24 hours resulted in a 1.3-fold induction of the *PPARγ* expression ($P < 0.01$), whereas 24 h incubation period with 400 μM DHA resulted in a 2.0-fold induction of the *PPARγ* expression ($P < 0.01$). In support of previous findings, 400 μM DHA increased the *PPARγ* gene expression to a greater extent than 400 μM EPA.

3.4. Effect of n-3PUFA on the IκBα/NF-κB Signaling Pathway and Total Protein Degradation in C2C12 Myotubes for PPARγ Knockdown.
To confirm that the inhibition of the IκBα/NF-κB signaling pathway and total protein degradation by n-3PUFA is mediated via activating the *PPARγ* mRNA expression, we examined the effect of n-3PUFA on the IκBα/NF-κB pathway in C2C12 myotubes for PPARγ knockdown.

The C2C12 myotubes transfected with either negative control Stealth RNAi oligonucleotide or PPARγ Stealth RNAi oligonucleotide were incubated for 48 h, respectively. Transfection of Stealth RNAi for PPARγ knockdown in C2C12 myotubes significantly decreased protein expression (Figures 4(a) and 4(b)) and PPARγ mRNA (Figure 4(c)) to approximately 82% and 86% ($P < 0.01$), respectively. Negative control Stealth RNAi treatment had no influence on PPARγ mRNA and protein expression ($P > 0.05$).

In C2C12 myotubes transfected with PPARγ Stealth RNAi oligonucleotide, treatment with 400 μM EPA or 400 μM DHA for 24 h did not affect the levels of p-IκBα or total IκBα (Figure 5(a)), NFκB DNA binding activity (Figure 5(b)), compared with BSA ($P > 0.05$). Remarkably, Figure 6 showed that in C2C12 myotubes transfected with either negative control Stealth RNAi oligonucleotide, 24 h incubation period with 400 μM EPA and DHA, respectively, caused 30% and 49% reduction in the rate of total protein degradation compared with BSA ($P < 0.01$). However, in C2C12 myotubes transfected with PPARγ Stealth RNAi oligonucleotide, 24 h incubation period with 400 μM EPA still caused a 17% reduction in the rate of total protein degradation ($P < 0.01$), whereas 24 h incubation period with 400 μM DHA resulted
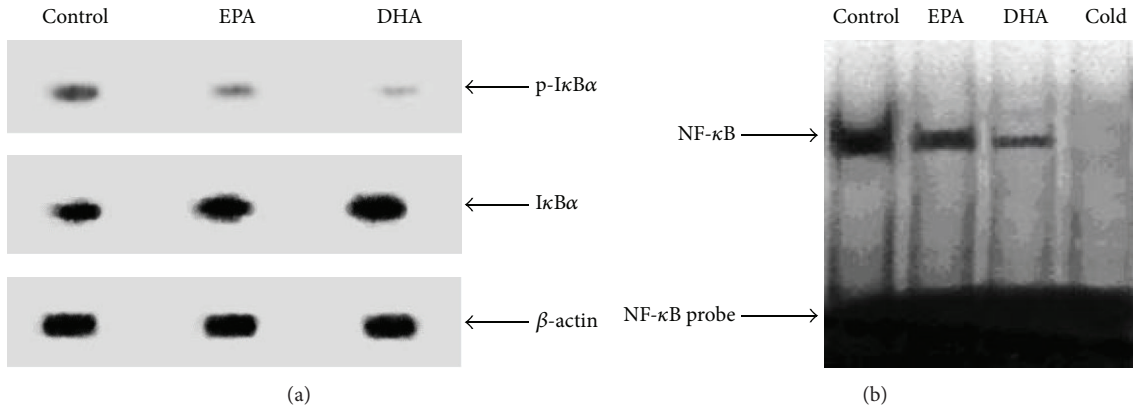
(a)

(b)

