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PPARγ as a molecular target of EPA anti-inflammatory activity during TNF-α-impaired skeletal muscle cell differentiation☆

Peter Magee[a], Stephen Pearson[b], Jayde Whittingham-Dowd[a], Jeremy Allen[a,*]

[a]Centre for Biomedical Research, University of Salford, M5 4WT Manchester, UK
[b]Centre for Rehabilitation and Human Performance Research, University of Salford, M5 4WT Manchester, UK

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Abstract

Activated skeletal muscle satellite cells facilitate muscle repair or growth through proliferation, differentiation and fusion into new or existing myotubes. Elevated levels of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) impair this process and are documented to have significant roles in muscle pathology. Recent evidence shows that the ω-3 polyunsaturated fatty acid eicosapentaenoic acid (EPA) can block TNF-mediated suppression of progenitor cell differentiation, but the nature of this activity and its significance for local regulation of inflammation are not known. In the current study, we examined differentiation of the C2C12 myoblast line during treatment with TNF-α and EPA and measured the expression, activation and inhibition of peroxisome proliferator-activated receptor-γ (PPARγ), as several studies have shown its involvement in mediating EPA activity and the inhibition of nuclear factor (NF)-κB inflammatory gene activation. We found that TNF-α treatment increased NF-κB activity and reduced expression and activation of PPARγ, resulting in impaired myotube formation. EPA treatment attenuated these effects of TNF-α and was associated with up-regulation of PPARγ. Furthermore, EPA inhibited TNF-α-mediated transcription and secretion of interleukin (IL)-6, a key target gene of TNF-mediated NF-κB transcriptional activity. Pretreatment with a PPARγ selective antagonist inhibited some of the actions of EPA but was only partially effective in reversing inhibition of IL-6 production. These results show that EPA activity was associated with altered expression and activation of PPARγ, but exerted through both PPARγ-dependent and PPARγ-independent pathways leading to suppression of the proinflammatory cellular microenvironment.

Keywords: Eicosapentaenoic acid; Peroxisome proliferator-activated receptor-γ; C2C12; Interleukin-6; Tumor necrosis factor-α

1. Introduction

Skeletal muscle satellite cells are a mixed population of stem and progenitor cells located under the muscle fiber sarcolemma. They respond to damage induced by disease or exercise, becoming activated and undergoing proliferation, differentiation and fusion to form new, or repair existing, myotubes. Their successful participation in this vital process is influenced by their immediate cellular microenvironment. Cellular mediators such as tumour necrosis factor (TNF)-α are known to be key regulators of skeletal muscle cell responses to injury. Whereas physiological levels of TNF-α are transiently up-regulated during myoblast regenerative responses to injury and stimulate differentiation [1], sustained high levels of TNF-α are associated with chronic inflammatory diseases and have been identified as playing a significant role in mechanisms of muscle pathology associated with impairment of differentiation and muscle wasting [2].

Our group has previously reported that eicosapentaenoic acid (EPA), an ω-3 polyunsaturated fatty acid (PUFA) mainly found in fish oil, was able to attenuate the inhibitory effects of pathologic levels of TNF-α on skeletal muscle cell differentiation [3]. EPA has been found to reduce symptoms in chronic inflammatory diseases, for example, rheumatoid arthritis [4] and inflammatory bowel disease [5], and has reported health benefits for chronic diseases such as cardiovascular disease, cancer and insulin resistance [6], where there is significant elevation of proinflammatory cytokines. It has been suggested that blocking the actions or production of proinflammatory mediators is one beneficial effect of ω-3 PUFAs [7]. In addition to anti-inflammatory activity, EPA may have anticachetic effects. In animal models and *in vitro*, it has been found that EPA reduces muscle wasting, a feature of aging in humans [8–11], and it is able to prevent myonecrosis of dystrophic muscle [12].

TNF-α has been found to exert many of its effects on skeletal muscle through nuclear factor (NF)-κB signaling [13]. Constitutive NF-κB resides as a complex of p50 and p65 DNA-binding proteins bound to inhibitory protein κBα (IκBα) in the cytoplasm. Activation of NF-κB requires phosphorylation of IκBα, which becomes ubiquitinated and subsequently degraded, followed by translocation of the NF-κB dimer to the nucleus where specific inflammatory target genes, such as interleukin (IL)-6 and TNF-α, are activated via promoter-

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* Corresponding author. Centre for Biomedical Science, University of Salford, The Crescent, M5 4WT Manchester, UK. Tel.: +44 0 1612950728; fax: +44 0 1612950515.
E-mail address: j.t.allen@salford.ac.uk (J. Allen).

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region binding sites. Although systemic pathologic levels of inflammatory mediators are likely to derive mainly from immune cells, it is recognized that other tissues, such as skeletal muscle, may be important sources of these and could exert both local and systemic biological effects. TNF-α-mediated activation of NF-κB appears to be causally related to muscle atrophy; in a murine model, NF-κB activity leads to muscle wasting that resembles clinical cachexia [14], whereas inhibition of NF-κB activation blocks inflammation-induced protein degradation of *in vitro* cultures of skeletal muscle myotubes [15].

Peroxisome proliferator-activated receptor-γ (PPARγ), a major transcriptional regulator of lipid metabolism with anti-inflammatory properties, inhibits NF-κB-mediated gene activation in skeletal muscle [16], and its expression can be induced by EPA in adipocytes [17] and in human skeletal muscle cells [18]. EPA binds efficiently to the ligand-binding domain of PPARγ and has been shown to activate PPAR response element-reporter assays in a number of cell lines [19]. It remains to be seen whether EPA itself or a metabolite, such as a prostaglandin, is the cause of this PPARγ transactivation. Oxidized forms of EPA are known to serve as endogenous ligands for PPARγ that can inhibit NF-κB DNA-binding activity [20,21], although a recent study has shown, in formed myotubes, that EPA inhibits NF-κB through PPARγ-mediated prevention of IκBα phosphorylation without altered NF-κB DNA-binding activity [22]. An earlier study of EPA inhibition of TNF-α expression and secretion from a human monocytic cell line also showed that EPA had an inhibitory effect on IκBα phosphorylation [7].

These findings led us to hypothesize that EPA was blocking the activity of TNF-α on skeletal muscle cell differentiation through interference with NF-κB signaling involving PPARγ. This was investigated in C2C12 skeletal muscle cells, which provide a well-established *in vitro* model system with which to evaluate skeletal muscle differentiation. The main goals of the study were to evaluate the effects of EPA treatment on TNF-α-mediated NF-κB activation and target-gene expression and to determine whether EPA anti-inflammatory activity was dependent on and exerted through altered expression and activation of PPARγ.

