Arachidonic acid supplementation enhances in vitro skeletal muscle cell growth via a COX-2-dependent pathway

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Markworth JF, Cameron-Smith D. Arachidonic acid supplementation enhances in vitro skeletal muscle cell growth via a COX-2-dependent pathway. Am J Physiol Cell Physiol 304: C56–C67, 2013. First published October 17, 2012; doi:10.1152/ajpcell.00038.2012.—Arachidonic acid (AA) is the metabolic precursor to a diverse range of downstream bioactive lipid mediators. A positive or negative influence of individual eicosanoid species [e.g., prostaglandins (PGs), leukotrienes, and hydroxyeicosatetraenoic acids] has been implicated in skeletal muscle cell growth and development. The collective role of AA-derived metabolites in physiological states of skeletal muscle growth/atrophy remains unclear. The present study aimed to determine the direct effect of free AA supplementation and subsequent eicosanoid biosynthesis on skeletal myocyte growth in vitro. C2C12 (mouse) skeletal myocytes induced to differentiate with supplemental AA exhibited dose-dependent increases in the size, myonuclear content, and protein accretion of developing myotubes, independent of changes in cell density or the rate/extent of myogenic differentiation. Nonselective (indomethacin) or cyclooxygenase 2 (COX-2)-selective (NS-398) nonsteroidal anti-inflammatory drugs blunted basal myogenesis, an effect that was amplified in the presence of supplemental free AA substrate. The stimulatory effects of AA persisted in preexisting myotubes via a COX-2-dependent (NS-389-sensitive) pathway, specifically implying dependency on downstream PG biosynthesis. AA-stimulated growth was associated with markedly increased secretion of PGE2 and PGF2α; however, incubation of myocytes with PG-rich conditioned medium failed to mimic the effects of direct AA supplementation. In vitro AA supplementation stimulates PG release and skeletal muscle cell hypertrophy via a COX-2-dependent pathway.

Arachidonic acid; C2C12; growth; nonsteroidal anti-inflammatory drug; skeletal muscle

ARACHIDONIC ACID (AA) is a polyunsaturated ω-6 fatty acid [20:4(ω6)], present in the diet, that is incorporated into cell membrane phospholipids (PLs) (19). Dietary change elicits rapid alterations in PL fatty acid composition, with supplemental AA increasing plasma PL abundance within days (42). In response to cellular perturbation (e.g., mechanical trauma, cytokines, or growth factors), AA is cleaved from PL molecules via the action of the enzyme PLA2. Free intracellular AA serves as a key transient cell-signaling intermediate, undergoing rapid enzymatic conversion to a diverse array of inflammatory autocrine/paracrine eicosanoid lipid mediators. Parallel cyclooxygenase (COX-1/COX-2) and lipxygenase (5-LOX/12-LOX/15-LOX) pathways catalyze the oxidation of AA to ultimately produce the PGs (PGE2, PGE5, PGF2α, PGI2, and thromboxane A2) and the leukotriene (LT)/lipoxin (LX) families of eicosanoids, respectively. Nonsteroidal anti-inflammatory drugs (NSAIDs) are thought to exert their anti-inflammatory, analgesic, and antipyretic action, specifically by inhibition of the COX branch of the AA pathway, indicative of a central role of PG species in mediating the inflammatory response.

Skeletal muscle cells express the COX genes COX-1 and COX-2 (49) and locally synthesize/release AA-derived PGs, including PGD2 (56), PGE2/PGF2α (33, 40, 41, 54), and PGI2 (2). NSAID treatment is associated with deleterious effects on adaptive skeletal muscle growth/regeneration (3, 4, 31, 39–41, 46), consistent with the inflammatory response being an important process in adaptive myofiber remodeling (7, 40, 50). NSAID treatment (26, 39) and genetic COX-2 deficiency (4, 41) also inhibit in vitro myogenesis, specifically implicating COX-2-derived PGs as locally secreted autocrine/paracrine factors necessary for normal control of skeletal muscle cell growth and development. Furthermore, skeletal muscle cells express LOX enzymes (60) and have the capacity to locally metabolize free intracellular AA through the LOX pathways to form 5-, 12-, and 15-hydroxyeicosatetraenoic acids (HETEs) (43).