FIGURE 2: Effect of n-3 PUFA on the IκBα/NFκB complex in C2C12 myotubes. The C2C12 myotubes were incubated with 600 μM BSA, 600 μM EPA, or 600 μM DHA for 24 hours, respectively. Protein extracts from C2C12 myotubes were assayed for western blot analysis with p-IκBα, IκBα, or β-actin (Figure 2(a)). The band on the western blot represented a protein with a molecular mass of ∼37 kDa as determined by the molecular mass markers included in the experiment. Total nuclear protein was subsequently isolated and analyzed by EMSA for NFκB DNA binding activity using a ^{32}P-labeled double-stranded oligonucleotide of the NFκB (Figure 2(b)). An additional unlabeled probe was added in the competition assay (cold). Data are representative of three experiments. BSA = bovine serum albumin, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, and p-IκBα = phosphorylated IκBα.
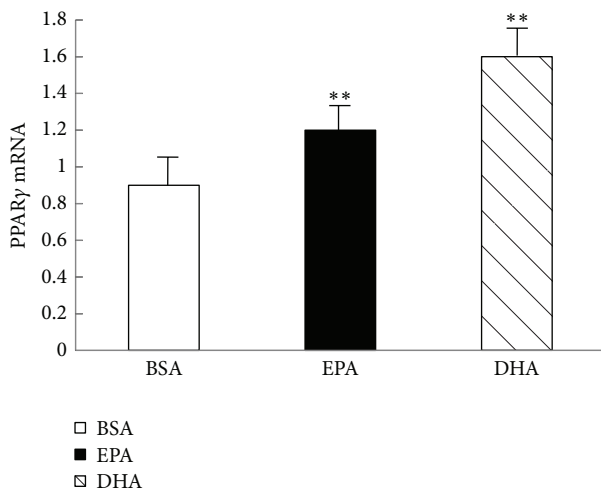

FIGURE 3: Effects of n-3 PUFA on *PPARγ* gene expression. C2C12 myotubes were incubated with 600 μM EPA, 600 μM DHA, or 600 μM BSA for 24 hours. The *PPARγ* mRNA was determined using real-time PCR analysis and relative abundance of mRNA was calculated after normalization to β-actin. *PPARγ* = peroxisome proliferator-activated receptor γ, BSA = bovine serum albumin, DHA = docosahexaenoic acid, and EPA= eicosapentaenoic acid. Data are expressed as mean ± S.D. of 3 different experiments. $^{**}P <$ 0.01 versus control.

in a 29% reduction in the rate of total protein degradation, compared with BSA ($P < 0.01$).

4. Discussion

Previous studies have found that eicosapentaenoic acid (EPA, C20:5n-3) decreased gene expression of the muscle RING finger 1 (*MuRF1*), which has been demonstrated to have

ubiquitin-ligase activity [13]. In the present study, it was further observed that total protein degradation was decreased by EPA at concentrations ranging from 400 μM to 600 μM and docosahexaenoic acid (C22:6n-3) at a wider dose range (300 μM to 700 μM), respectively. These results demonstrated that the effect of EPA and DHA on total protein degradation may be dependent on the concentration of fatty acids. Remarkably, in contrast to the fatty acid-free BSA control, the maximal effect for the inhibition of total protein degradation was observed at concentration of 500 μM EPA and 400 μM DHA, respectively. The results revealed that long chain DHA can more efficiently decrease total protein degradation than EPA. However, whether the suppressive effects of EPA and DHA on the total protein degradation depend on the chain length, it remains to be seen.