2. Materials and methods

2.1. Cell culture

The murine skeletal muscle cell line C2C12 [23] was obtained from the European Centre for Animal Cell Culture (Porton Down, UK). C2C12 myoblasts are able to undergo differentiation into spontaneously contracting myotubes on withdrawal of growth factors [24]. Thus, myoblasts were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (both from Lonza Biologics, Slough, UK) containing antibiotics (10,000 U/ml penicillin G, 10 mg/ml streptomycin sulfate and 25 μg/ml amphotericin B from Sigma-Aldrich, Poole, UK), referred to as growth media (GM). Myoblasts were seeded at approximately 10^4 cells/cm^2 onto uncoated tissue culture plastic flasks or multiwell plates (Greiner Bio-One, Stonehouse, UK) for 24 h at 37°C and 5% CO_2, at which point they had reached approximately 70% confluency. At this time, myoblasts were induced to differentiate by briefly rinsing cells with phosphate-buffered saline (PBS) and replacing GM with DMEM containing antibiotics, supplemented with 2% (v/v) heat-inactivated horse serum, referred to as differentiation media (DM). To evaluate their effects on myogenic differentiation, murine recombinant TNF-α (Peprotech Ltd., London, UK) or EPA (IDS Ltd., Boldon, Tyne & Wear, UK) were added to cell cultures directly following induction of differentiation. EPA or, for certain experiments, oleic acid (OA) or linoleic acid (LA) was first complexed with fatty-acid-free bovine serum albumin (BSA) (Sigma) as described previously [17], Briefly, PUFA stock solutions (50 mM) were prepared in absolute ethanol and stored at −20°C in a glass vial in the dark. Working solutions were prepared by adding 50 mM PUFA stock solution to prewarmed (37°C) DMEM containing 4% (w/v) fatty-acid-free BSA. PUFA/BSA was conjugated at 37°C for at least 1 h before preparation of working solutions by dilution into cell culture media. The molar ratio of EPA/BSA for these studies was 0.08:1. A large molar excess of BSA was used to ensure maximum conjugation and to limit free PUFA available in order to mitigate any possible cytotoxicity due to free PUFA undergoing lipid peroxidation. The final concentration of ethanol in cultures was always below 0.1%. Myoblasts were cultured in DM for up to 5 days after the treatment(s), receiving fresh media after every 48 h. All treatments were replenished at this time.

2.2. Assessment of myogenic differentiation

To study the effect of TNF-α and EPA treatments on differentiation of myotubes, immunocytochemistry was performed. Cultures were briefly rinsed with PBS and then fixed with ice-cold methanol for 2 min. Cells were then washed three times with PBS for 3 min, blocked with 1% BSA in PBS for 30 min and then incubated with 38 μg/ml of MF-20 antibody (Developmental Studies Hybridoma Bank, University of Iowa, USA) to detect myosin heavy chain (MyHC) protein at a dilution of 1:250 in 0.5% BSA/PBS for 1 h. After washing with PBS, cells were incubated with 2 μg/ml goat anti-mouse Alexa Fluor 546 IgG at a dilution of 1:2000 in PBS for 1 h in the dark. After washing with PBS, cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The stained cells were analyzed under a Nikon TE2000 inverted fluorescence microscope, and DAPI and MyHC images were captured with a Hamamatsu Orca camera and merged using Image-Pro Lab v3.7 image analysis software (Nikon UK Ltd., Kingston upon Thames, UK). Myotube metrics were also quantified using Image-Pro software to determine myotube diameter and a myoblast fusion index. Average myotube width (MW) was evaluated as the mean of five approximately equidistant measurements taken along the length of the myotube. For each treatment, 10 fields of view were chosen randomly, and 10 myotubes were measured in each field. A myogenic index (MI) was also calculated to indicate myotube fusion. Using 10 images from randomly chosen microscope fields of DAPI- and MyHC-stained cells for each treatment, the total number of nuclei and the number of nuclei incorporated into myotubes were counted. The MI was calculated as the percentage of nuclei incorporated into myotubes (defined as containing at least two nuclei) relative to the total number of nuclei.

2.3. PPAR γ nuclear translocation

To study the effect of TNF-α and EPA treatments on PPARγ nuclear translocation, immunocytochemistry was performed. Cells were grown to 70% confluence in 24-well plates, serum-deprived in DM for 24 h and then treated with either vehicle (0.1% ethanol) as a control, GW9662 (10 μM; based on Ref. [25]), rosiglitazone (RGZ) (10 μM; not shown to be cytotoxic at up to 50 μM based on ref. [25]), TNF-α (20 ng/ml; based on Ref. [3]) or EPA (50 μM; based on Ref. [3]), which was added either together with TNF-α or as a separate 2-h pretreatment after which it was withdrawn and replaced by TNF-α for 24 h. Cultures were briefly rinsed with PBS and then fixed with ice-cold methanol for 2 min. Cells were then washed three times with PBS for 3 min and blocked with 2% BSA in PBS for 1 h. A primary PPARγ antibody (N-20 goat polyclonal IgG, Santa Cruz Biotechnology) was applied at a 1:250 dilution for 1–2 h. After washing with PBS three times, cells were then incubated with a secondary antibody (donkey anti-goat IgG-FITC, Santa Cruz Biotechnology) at a 1:200 dilution in PBS for 1 h in the dark. After washing with PBS, cells were counterstained with DAPI. The labeled cells were analyzed under a Nikon TE2000 inverted fluorescence microscope, and DAPI and MyHC images were captured using separate filters with a Hamamatsu Orca camera and merged using Image-Pro Lab software.

2.4. Quantitative polymerase chain reaction (PCR)

PCR primers and probes were obtained as Taqman gene expression assays from Applied Biosystems (Warrington, UK) targeting mRNA expression of interleukin-6 (IL-6; Mm00446191_m1) and PPARγ: (Mm00440945_m1). A β-actin primer/probe was obtained from PrimerDesign (Southampton, UK), selected as the exogenous housekeeping gene for normalization of results on the basis of previous published work with the C2C12 model and was used in all real-time analyses. After treatments, C2C12 were lysed using an RNeasy kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions and stored at −80°C prior to RNA extraction. Briefly, RNA was extracted, and genomic DNA was removed, by spin-column purification according to the supplier's recommendations. RNA yield and purity were evaluated spectrophotometrically. Total RNA (1 μg) was used in a reverse transcription reaction and cDNA synthesis performed using a precision reverse transcription kit according to the supplier's instructions (PrimerDesign). The cDNA (2.5 μl), diluted 1:10, was used in each PCR, performed in triplicate on an Applied Biosystems 7500 Fast system. All PCRs were prepared using Precision Mastermix with low ROX (PrimerDesign), according to supplier's recommendations. PCR conditions were as follows for all genes: 95°C for 10 min and then 50 cycles of 95°C for 15 s, 50°C for 30 s and 72°C for 15 s. Data were validated using the pairwise fixed reallocation randomization test, and 10,000 randomizations were performed in the Relative Expression Software Tool [26].