Skeletal muscle cell growth typically involves parallel changes in myofiber size and myonuclear addition. Myonuclear accretion, characteristic of myofiber regeneration and hypertrophy, depends on the activation, proliferation, differentiation, and fusion of satellite cell-derived myoblasts with the growing multinucleated cell (20, 25, 30, 38). High exogenous concentrations of individual PGs elicit class-specific physiological effects on multiple stages of skeletal muscle cell growth. For example, PGI2 (2) and PGE2 (15, 17) enhance early and late muscle cell fusion events during myogenesis, respectively, leading to enhanced myonuclear accretion and increased myotube size. Conversely, PGD2 exerts negative effects on early skeletal myogenesis, characterized by impairment of myocyte differentiation/fusion (56). Similarly, PGF2α and PGE2 have opposing effects on skeletal muscle cell protein turnover in preexisting myotubes/myofibers, acting to increase rates of protein synthesis and protein degradation, respectively (37). Although the molecular mechanisms by which PGs may influence myogenesis/protein turnover remain poorly understood, the type F prostanoid receptor is coupled to intracellular skeletal muscle cell growth signaling via the nuclear factor of activated T cells C2 (15, 17) and mammalian target of rapamycin (mTOR) (23) pathways. In addition to COX-derived PGs, LOX metabolites of AA have been implicated in the control of skeletal muscle cell growth. Exogenous provision of the 5-LOX product LTB4 enhances myoblast proliferation/differentiation during myogenesis (48). On the other hand, the 15-LOX product 15-HETE has been implicated in mediating the skeletal muscle protein degradation response to the cata-

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bolic proteolysis-inducing factor (43, 57, 59). The collective role of the diverse and opposing effects of individual eicosanoids on muscle cell biology in physiological states of skeletal muscle hypertrophy and atrophy remains unclear.

Under physiological circumstances, multiple eicosanoid species are synthesized from free fatty acid substrates and act in concert to induce a local cellular response. Eicosanoid synthesis is dependent on membrane PL composition, local expression of COX/LOX isoforms, and downstream class-specific synthase enzymes and may vary widely across different tissues and/or physiological states. Mechanical stimulation of skeletal muscle cells promotes local membrane PL cleavage, free intracellular AA accumulation, PG synthesis/release, and cell growth (45, 54, 55). Consistently, inhibition of PG synthesis via NSAID administration in young healthy humans in vivo blunts adaptive myofiber protein synthesis (52, 53) and satellite cell proliferation (22, 27, 28) responses to resistance exercise. In contrast, skeletal muscle wasting associated with chronic inflammatory conditions, including cancer cachexia (24, 44, 47), arthritis (10), and aging-associated sarcopenia (36, 51), has been reported to be improved by systemic NSAID treatment. Thus, unlike the apparent positive collective role of the COX/PG pathway in adaptive muscle growth/regeneration in vivo, evidence is also indicative of a deleterious role of this pathway in the maintenance of skeletal muscle mass under conditions of chronic systemic low-grade inflammation.

The divergent reports of NSAIDs on skeletal muscle mass in vivo may potentially be explained by the diverse effects of the individual eicosanoid species discussed above. The net effect of heightened free AA availability appears to depend on the cumulative effects of the individual eicosanoids synthesized. For example, treatment of isolated rodent skeletal muscle tissue with AA was reported to promote rapid synthesis/release of PGF$_{2\alpha}$ and PGE$_2$ and stimulate muscle protein turnover (synthesis and degradation) (37, 45). Under ex vivo conditions, however, supplemental AA increased protein degradation in absolute terms to a greater extent than protein synthesis, the net result being skeletal muscle protein catabolism (37). Despite the importance of satellite cell myogenesis in skeletal muscle regeneration/hypertrophy (20, 25, 30, 38) and recent studies implicating specific individual PGs as important mediators of myoblast survival (17), proliferation (4, 26, 33, 39), differentiation (26, 39, 56), and fusion (2, 15, 26, 41), no study has investigated the direct net effect of heightened free AA availability on these processes.