Despite increasing evidence of the effect of long chain n-3 polyunsaturated fatty acid (n-3 PUFA) on total protein degradation, little is known concerning the mechanisms by which long chain n-3PUFAs regulate muscle protein degradation. As a result of 400 μM EPA and DHA, both can inhibit total protein degradation. Therefore, in the current study, to test whether the effect of EPA or DHA on muscle protein degradation affected NFκB activity, we investigated the effect of 400 μM EPA or DHA on NFκB activity. The key to NFκB regulation is the IκBα protein, which retain in the NFκB cytoplasm. Phosphorylation of IκBα by IκB kinases triggers its polyubiquitinylation and degradation, thereby releasing NFκB, which translocates to the nucleus. As expected, incubation of C2C12 myotubes with 400 μM EPA for 24 h decreased the IκBα phosphorylation and resulted in a 47% induction of the NFκB DNA binding activity, whereas C2C12 myotubes incubated in the presence of 400 μM DHA for 24 h decreased the IκBα phosphorylation and caused a 68% reduction in the levels of the NFκB DNA binding activity. These results demonstrated that DHA can more efficiently inhibit NFκB activity than EPA. The results are
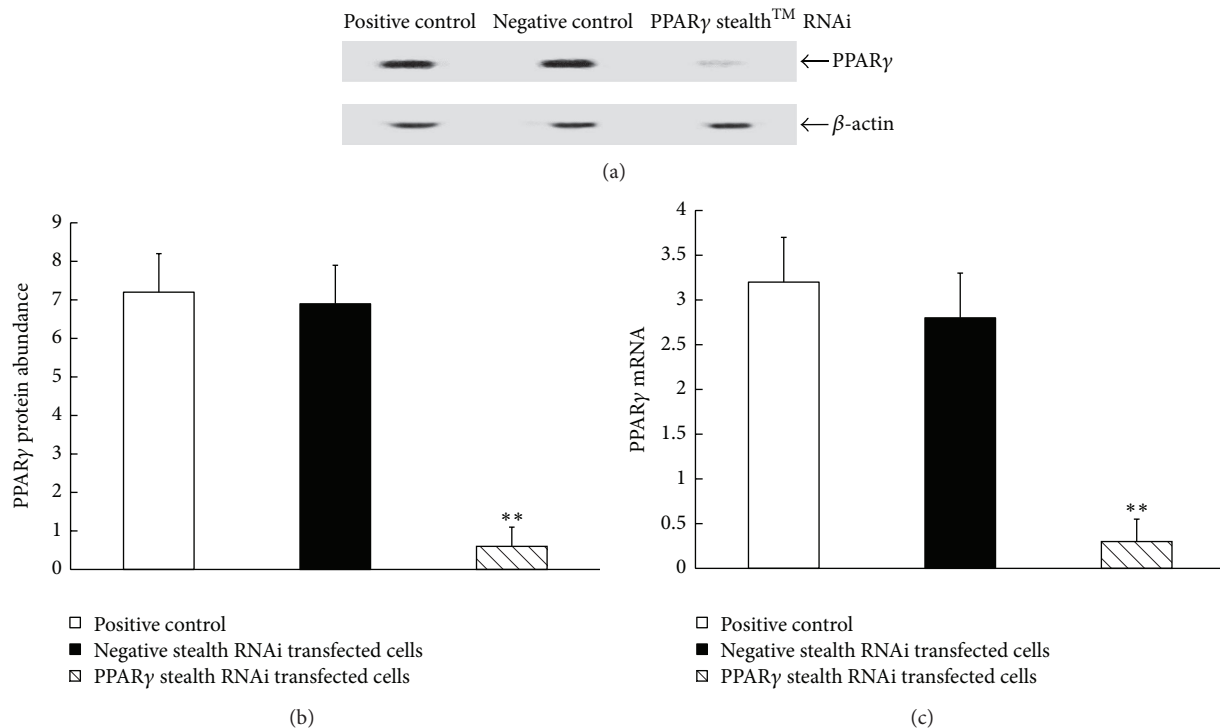
(a)

(b)

(c)

FIGURE 4: Transfection of Stealth RNAi for PPARγ knockdown in C2C12 myotubes. The C2C12 myotubes transfected with either negative control Stealth RNAi oligonucleotide or PPARγ Stealth RNAi oligonucleotide were incubated for 48 h, respectively. Protein extracts from C2C12 myotubes were assayed for western blot analysis with PPARγ (Figure 4(a)). The band on the western blot represented a protein with a molecular mass of ~55 kDa as determined by the molecular mass markers included in the experiment. PPARγ protein expression was determined by western blot and relative abundance of protein was calculated after normalization to β-actin (Figure 4(b)). The *PPARγ* mRNA was determined using real-time PCR analysis and relative abundance of mRNA was calculated after normalization to β-actin (Figure 4(c)). Data are expressed as mean ± S.D. of 3 different experiments. $^{**}P < 0.01$ versus positive control group. PPARγ = peroxisome proliferator-activated receptor γ.

(a)