2.5. NF-κB transcriptional activity

C2C12 stably transfected with an NF-κB-luciferase reporter gene construct was kindly provided by Dr. R. Langen (Department of Respiratory Medicine, Maastricht University, Maastricht, the Netherlands). The NF-κB reporter cells were plated in 35-mm dishes and allowed to grow to 70%–80% confluence. Growth medium was replaced with DM for up to 24 h with treatments; for assessment of NF-κB transcriptional activation, EPA (50 μM) was added as a co-treatment with TNF-α (20 ng/ml) or as a 2-h pretreatment, after which it was withdrawn and treatment continued with TNF-α only. Both treatments were also applied separately. Assessment of luciferase activity was performed using the Promega Dual-Luciferase Assay according to the manufacturer's instructions (Promega, Madison, WI, USA) and

corrected for total protein content using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Briefly, after the appropriate incubation times, cells were washed twice with cold PBS and subsequently lysed in (100 μl) buffer on ice for 10 min. Cell lysates were centrifuged (13,000g, 1 min), and supernatants were either snap-frozen and stored at −80°C for later analysis or placed on ice for immediate analysis.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Conditioned media were collected for ELISA analysis of IL-6; C2C12 seeded onto 24-well plates at a density of 40,000 cells per well were maintained in 10% DMEM for 24 h prior to treatment until 70% confluent. GM were replaced with DM containing TNF-α (20 ng/ml) or EPA (50 μM). EPA was added, either together with TNF-α or as a separate 2-h pretreatment, after which it was withdrawn and replaced by TNF-α only. Treatments were refreshed every 48 h, and media were collected from cells at 72, 96 and 120 h, respectively. ELISAs were performed on 96-well plates (Grenier Bio-One, Stonehouse, UK). Wells were coated with 50 μl of IL-6 capture antibody (BD OptEIA, BD Bioscience, San Diego, CA, USA) at a dilution of 1:250 in coating buffer (0.1 M sodium carbonate buffer pH 9.5, BD OptEIA). The plate was sealed and incubated at 4°C for 24 h. Wells were aspirated and washed three times using a PBS wash buffer containing 0.05% Tween 20. Residual wash buffer was removed by blotting the plate on absorbent paper, and the plate was blocked with 50 μl of assay diluent (PBS containing 10% FBS pH 7.0, BD OptEIA) for 1 h at room temperature. Wells were aspirated and washed three times using wash buffer, and then 50 μl of standards and samples was aliquoted in triplicate onto the plate. Recombinant lyophilized mouse IL-6 (BD OptEIA) was prepared and dispensed onto the plate in a serial dilution starting at 1000 pg/ml in assay diluent. Samples and standards were incubated on the sealed plate for 2 h at room temperature. Following incubation, the samples/standards were aspirated from the plate, and the wells were washed five times with wash buffer. Fifty microliters of working detector (biotinylated anti-mouse IL-6 and streptavidin-horseradish peroxidase conjugate, BD OptEIA) at a dilution of 1:500 in assay diluent was added to each well, and the plate was sealed and incubated at room temperature for 1 h. The plate was aspirated and washed seven times for 30 s per wash before incubating in 50 μl substrate solution (1:1 ratio of hydrogen peroxide+3,3′,5,5′ tetramethylbenzidine, BD OptEIA). The unsealed plate was incubated in the dark for 30 min before adding 50 μl of stop solution (1 M H_3PO_4, BD OptEIA). Absorbances were read at 450 nm and 570 nm using a Thermo Fisher Multiskan FC plate reader (Fisher Scientific, UK). The absorbance at 570 nm was used as a correction value and was subtracted from the value taken at 450 nm. A standard curve was used to estimate IL-6 concentrations of samples.

2.7. Statistical analysis

Statistical significance was performed using two-way analysis of variance (ANOVA) for multiple comparison testing and/or one-way ANOVA with Bonferroni post hoc analysis for between-groups comparisons. Statistical significance was accepted when $P<.05$.

3. Results

3.1. PPARγ mediates EPA restoration of TNF-impaired C2C12 morphology

After 5 days of differentiation, the morphological appearance of formed myotubes was examined, qualitatively, by immunocytochemistry of MyHC expression (Fig. 1A images A–H) and, quantitatively, by calculation of MI and MW (Fig. 1B). The results show that TNF-α suppressed myotube fusion (Fig. 1A image A), which was confirmed by analysis of MI which declined to approximately 40% of control ($P<.05$ vs. untreated) and of MW which significantly decreased to approximately 50% of control ($P<.05$ vs. untreated) with TNF-α treatment. These effects were overcome by EPA administration. By 5 days, EPA, delivered either as a co-treatment (Fig. 1A image B) or as a 2-h pretreatment (data not shown), completely prevented the inhibitory actions of TNF-α on myotube formation. This was reflected in restoration of MI and MW markers back to control levels [not significant (NS) vs. untreated]. In parallel experiments, docosahexaenoic acid (DHA) was as effective as EPA (data not shown). In contrast to EPA, both OA and LA impaired myotube formation, as shown by images C and D (Fig. 1A), respectively. Corresponding MIs were reduced significantly to 40% and 60% of control for OA and LA, respectively ($P<.05$ vs. untreated). Those myotubes that did form were of approximately normal widths for both these treatments (≫NS vs. untreated). When OA and LA were applied in combination with TNF-α, there were further reductions in myotube formation, shown in images E and F (Fig. 1A), respectively. Fewer myotubes were formed; MIs were reduced significantly to only 20% of controls and were lower than those for PUFA alone ($P<.05$ vs. OA or LA alone), suggesting an additive effect. However, when administered with TNF-α, OA and LA reduced MW to approximately 50% of controls ($P<.05$ vs. OA or LA alone), although there was no observable additive effect. These changes were not due to PUFA cytotoxicity as viability was not significantly altered in the presence of OA or LA (data not shown). To investigate the mechanism of EPA activity, a specific PPARγ antagonist (GW9662) was administered as a 2-h pretreatment to block any PPARγ-mediated effect of EPA. GW9662 alone had no detrimental effect on the appearance of myotubes (Fig. 1A image G) or their formation (NS vs. untreated) and was not found to have any significant effects on cell viability using an adenosine triphosphate bioassay (data not shown). However, GW9662 pretreatment was effective in blocking the restorative effects of EPA following its co-treatment with TNF-α (Fig. 1A image H); MI was reduced to 50% of control ($P<.05$ vs. TNF+EPA) and MW was reduced to 60% of control ($P<.05$ vs. TNF+EPA), suggesting that a significant proportion of EPA activity was mediated through PPAR pathways.