The present study aimed to examine the impact of supplementation with exogenous free AA on skeletal myogenesis. It was demonstrated that AA enhances myotube growth and myonuclear accretion during myogenesis. Furthermore, the hypertrophic effects of AA supplementation persist postdifferentiation in preexisting myotubes. These effects are dependent on metabolism of supplemental AA and require COX-2 enzyme activity (i.e., are NSAID-sensitive), implying a role for downstream PG lipid mediators. Nevertheless, secreted factors alone, at the concentrations produced in response to AA supplementation, do not appear sufficient to account for the effect of AA on skeletal muscle cell growth.

**MATERIALS AND METHODS**

**Muscle cell culture.** Myoblasts from the skeletal muscle-derived C2C12 cell line were obtained from American Type Culture Collection. Proliferating myoblasts were cultured at 37°C in 5% CO$_2$ in DMEM [high-glucose, with phenol red and L-glutamine (GIBCO)] supplemented with 10% FBS (GIBCO) and antibiotics [100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO)]. Cells were grown to 100% confluence and then induced to differentiate by transfer to DMEM (high-glucose, with phenol red and L-glutamine) containing antibiotics and 2% horse serum (GIBCO).

**AA supplementation.** To assess the effect on myogenic differentiation and myotube development, AA (Cayman Chemicals) or vehicle control (0.1% ethanol final concentration) was added to the differentiation medium at the onset of differentiation. To assess the effect of AA supplementation on preexisting myotubes, C2C12 myoblasts were allowed to differentiate under typical conditions for 72 h (without a medium change) prior to initiation of AA supplementation (in fresh differentiation medium). After initiation of AA supplementation, at 24-h intervals, the culture medium was removed and replaced with fresh differentiation medium with or without AA. At this time, the medium bathing the cells (conditioned medium) was collected, snap-frozen, and stored at −80°C until further analysis.

**Nonmetabolizable AA analog.** Eicosatetraynoic acid (ETYA; Cayman Chemicals), a structural, yet nonmetabolizable, analog of AA, was used as previously described to assess the requirement of downstream AA metabolism in the cellular response to AA treatment (1, 8, 11, 18). Cells were treated in parallel with equivalent doses of ETYA or AA in select experiments.

**COX inhibitor studies.** The nonselective NSAID indomethacin and the COX-2-selective drug (COXIB) NS-398 were purchased from Cayman Chemicals. NSAIDs or the respective solvent vehicles (±0.1% ethanol for indomethacin and ±0.1% DMSO for NS-398) were added to the culture medium in the presence or absence of supplemental AA.

**ELISA.** ELISA was used to evaluate the conditioned differentiation medium collected from cells every 24 h. PGF$_{2\alpha}$ and PGE$_2$ levels were determined as suggested by the manufacturer’s protocol (PGF$_{2\alpha}$ and PGE$_2$ ELISA kits, Cayman Chemicals).

**Immunocytochemistry.** At the indicated times, culture medium was removed and cell monolayers were washed with PBS and fixed in 4% paraformaldehyde-PBS. Fixed cells were permeabilized with 0.1% Triton X-100, blocked in 1% BSA-PBS for 1 h at room temperature, and incubated with a primary antibody against sarcomeric myosin (MF20 supernatant, Developmental Studies Hybridoma Bank; 1:25 dilution) overnight at 4°C. Cells were washed in PBS and then incubated in goat anti-mouse IgG conjugated to Alexa Fluor 488 secondary antibody (1:250 dilution) and 4′,6-diamidino-2-phenylindole (DAPI; to counterstain myonuclei) for 1 h at room temperature. Cells were visualized with an inverted Olympus IX70 fluorescence microscope, and digital images were collected using a Spot RT slider camera and Magnifire software (Olympus). Global adjustments to image fluorescent signal brightness/contrast were made in Adobe Photoshop, and images were overlayed as red-green-blue channels [sarcomeric myosin (green) and DAPI (blue)] to facilitate quantitative morphological analysis.