(b)
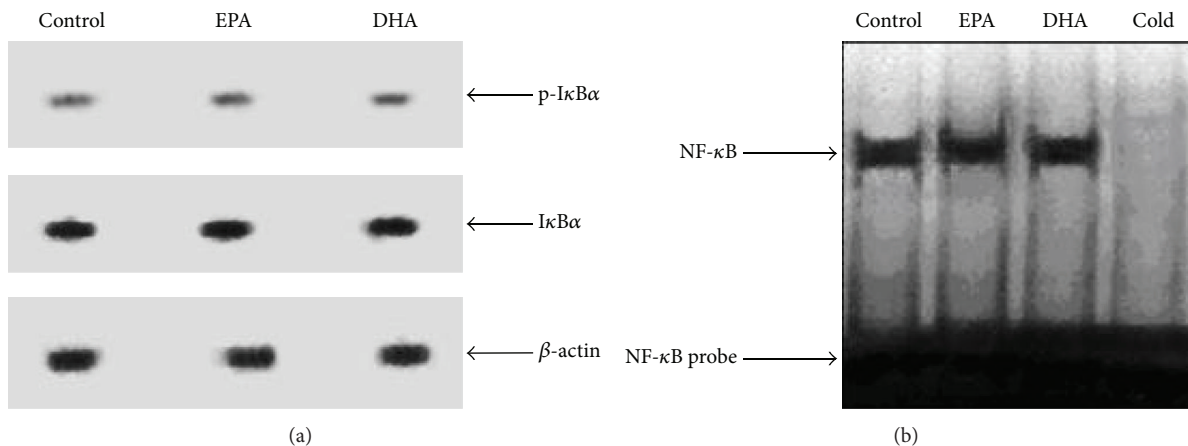
FIGURE 5: Effect of n-3 PUFA on the IκBα/NFκB complex in C2C12 myotubes knockdown of PPARγ. The C2C12 myotubes transfected with PPARγ Stealth RNAi oligonucleotide were incubated with 600 μM EPA or 600 μM DHA for 24 hours, respectively. BSA was used as fatty acid-free control. Protein extracts from C2C12 myotubes were assayed for western blot analysis with p-IκBα, IκBα, or β-actin (Figure 5(a)). The band on the western blot represented a protein with a molecular mass of ~37 kDa as determined by the molecular mass markers included in the experiment. Total nuclear protein was subsequently isolated and analyzed by EMSA for NFκB DNA binding activity using a ^{32}P-labeled double-stranded oligonucleotide of the NFκB (Figure 5(b)). An additional unlabeled probe was added in the competition assay (cold). Data are representative of three experiments. BSA = bovine serum albumin, DHA = docosahexaenoic acid, EPA = eicosapentaenoic acid, and p-IκBα = phosphorylated IκBα.
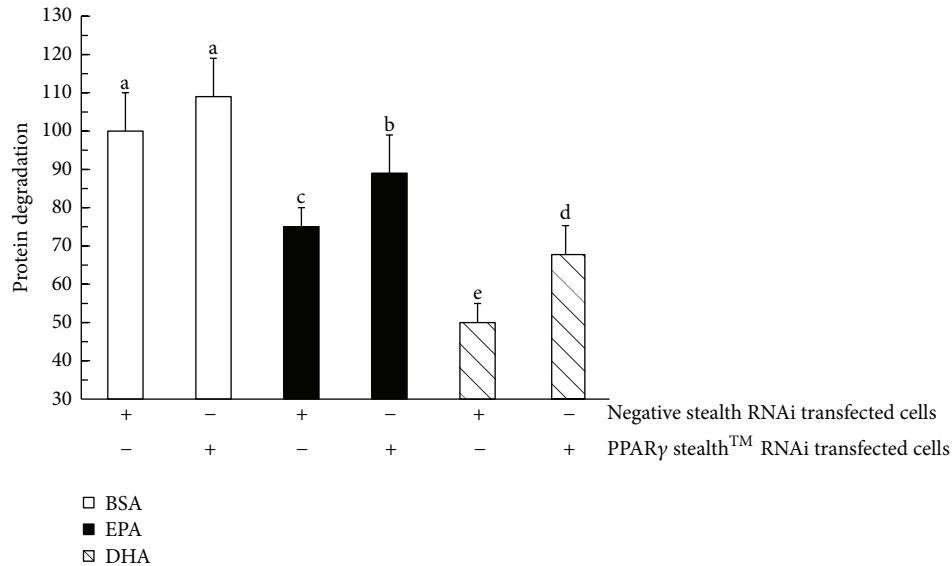
FIGURE 6: Effect of n-3 PUFA on protein degradation in C2C12 myotubes knockdown of PPARγ. After the transfection of C2C12 myotubes with either the negative control Stealth RNAi oligonucleotide or the PPARγ Stealth RNAi oligonucleotide for 48 h, C2C12 myotubes were incubated with 400 μM EPA or 400 μM DHA for 24 h. BSA was used as the fatty acid-free control. Data are expressed as mean ± S.D. of 8 different experiments. BSA = bovine serum albumin, DHA = docosahexaenoic acid, and EPA = eicosapentaenoic acid. Means without a common letter differ, $P < 0.01$.

in agreement with other studies showing that EPA inhibits NFκB activation by preventing IκBα phosphorylation and further reducing degradation of the inhibitory IκBα protein [18, 19].