3.2. PPARγ expression and activation are inversely regulated by TNF-α and EPA

PPARγ mRNA expression was examined using real-time quantitative PCR (Fig. 2A) and was normalized against β-actin, as described in Materials and Methods. Following treatment with TNF-α, expression of PPARγ was significantly down-regulated at 6- and 24-h time points compared to respective untreated controls ($P<.05$ vs. untreated). In contrast, EPA delivered either as a co-treatment or as a pretreatment with TNF-α significantly up-regulated PPARγ expression in a time-dependent manner ($P<.01$). EPA induced a maximal four- to sixfold increase in PPARγ expression compared to TNF-α by 24 h. A 2-h pretreatment with GW9662, before co-treatment with EPA and TNF-α, partially but significantly inhibited the expression of PPARγ by 24 h compared to EPA treatments ($P<.01$).

To further investigate and confirm the involvement of the PPAR pathway in mediating EPA activity, immunocytochemical staining for PPARγ expression and cellular localization with a specific PPARγ antibody was performed (Fig. 2B) on differentiating myoblasts up to 24 h following treatments. Under basal conditions, PPARγ appeared to be moderately expressed and was detectable in cell cytoplasm, with little present in nuclei (Fig. 2B images A and B). However, in cells treated with the PPARγ ligand RGZ as a positive control for expression and activation of PPARγ, overall expression was observed to increase throughout the cells, and notably, both perinuclear and nuclear stainings for PPARγ were increased within 2 h, with maximal effects observed at 24 h (Fig. 2B images C and D). When the GW9662 antagonist was applied prior to RGZ, the overall expression of PPARγ was observed to decrease, and nuclear localization was reduced (Fig. 2B images E and F), although still apparent, compared to controls. Treatment with TNF-α appeared to reduce the overall expression of PPARγ compared to controls, and there was no nuclear PPARγ presence (Fig. 2B images G and H). In contrast, EPA co-treatment (Fig. 2B images I and J) and pretreatment (Fig. 2B images K and L) together with TNF-α showed increased overall PPARγ expression and activation indicated by nuclear translocation compared with TNF-α alone (Fig. 2B images G and H). These EPA-induced changes in PPARγ expression and localization were prevented by GW9662 pretreatment; notably, nuclear translocation was not observed (Fig. 2B images M and N).

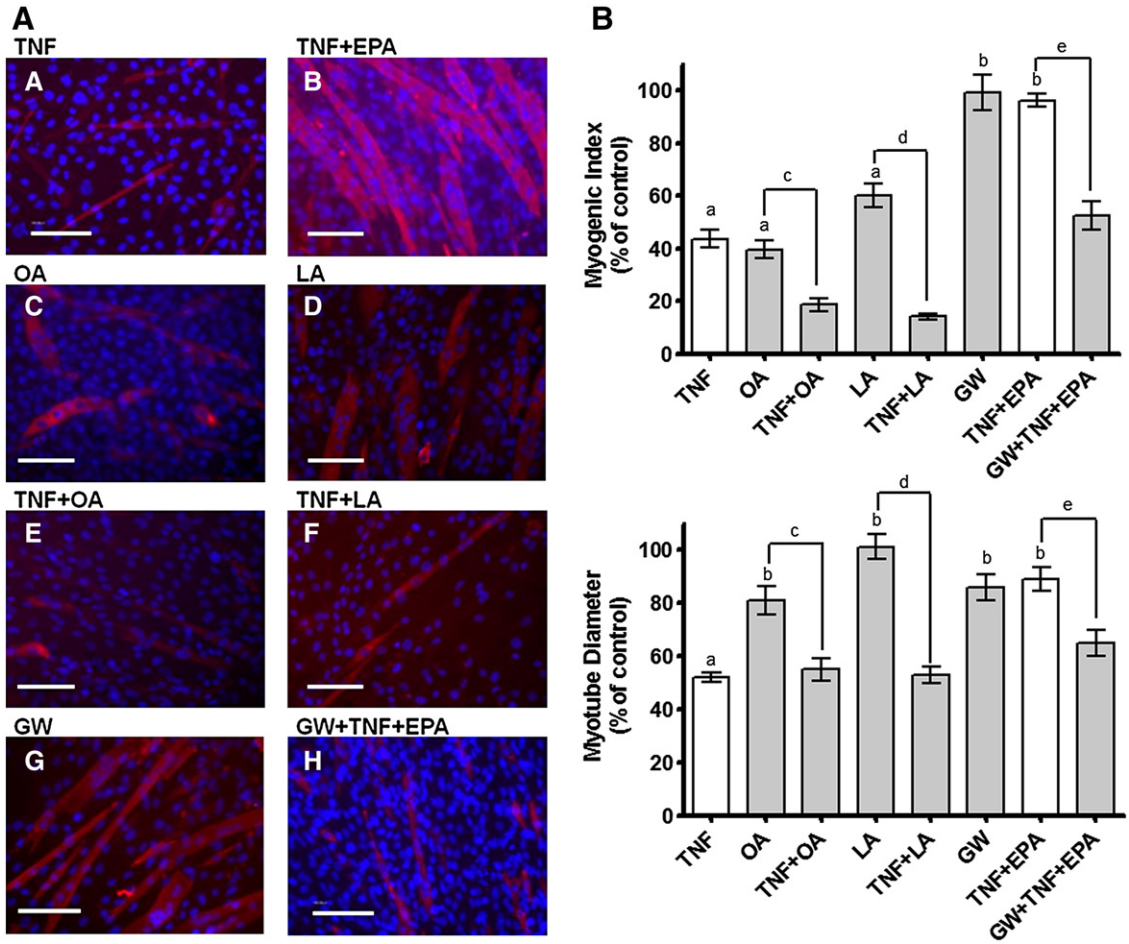


Fig. 1. PUFA-mediated protection from the damaging effects of TNF-α on C2C12 myotube formation is ω-3 specific and is blocked by a PPARγ antagonist. Images in (A) (images A–H) show immunofluorescence detection of Alexa Fluor 546-conjugated anti-MF20 antibody against MyHC (pink) and DAPI counterstaining of nuclei (blue) to detect myotubes. Images were obtained after 5 days of myotube differentiation following interventions with 20 ng/ml TNF-α alone (image A), TNF-α with 50 μM EPA (image B), 50 μM oleic acid alone (image C), 50 μM linoleic acid alone (image D), TNF-α with oleic acid (image E), TNF-α with linoleic acid (image F), 10 μM GW9662 (GW) pretreatment for 2 h (image G), GW pretreatment before TNF-α with EPA (image H). Quantitative markers of myotube metrics are shown in (B). Myogenic indices (upper graph) and MWs (lower graph) were calculated for each intervention. Significant differences between interventions are shown by letters: [a]$P<.05$ vs. untreated; [b]NS vs. untreated; [c]$P<.05$ vs. oleic acid; [d]$P<.05$ vs. linoleic acid; [e]$P<.05$ vs. TNF-α with EPA. Images are representative of three independent experiments. Data are expressed as means±S.E.M. from three independent experiments. Scale bars are 100 μm.