**Morphological analysis.** To assess average myotube diameter, 50 myotubes per well were analyzed (3–4 independent culture wells per group). From each well, 5 fields of view were randomly selected, and the diameters of the 10 largest sarcomeric myosin-positive multinucleated cells in each field were measured at their widest uniform point along the middle portion of the myotube using ImageJ software (National Institutes of Health, Frederick, MD). For branching myocytes, each branch was measured as a separate myotube, and the region where the branches converge was excluded. To assess cell number, the mean total number of DAPI-stained nuclei in each field of view was quantified by counting all nuclei within 15–20 fields (5
fields in each of 3–4 individual culture wells) per group). To assess myogenic differentiation, the number of these DAPI-stained nuclei located within sarcomeric myosin-positive cytoplasm was determined and expressed as a percentage of total nuclei analyzed (%differentiation). The fusion index (%fusion) was quantified as the percentage of total DAPI-stained nuclei located within multinucleated cells [sarcomeric myosin-positive cells with ≥2 nuclei (thus having undergone fusion)]. Myonuclear accretion was additionally analyzed by performing myotube/nuclear number assays (5 fields of view per well, 3–4 wells per group). Results are expressed as myotube number (total number of myotubes per field of view), average number of nuclei per myotube, and relative percentage of nascent (2–4 nuclei) and mature (≥5 nuclei) myotubes.

Protein content quantification. Cells were harvested by scraping in ice-cold 1× radioimmunoprecipitation assay (RIPA) lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, and 1 mM EDTA with protease inhibitors (1 mM PMSF, 10 μg/ml aproamin, 10 μg/ml leupeptin, and 2 mM Na3VO4)] and rotated end-over-end for 1 h at 4°C. Cell lysates were centrifuged at 13,000 g at 4°C for 15 min, and the supernatant was stored at −80°C until further analysis. Total protein content of cell culture lysates was determined with a bicinchoninic acid protein kit (Thermo Scientific) according to the manufacturer’s protocol.

DNA extraction/quantification. Cells were harvested by scraping in TRI Reagent (Ambion). Chloroform was added to the solution, which was thoroughly mixed and incubated on ice for 10 min prior to centrifugation at 13,000 g at 4°C, the supernatant was removed, and the DNA pellet was washed thoroughly in 75% ethanol. Samples were centrifuged at 2,000 g for 5 min at 4°C, the supernatant was removed, and the DNA pellet was air-dried at room temperature for 5–10 min. The DNA pellet was dissolved in 8 mM NaOH, and DNA concentration was determined using a spectrophotometer (NanoDrop 1000, Thermo Scientific) following the manufacturer’s instructions.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 4.1. Values are means ± SE. Statistical significance was determined using one- or two-way ANOVA, as appropriate. After a significant main or interaction effect was found, pair-wise comparisons were determined with Bonferroni’s post hoc tests. Differences were considered significant at P < 0.05.

RESULTS
AA supplementation increases developing muscle cell size and protein accretion during myogenic differentiation. We initially sought to determine the effect of exogenous AA supplementation on muscle cell growth/development during myogenic differentiation. Myoblasts were grown to a high cell density and induced to differentiate in the presence or absence of supplemental AA. Culture treatment medium was replenished every 24 h, and cells were fixed and immunostained for sarcomeric myosin to visualize myotube structure 3 days after induction of differentiation (Fig. 1). Myotubes that formed in the presence of increasingly high doses of supplemental AA were noticeably larger and exhibited a shortened/thickened phenotype (Fig. 1A). The effect of supplemental AA on muscle cell size was dose-dependent, with 25 μM AA inducing the maximal response. Quantitative analysis of myotube diameter found statistically significant increases in cell size compared with control myotubes with ≥3.25 μM AA (P < 0.05; Fig. 1B). The maximal effective dose tested (25 μM AA) promoted a mean increase in myotube diameter of 80.6% (P < 0.001 vs. 0 μM; Fig. 1B). Analysis of the total protein concentration of lysates of cells treated with AA (20 μM) showed significant increases in total protein content per well at day 1 (P < 0.05), day 2 (P < 0.01), and day 3 (P < 0.001) of differentiation (Fig. 1C). Exposure to higher tested doses (50–100 μM) of supplemental AA resulted in loss of the AA-induced growth response and progressive detachment of myotubes from the growth surface, possibly indicative of cytotoxicity at these doses (data not shown). These data suggest that AA supplementation during myogenesis can enhance skeletal myotube growth in a dose-dependent manner, and this effect is associated with heightened overall total protein accretion.