We then investigated the molecular mechanism by which EPA and DHA decreased NFκB activation and inhibited total protein degradation. Many researches reported that activation of the transcription factor PPARγ in the skeletal muscle can inhibit NFκB activity [20, 21]. It was noteworthy that Li et al. [22] reported that n-3PUFA such as EPA and DHA are known natural ligands of PPARγ, which were required in relatively high concentrations (approximately up to 100 μmol/L) for PPAR activation. In the present study, C2C12 myotubes incubated in the presence of 400 μM EPA for 24 hours resulted in a 1.3-fold induction of the *PPARγ* gene expression, whereas 24 h incubation period with 400 μM DHA resulted in a 2.0-fold induction of the *PPARγ* gene expression. These results supposed that the effect of n-3PUFA on *PPARγ* gene expression is likely to depend on the chain length of fatty acids, which was in line with a previous report by Huang et al. [13], who found that C2C12 myotubes incubated in the presence of EPA (600 μM, 24 h) compared with the BSA control resulted in a 1.47-fold induction of the *PPARγ* gene expression, while α-linolenic acid (ALA; C18:3n-3) with shorter chain lengths was not able to affect *PPARγ* gene expression. Taken together, these results supposed that long chain EPA and DHA can both decrease NFκB activation and inhibit skeletal muscle degradation by activating *PPARγ* gene expression in a chain length dependent manner. However, because we did not test some other fatty acids, it is premature to give this conclusion without further study.

In order to investigate whether EPA inhibited the IκBα/NF-κB pathway and muscle protein degradation via

activating the *PPARγ* mRNA expression, PPARγ knockdown by RNA interference (RNAi) decreased PPARγ mRNA and protein expression to approximately 86% and 82% in C2C12 myotubes. Interestingly, it was further observed that treatment with 400 μM EPA or DHA for 24 h did not affect the levels of p-IκBα and total IκBα and NFκB DNA binding activity in C2C12 myotubes for PPARγ knockdown. These results demonstrated that PPARγ knockdown by RNAi abolished the suppressive effects of the EPA or DHA on the IκBα/NFκB pathway in C2C12 myotubes, supporting that the EPA or DHA effects are mediated via PPARγ activation.

Remarkably, in contrast to the fatty acid-free BSA control, 24 h incubation period with 400 μM EPA and DHA, respectively, can cause 30% and 49% reduction in the rate of total protein degradation in C2C12 myotubes without PPARγ knockdown. However, in C2C12 myotubes for PPARγ knockdown, 24 h incubation period with 400 μM EPA still caused a 17% reduction in the rate of total protein degradation, whereas 24 h incubation period with 400 μM DHA resulted in a 29% reduction in the rate of total protein degradation. The experiments founded that EPA and DHA both still decreased the total protein degradation in C2C12 myotubes, although PPARγ knockdown by RNAi attenuated the suppressive effects of the EPA or DHA on the total protein degradation. These results revealed that the mechanism by which n-3PUFA regulated total protein degradation may be involved in other signaling pathway, except for the *PPARγ*/IκBα/NFκB signalling pathway in C2C12 myotubes.

Taken together, these results revealed that DHA, more efficiently than EPA, inhibited the protein degradation by regulating IκBα/NFκB signaling pathway in C2C12 myotubes by activating PPARγ gene expression.

Abbreviations

ALA: α-Linolenic acid (C18:3n-3)
DHA: Docosahexaenoic acid (C22:6n-3)
EPA: Eicosapentaenoic acid (C20:5n-3)
EMSA: Electrophoretic mobility shift assay
BSA: Bovine serum albumin
MuRF1: Muscle RING finger 1
n-3PUFAs: N-3 polyunsaturated fatty acids
NF-κb: Nuclear factor-kappa B
p-IκBα: Phosphorylated IκBα
PPARγ: Peroxisome proliferator-activated receptor γ
RNAi: RNA interference.

Conflict of Interests

The author(s) declare that they have no conflict of interests.

Authors' Contribution

Fei-ruo Huang and Yue Wang conceived and designed the experiments. Yue Wang, Qiao-wei Lin, Pei-pei Zheng, and Jian-song Zhang carried out the experiment. Yue Wang analyzed the data. Fei-ruo Huang contributed reagents/materials/analysis tools. Yue Wang wrote the paper. All authors read and approved the final paper.

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