3.3. EPA inhibits TNF-α induced NF-κB transcriptional activation

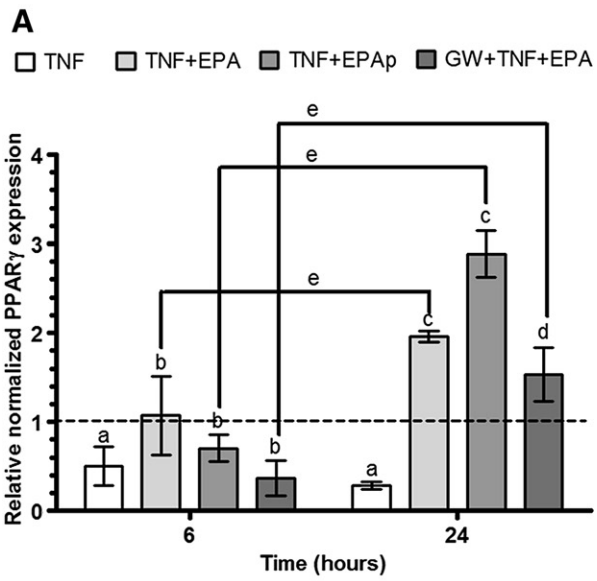
To investigate whether EPA activity inhibits TNF-α-mediated NF-κB activation, C2C12 cells stably transfected with an NF-κB luciferase reporter were utilized (Fig. 3). A time course of transcriptional activity up to 24 h in response to TNF-α and EPA showed that whereas EPA alone had no effect on NF-κB transcriptional activity (NS vs. untreated), treatment with TNF-α induced significant increases in NF-κB activation as early as 2 h, sustained through 24 h ($P<.05$ vs. untreated). The magnitude of the increased activity was approximately threefold at both times (NS vs. time points). Data are also shown for the blocking effect of EPA at 24 h (lower graph). NF-κB activity was dramatically reduced with EPA co-treatment to only 11% of the maximum induced by TNF-α ($P<.05$ vs. TNF). A similar but enhanced effect was observed with EPA pretreatment ($P<.05$ vs. TNF+EPA), which completely abrogated the TNF-α-increased NF-κB activity ($P<.05$ vs. TNF). These data show that EPA potently inhibits TNF-α-induced NF-κB transcriptional activity in differentiating skeletal muscle cells.

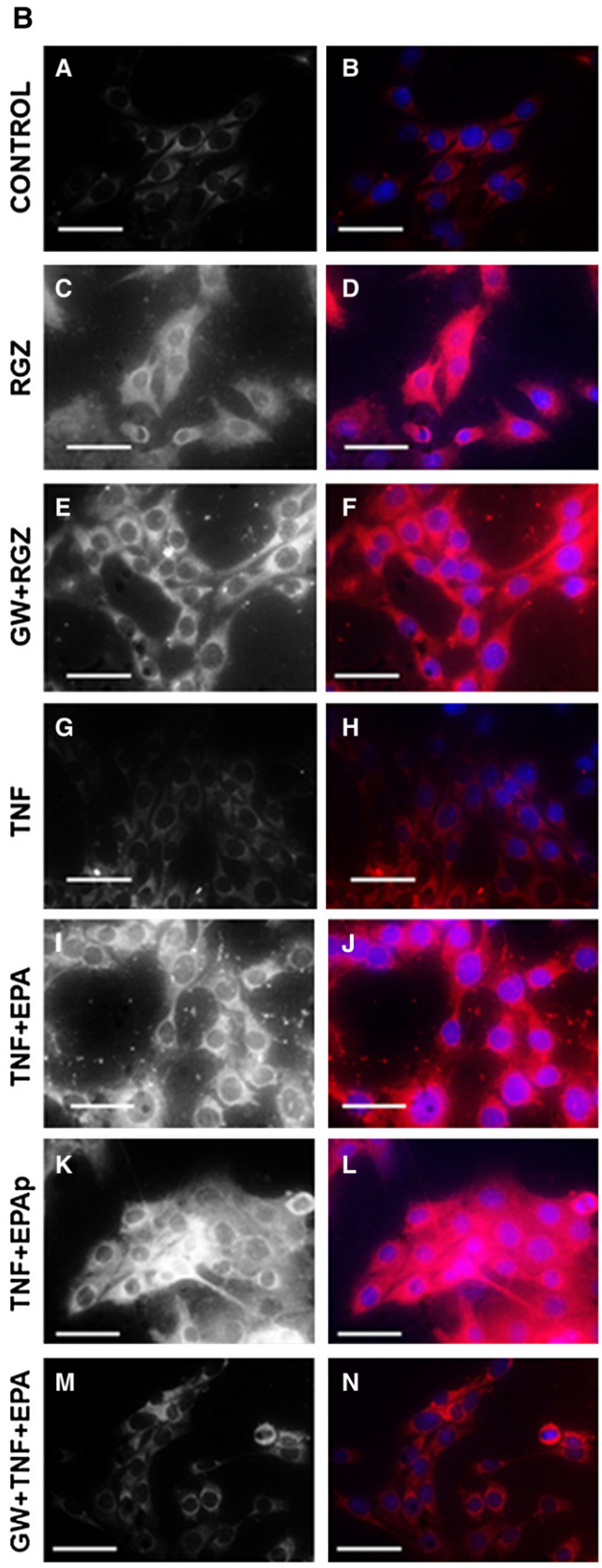
3.4. EPA inhibition of the NF-κB target gene, IL-6

To show that the effects of EPA on NF-κB result in functional outcomes, experiments were performed to measure the expression of a known proinflammatory target of NF-κB activation, namely, IL-6. Gene expression was examined using real-time quantitative PCR (Fig. 4A) and was normalized against β-actin, and IL-6 protein was measured in cell-conditioned media by ELISA (Fig. 4B), as described in Materials and Methods. In response to treatment with TNF-α, mRNA expression of IL-6 was significantly increased by approximately 3.5-fold, which was sustained through at least 24 h ($P<.05$ vs. respective control). EPA, administered either as a TNF-α co-treatment or as a pretreatment, significantly inhibited IL-6 expression compared to TNF-α alone at 6 h ($P<.05$) and at 24 h ($P<.05$), with some time-dependent enhancement of the EPA effect at 24 h, compared to 6 h, with co-treatment ($P<.01$). By 24 h, EPA co-treatment had reduced IL-6 expression to a level comparable with that in untreated controls. To determine whether this effect was dependent on EPA acting through induction of PPARγ, experiments were repeated with GW9662 pretreatment. Subsequent to GW9662 pretreatment, EPA suppression of the TNF-α-induced expression of IL-6 was unaltered at 6 h but removed by 24 h ($P<.01$).