AA-induced skeletal muscle cell hypertrophy does not involve changes in myoblast proliferation/survival or extent of myogenic differentiation. To further assess the effect of AA supplementation on various stages of myogenesis, cultures were fixed, immunostained for sarcomeric myosin, and counterstained for DAPI (to visualize myonuclei) at confluence and then every 24 h throughout differentiation. Whole control and AA-treated cultures appear morphologically indistinguishable at confluence, after 48–72 h, obvious visual differences in the morphology of developing myotubes become apparent (Fig. 2A). To assess whether increased myotube size in response to supplemental AA may be attributable to changes in cell density as a result of altered myoblast proliferation and/or survival, the total number of DAPI-stained nuclei per field of view was counted (Fig. 2B). As a second measure of cell density, the total amount of DNA per well was determined (Fig. 2B). Neither number of nuclei nor DNA content per well significantly changed throughout 3 days of differentiation under normal culture conditions. Furthermore, at no time point did number of nuclei or DNA content differ between vehicle- and AA-treated cultures, indicating that cell density is not influenced by AA (Fig. 2B). Thus the myotube growth response to AA supplementation does not appear to be associated with net changes in cell density.

To assess myoblast differentiation and fusion, the percentage of total nuclei located within sarcomeric myosin-positive cytoplasm (differentiation index) and the percentage of total nuclei within multinucleated (fused) cells (≥2 nuclei; fusion index) were determined (Fig. 2B). At confluence, some degree of spontaneous differentiation (~10% of cells) was apparent in control cells, with a minimal amount of cell fusion (~5% of cells). At 24 h after serum withdrawal, ~30% of the myoblast population had undergone differentiation, and small nascent myotubes (2–4 nuclei) were predominant. After 48–72 h, the differentiation index was maximal, with ~50% of total nuclei located within sarcomeric myosin-positive cells, the vast majority of which have fused. At all times, differentiation and fusion indexes were comparable between vehicle- and AA-treated cultures (Fig. 2B). These findings suggest that AA-treated cultures are comparable to control in terms of total cell number, as well as the rate/extent of myogenic differentiation and early myoblast-myoblast fusion to form small nascent multinucleated myotubes.

AA-induced myotube growth is associated with heightened myonuclear accretion. Despite a comparable proportion of the total cell population undergoing differentiation and fusion, visual differences in the extent of myotube multinucleation (number of nuclei per myotube) in AA-supplemented cultures...
were apparent (Fig. 2A). To quantitatively determine changes in myonuclear accretion that may contribute to augmented myotube size, the number of myotubes (sarcomeric myosin-positive cells with \( \geq 2 \) nuclei) per microscopic field of view and the average number of myonuclei per myotube (number of fused nuclei per myotube) were counted (Fig. 2C). Compared with control, AA-treated cultures contained approximately half the total number of myotubes per field of view at day 3 of differentiation (Fig. 2C). As the total number of fused cells was comparable to control (see above), myotubes that formed in the presence of supplemental AA contained, on average, approximately twice as many myonuclei per myotube (Fig. 2C). Nuclear number assays revealed that the lower myotube number was predominantly attributable to the presence of fewer small nascent myotubes (2–4 nuclei per myotube) in AA-treated cultures. Therefore, clustering of fused myonuclei into larger, less numerous myotubes was accompanied by a reduction in the relative percentage of small myotubes (Fig. 2C). These data indicate that AA supplementation specifically enhances cell recruitment and myonuclear accretion by the growing myotubes.