Measurement of IL-6 protein in cell-conditioned media was performed by ELISA at 72-h, 96-h and 120-h time points. Untreated differentiating myotubes produce only small quantities of IL-6, and this does not alter during the experiment (NS vs. control 72 h). Similarly, EPA did not induce any additional secretion of IL-6 into

media throughout the duration of the experiments (NS vs. respective control). However, TNF-α dramatically up-regulated IL-6 secretion, >40-fold at all time points ($P<.01$ vs. respective control) and further increased with time, peaking at 96 h ($P<.01$ vs. 72-h TNF) before falling back slightly at 120 h, though still significantly higher than at 72 h ($P<.01$ vs. 72-h TNF). The ability of n-3 and other PUFA to





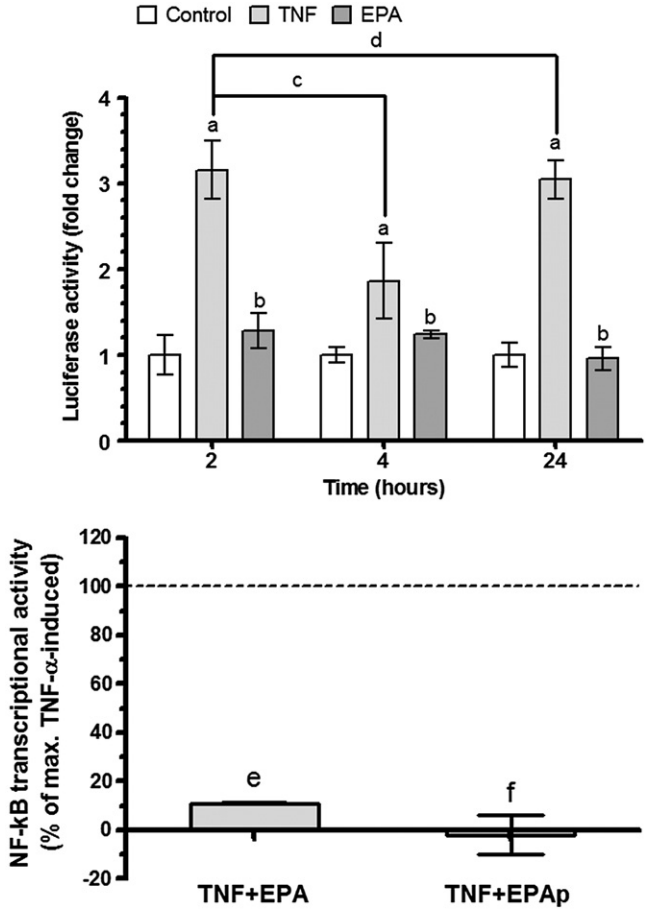


Fig. 3. EPA inhibits TNF-α-induced NF-κB transcriptional activity. Myoblasts from the NF-κB sensitive reporter stable cell line were differentiated for up to 24 h and were left untreated (control) or were treated with 20 ng/ml TNF-α alone, 50 μM EPA alone or a combination of treatments: TNF-α with EPA co-treatment; TNF-α with EPA pretreatment for 2 h then withdrawal. At 2 h and 24 h, cells were lysed, and luciferase activity was determined and normalized to total protein content. Time-dependent responses to TNF-α or EPA are expressed as the relative change in luciferase activity (upper graph), and the effect of EPA with TNF-α is shown as the % of maximum TNF-α-induced NF-κB activity (lower graph). Significant differences between interventions are shown by letters: [a]$P<.05$ vs. respective control; [b]NS vs. controls; [c]NS; [d]$P<.05$ vs. TNF-α; [e]$P<.05$ vs. TNF-α; [f]$P<.05$. Data are expressed as means±S.D. from three independent experiments.

suppress TNF-α-mediated IL-6 production is shown in the lower graph as a % of the maximum IL-6 production. EPA suppressed TNF-α-mediated IL-6 secretion, completely (approximately 96%) when delivered prior to TNF-α treatment ($P<.01$ vs. TNF-α) and severely (approximately 80%) when delivered in combination with TNF-α ($P<.01$ vs. TNF-α). Pretreatment with GW, before TNF+EPA co-treatment, ameliorated the effectiveness of EPA by approximately 50% ($P<.01$ vs. TNF+EPA). In contrast to EPA, neither OA nor LA had much impact on TNF-α-induced IL-6 production. Although there was a statistically significant decrease following OA treatment ($P<.01$ vs. TNF-α), this was very small (approximately 3%), whereas there was statistically significant, but relatively small, increase (approximately 10%) in IL-6 production with LA ($P<.01$ vs. TNF-α).

4. Discussion

The present study indicates that in response to TNF-α, skeletal muscle cell differentiation was severely impaired at a morphological level and was associated with increased NF-κB transcriptional activity and inhibition of PPARγ expression and activation. EPA treatment attenuated these damaging effects of TNF-α and was associated with inhibition of NF-κB transcriptional activity and stimulation of PPARγ expression and activation. Furthermore, EPA decreased transcript levels of known NF-κB target genes, IL-6 and TNF-α. This anti-inflammatory activity of EPA was found to be at least partially dependent on PPARγ activation. The ability of PUFA to block the effects of TNF-α on differentiation was selective for n-3 PUFA; an n-6, LA, and an n-9, OA, failed to restore myotube morphology or inhibit IL-6 production. However, parallel experiments with DHA showed that this n-3 PUFA was equally as effective as EPA (data not shown). Injury to skeletal muscle activates processes for local regeneration that involve activation of quiescent satellite cells to myoblasts that undergo differentiation to form new myotubes or contribute to repair of existing myotubes. Impairment of differentiation can contribute to muscle pathology found in chronic inflammatory diseases [2] and is associated with high systemic levels of proinflammatory mediators such as TNF-α. TNF-α is a pleiotropic cytokine that exerts many of its effects on skeletal muscle through NF-κB signaling [13]. TNF-α has been shown to induce further expression of inflammatory cytokines via transcriptional activation of NF-κB [27]. In particular, IL-6, an often-cited cytokine in muscle damage studies, has been reported to be targeted by TNF-α, resulting in a potentiation of IL-6 via the NF-κB pathway [28]. In a previous study, our group reported that EPA could block the inhibitory activity of TNF-α on myoblast differentiation [3]; however, the mechanism of this activity was not investigated. In the present study, the C2C12 model was employed as an established experimental model for investigating cytokine signaling pathways during skeletal muscle differentiation [29]. It was shown here that EPA, administered at 50 μM, prevented TNF-α-mediated NF-κB activation and target-gene expression and, additionally, that EPA anti-inflammatory activity was partially dependent on altered expression and activation of PPARγ.