A nonmetabolizable analog fails to mimic the effect of AA. In addition to being the metabolic precursor in the biosynthesis of eicosanoids, AA can directly influence certain aspects of cellular physiology (5). ETYA is a structural analog of AA in which the four alkene bonds are replaced with alkyne bonds. As a nonmetabolizable analog, ETYA acts as a nonspecific COX/LOX inhibitor by competing with AA for enzyme binding. However, certain physiological effects induced by AA have also been reported to be mimicked by ETYA (1, 8, 11, 18). The effect of AA in such cases has been considered to be independent of its metabolism. To investigate whether the effect of AA on myotube growth is a direct effect of supplemental AA or, alternatively, is mediated downstream of AA
metabolism, myoblasts were induced to differentiate in the presence of vehicle (0.1% ethanol), the maximally effective dose (25 μM) of AA, or an equivalent dose (25 μM) of ETYA (Fig. 3A). AA supplementation enhanced myotube size (Fig. 3B) and myonuclear accretion (Fig. 3 C and D). Conversely, ETYA failed to mimic the effects of supplemental AA. Rather, myotubes that formed in the presence of ETYA exhibited significantly reduced myotube diameter, a decreased percent-
age of myotubes with five or more nuclei, and increased myotube number compared with vehicle cultures (Fig. 3, B–D). These data suggest that AA does not directly influence myotube growth; rather, this effect is secondary to its downstream metabolism. Furthermore, blunted basal growth in the presence of ETYA may be indicative of competition for COX/LOX binding, with endogenous free AA present under typical control cell culture conditions.

**COX-2 activity is required for basal myogenesis and AA-stimulated skeletal muscle cell hypertrophy during differentiation.** The COX enzymes COX-1 and COX-2 catalyze the synthesis of the PG class of lipid mediators from free intracellular AA substrate. To assess the requirement for COX activity in basal and AA-induced C2C12 skeletal muscle cell growth, COX-inhibiting NSAIDs were utilized. Confluent myoblasts were induced to differentiate in the presence or absence of the nonselective COX inhibitor indomethacin or the COX-2-selective inhibitor NS-398 (Fig. 4A). Indomethacin and NS-398 dramatically impaired basal C2C12 myotube formation at the higher doses tested. These findings show that nonselective and, more specifically, COX-2-selective NSAIDs inhibit in vitro C2C12 myogenesis similarly to that previously reported in primary skeletal muscle cell culture (26, 39).

To assess the requirement of COX activity in AA-induced skeletal muscle cell growth, confluent myoblasts were induced to differentiate with or without supplemental AA in the presence or absence of NSAIDs. Quantitative analysis revealed that cultures exposed to indomethacin (200 μM) or NS-398 (50 μM) alone throughout myogenesis displayed significantly impaired differentiation at day 3 (Fig. 4C). Additionally, indomethacin alone significantly reduced nuclear density compared with vehicle (Fig. 4B). In the presence of indomethacin or NS-389, AA failed to enhance myotube formation. Rather, defects in nuclear density and percent differentiation were significantly greater when NSAIDs were combined with supplemental AA than when NSAIDs were given alone (Fig. 4, B and C). These data suggest that COX-2 activity is required for successful early C2C12 myogenesis and that the deleterious effects of COX-2 blockade on myoblast differentiation are amplified at a given NSAID dose in the presence of a heightened availability of free AA substrate.