Suppression of NF-κB activity appears to be a key aspect of how EPA exerts its anti-inflammatory activity [7]. It has been suggested this may occur via a reduction in phosphorylation and thus reduced degradation of the inhibitory IκB complex [30–32]. Others have suggested that the highly polyunsaturated EPA could be readily oxidized and that oxidized EPA then interferes with NF-κB activation [21]. A further possibility is that EPA suppressive activity on NF-κB is mediated through PPARs. These are a group of nuclear receptors that can participate in many physiological processes through binding with specific PPAR-response elements in target genes and have been found to exert anti-inflammatory effects in several cell types, including skeletal muscle [33]. All three different PPARs — PPARα, PPARδ and PPARγ — are present in C2C12 [34], although only PPARγ has been found to inhibit cytokine-induced NF-κB activity in C2C12 cells [16]. Data presented in this study show that TNF-α inhibits PPARγ mRNA expression and nuclear translocation in differentiating C2C12 and that EPA treatment was associated with increased expression of PPARγ transcripts and nuclear translocation of

Fig. 2. PPARγ expression is inversely regulated by TNF-α and EPA. Myoblasts were differentiated for up to 24 h and were left untreated (control) or were treated with 20 ng/ml TNF-α alone, 10 μM RGZ alone or a combination of treatments: TNF-α with EPA co-treatment; TNF-α with EPA pretreatment for 2 h then withdrawal; 10 μM GW9662 (GW) pretreatment for 2 h before RGZ; GW pretreatment for 2 h before TNF-α with EPA. At 6-h and 24-h time points, expression of PPARγ mRNA was assessed by real-time PCR and normalized to β-actin. PPARγ expression levels are shown relative to untreated control as means±S.E.M. from two independent experiments (A). Significant differences between interventions are shown by letters: [a]$P<.05$ vs. untreated and NS 6 h vs. 24 h; [b]NS vs. TNF-α 6 h; [c]$P<.05$ vs. TNF-α 24 h; [d]NS vs. TNF+EPA 24 h; [e]$P<.01$. Images A–N (B) show immunofluorescence detection of PPARγ only (left column) and for the same field of view both PPARγ (pink) and Hoechst (blue) detection of nuclei merged together (right column). Images are representative of three independent experiments. Scale bar=100 μm.

PPARγ. These data are consistent with findings that EPA increased PPARγ expression in human adipose tissue [35] and in colon cancer HT-29 cells [19]. In fact, it has been found that PUFAs including EPA and certain of their oxidized products are endogenous ligands for PPARs and can activate them [36]. Here, the specific PPARγ antagonist, GW9662, blocked EPA anti-inflammatory activity. The extent of this inhibition varied for different aspects of the EPA activity. Thus, complete blocking of EPA effects on myotube formation was apparent, as was the inhibition of PPARγ and IL-6 gene expression (6 h and 24 h, respectively) and PPARγ translocation. However, it was notable that GW9662 was only partially effective in releasing EPA-mediated inhibition of TNF-α-induced IL-6 protein production (as was MK886, a specific inhibitor of PPARα; data not shown), suggesting that some aspects of EPA anti-inflammatory activity also involve PPAR-independent pathways. GW9662 was also able to inhibit the activity of the PPARγ agonist RGZ on expression of PPARγ transcripts and nuclear translocation in this model. Inhibition of ligand-dependent PPARγ transcriptional activity by TNF-α is well known and appears to depend on the acute or chronic nature of the insult. In adipocytes, a chronic exposure (e.g., >16 h) inhibits PPARγ mRNA transcription. In acute exposures, gene transcription is not altered, but transcriptional activity of PPARγ is inhibited at the level of DNA binding either by the enhanced recruitment of nuclear corepressor protein [31] or by a failure to clear the repressor complexes [37]. Thus, in a delicate balancing act, PPARγ transcriptional activity depends on the presence of nuclear coactivators and corepressors that determine the outcome of DNA binding [31]. Whether this applies in skeletal muscle is unclear, but in a recent study with C2C12 myotubes treated for 4 h with a low dose of TNF-α (1 ng/ml), inhibition of PPARγ transcriptional activity was found not to be due to altered DNA binding [16]. In agreement, it was found that treatment of formed myotubes with EPA did not affect NF-κB DNA binding activity [22]. By comparison, in this report where the cells were treated during early differentiation, a pathologic dose of TNF-α was used (20 ng/ml), and the duration of treatment was for longer (24 h), at which time down-regulated expression of PPARγ transcripts was apparent. In the study by Huang et al. [22], a high 600-μM dose of EPA was used, and they found that lower doses were ineffective at regulating IκBα. They suggested that, under normal physiological conditions, higher concentrations of fatty acids were necessary for regulation, compared to pathophysiological conditions [22]. In this study, a lower (50μM) dose of EPA was effective under pathophysiological conditions, i.e., with proinflammatory TNF-α treatment, supporting this view. However, it must be acknowledged that any interpretation of physiological relevance of PUFA dosing is difficult and limited by the use of cell culture model systems. The 50-μM dosing used here is within the range employed in many other *in vitro* studies [9,10,17,38] and is physiologically achievable in

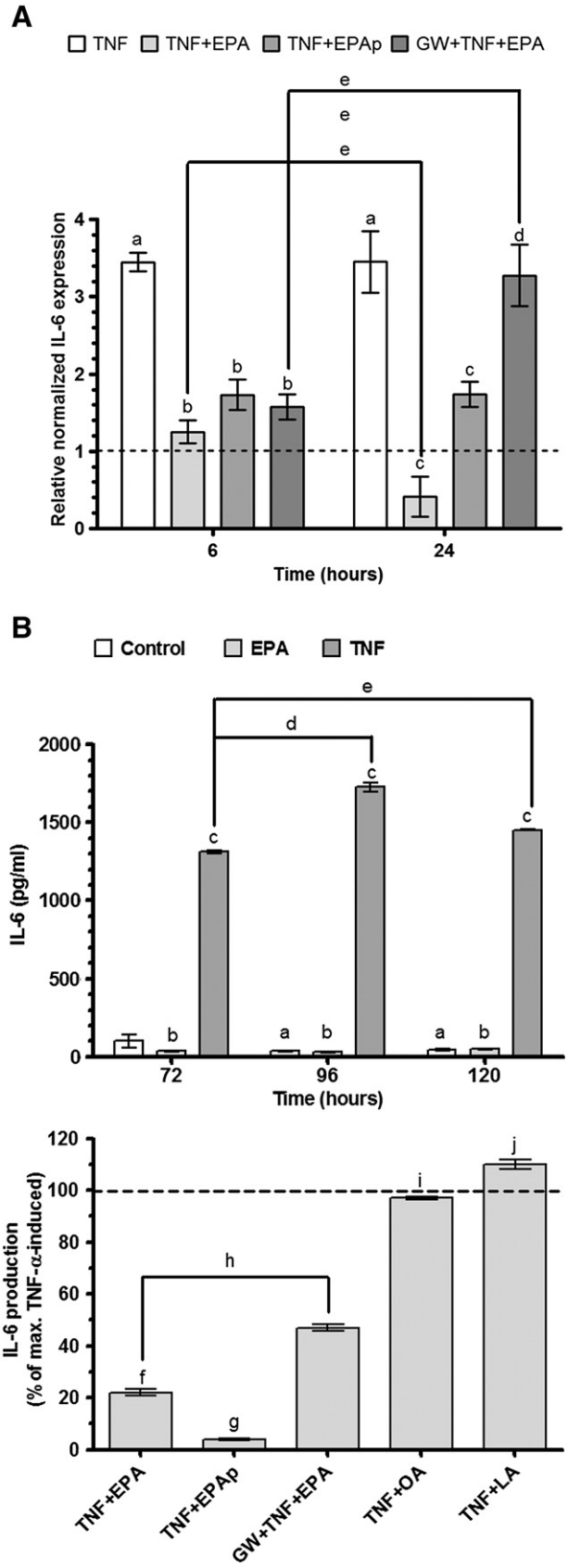


Fig. 4. EPA actions on proinflammatory NF-κB target gene expression are mediated via PPARγ. Myotubes were differentiated for up to 5 days and were left untreated (control) or were treated with 20 ng/ml TNF-α alone, 50 μM EPA alone or a combination of treatments: TNF-α with EPA co-treatment; TNF-α with EPA pretreatment for 2 h then withdrawal; TNF-α with 50 μM oleic acid; TNF-α with 50 μM linoleic acid; 10 μM GW9662 (GW) pretreatment for 2 h before TNF-α with EPA. At 6-h and 24-h time points, expression of IL-6 mRNA was assessed by real-time PCR and normalized to β-actin. IL-6 expression levels are shown relative to untreated control as means±S.E.M. from two independent experiments (A). Significant differences between interventions are shown by letters: [a]$P<.05$ vs. untreated and NS 6 h vs. 24 h; [b]$P<.05$ vs. TNF-α 6 h; [c]$P<.05$ vs. TNF-α 24 h; [d]NS vs. TNF-α 24 h; [e]$P<.01$. After 72 h and up to 120 h, conditioned media were collected for each intervention, and IL-6 levels were measured by ELISA (B). In the upper panel, a time course of IL-6 production is shown for forming myotubes, showing IL-6 levels in pg/ml. Significant differences between interventions are shown by letters: [a]NS vs. control 72 h; [b]NS vs. respective control; [c]$P<.01$ vs. respective control; [d]$P<.01$ vs. TNF-α 72 h; [e]$P<.01$ vs. TNF-α 72 h. In the lower panel, the ability of n-3 and other PUFA to suppress TNF-α-mediated IL-6 production is shown as a % of the maximum IL-6 production. Significant differences between interventions are shown by letters: [f]$P<.01$ vs. TNF-α; [g]$P<.01$ vs. TNF-α; [h]$P<.01$ vs. TNF+EPA; [i]$P<.01$ vs. TNF-α; [j]$P<.01$ vs. TNF-α. Data are expressed as means±S.E.M. from three independent experiments.

humans with supplementation [39]. Clearly, the actual concentration of free PUFA available at the cell surface will be much lower than this and will reflect experimental conditions, such as the PUFA/BSA molar ratio which is the principal determinant of the unbound fatty acid concentration [40]. In addition, the metabolic fate of the EPA was not considered as part of this study but may be important, as, for example, in many animal studies, it has been shown that consumption of dietary n-3 PUFA results in enrichment of the cellular membrane phospholipids [41]. In this study, a low EPA/BSA molar ratio was used to avoid any possible cytotoxicity, and although this would have resulted in a lower rate of EPA uptake, this was adequate to achieve efficacy.

To evaluate the functional relevance of these findings, the effects of EPA activation of PPARγ on the transcription of known endogenous NF-κB target genes was investigated. IL-6 transcripts and protein levels were increased in response to TNF-α treatment in this study. This supports a view that cytokines such as IL-6 and TNF-α may originate from skeletal muscle and modulate muscle synthesis *in vivo*. TNF-α is itself a target of NF-κB activity, and expression of TNF-α transcripts was also increased in this study (data not shown) after exogenous TNF-α treatment, confirming a previous report that TNF-α is able to modulate the expression of its own gene in this model [42]. IL-6 has both pro- and anti-inflammatory properties, and high levels of serum IL-6 are associated with injury, sepsis, cachexia and cancer [43] but also result from skeletal muscle production in exercising healthy individuals [44]. IL-6 produced by skeletal muscle may have trophic effects during skeletal muscle injury/repair [45,46] but may also contribute to muscle wasting [47,48], including sarcopenia [49]. Data presented here show that TNF-α-induced IL-6 production in differentiating cells was significantly reduced by EPA treatments. Supplementation of a resistance training program in older adults with the n-3 PUFA alpha-linolenic acid (ALA) reduced IL-6 levels in males and would be expected to increase EPA through metabolism of ALA [50,51]. These effects contrast with the reported activity of saturated fatty acids. Palmitate increases IL-6 expression in human skeletal muscle myotubes through NF-κB activation, and levels of palmitate correlate with serum IL-6 in individuals with obesity and type 2 diabetes [52]. Our data are in agreement with these findings, showing an n-3 specific reduction in IL-6 production compared to no effect or a small increase with OA and LA, respectively. As stated above, the actions of EPA on IL-6 protein were partially attenuated by GW9662, indicating that this activity may involve PPARγ-independent, as well as PPARγ-dependent, pathways. EPA has been found to attenuate IL-6 production in a PPARγ-dependent manner in C6 glioma cells [53]. Previous studies [38,54] have reported EPA-mediated effects on IL-12 cytokine production as being independent of PPARγ, but involving both activation of PPARγ expression and suppression of NF-κB, as reported for IL-6 here. The ability of EPA to suppress NF-κB activity may involve PPARγ. This is supported by findings from a recent study in which RNAi experiments were used to knockdown PPARγ in formed myotubes and, as a consequence, blocked the inhibitory activities of high (600 μM) doses of EPA against IκBα [22]. EPA may prevent phosphorylation and subsequent degradation of the IκBα subunit [7]. IκBα controls the nuclear corepressor HDAC3 and prevents its nuclear translocation [31]. Since HDAC3 binding with PPARγ inhibits its activity, the upstream activity of EPA on IκBα may result in enhanced PPARγ activity. Further experiments will be required to confirm and understand the nature of the EPA–PPARγ–NF-κB interactions that result in the observed anti-inflammatory actions of EPA.

In conclusion, it was found that EPA has potent anti-inflammatory activity against cytokine-induced impairment of C2C12 skeletal muscle cell differentiation, which is mediated through inhibition of NF-κB signaling and is associated with PPARγ expression and activation. EPA activity appears to be wholly PPARγ dependent for some aspects, whereas others, notably suppression of IL-6, involve PPARγ-independent mechanisms as well. The exact nature of this mechanism warrants further investigations since PUFAs such as EPA may represent a class of naturally occurring, low-toxicity PPAR ligands with potent anti-inflammatory properties that may be an interesting therapeutic avenue for treatment of inflammation-associated skeletal muscle damage in chronic disease states or aging.

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