**Conditioned medium from AA-supplemented cultures fails to mimic the effects of direct AA supplementation.** A number of secreted autocrine/paracrine factors, including growth factors, cytokines, and eicosanoids, have been implicated in the control of muscle cell growth. To determine whether the effect of AA on skeletal muscle cell growth is mediated by a secreted autocrine/paracrine factor, conditioned medium was collected from vehicle- and AA-supplemented cells every 24 h and fed to differentiating muscle cell cultures grown in parallel (Fig. 5). As expected, direct AA supplementation markedly enhanced muscle cell growth (Fig. 5A). AA-stimulated muscle cell growth was associated with marked increases in the secretion of PGE$_2$ by a COX-dependent (indomethacin-sensitive) pathway (Fig. 5C). Nevertheless, myotube diameter and the percentage of myotubes with five or more nuclei did not differ significantly between cells treated with conditioned medium from AA-supplemented cultures and those treated with conditioned medium from non-AA-supplemented cultures (Fig. 5B). Similarly, neither group of conditioned medium-treated cells differed in these markers of myotube growth compared vehicle donor cultures. These data suggest that stable secreted autocrines are critical for AA-induced skeletal muscle cell growth.
crine/paracrine factors at the concentrations synthesized and released by cells do not appear sufficient to mimic the effect of supplemental AA on skeletal muscle cell size or myonuclear accretion.

Postdifferentiation AA supplementation stimulates hypertrophy of preexisting myotubes by a COX-2-dependent pathway. Because of the requirement of COX-2 during early myogenesis, NSAIDs may block the effect of supplemental AA on later stages of muscle cell growth indirectly by preventing myotube formation and/or by limiting the number of differentiated myocytes available to support myotube hypertrophy. To further test the role of COX-2 in AA-stimulated skeletal muscle cell growth without the potential confounding influence of NSAIDs on early myogenesis, myotubes were allowed to develop for 3 days prior to treatment with AA with or without NSAIDs. After 72 h of AA supplementation (day 6 of differentiation), cultures were fixed and stained for sarcomeric myosin without the potential confounding influence of NSAIDs on early myogenesis, myotubes were allowed to develop for 3 days prior to treatment with AA with or without NSAIDs. After 72 h of AA supplementation (day 6 of differentiation), cultures were fixed and stained for sarcomeric myosin (Fig. 6A). Consistent with the hypothesis that AA-stimulated growth does not involve early myogenesis, supplementation of cells with AA from day 3 to day 6 postdifferentiation promoted dose-dependent increases in myotube size of comparable magnitude to that observed throughout early myogenesis (Fig. 6B). Postdifferentiation myotube hypertrophy was also associated with heightened myonuclear accretion (albeit to a lesser extent than during differentiation; Fig. 6D, bottom right). In contrast to the effects of NSAIDs during early myogenesis, exposure to indomethacin or NS-398 alone from day 3 to day 6 of differentiation did not influence myotube diameter (Fig. 6C), nuclear density (Fig. 6D, top left), percent differentiation (Fig. 6D, top right), or myotube number (Fig. 6D, bottom left). Nevertheless, in cotreatment experiments, indomethacin (100–200 μM) or NS-398 (50 μM) significantly blocked the effect of AA supplementation on postdifferentiation myotube hypertrophy (Fig. 6C) and myonuclear accretion (Fig. 6D, bottom right).

DISCUSSION

PL hydrolysis, leading to free AA release and intracellular accumulation, is a key cellular process in the control of inflammation and cell signaling (5). Unesterified intracellular AA is metabolized by multiple downstream COX and LOX pathways to form a diverse array of potential downstream bioactive lipid mediators (e.g., PGs, HETEs, LTs, and LXs). The present study was undertaken to determine the effect of heightened free AA availability on in vitro skeletal muscle cell growth. AA supplementation stimulated dose-dependent increases in myotube size and myonuclear accretion, without
influencing total cell number or the rate/extent of myogenic differentiation. These effects were associated with heightened PGE$_2$ and PGF$_{2\alpha}$ secretion, required metabolism of supplemental AA, and were prevented by nonselective or COX-2-selective NSAID administration. These findings show that heightened AA availability via supplementation enhances endogenous PG synthesis and stimulates net skeletal muscle cell hypertrophy via a COX-2-dependent pathway.

AA supplementation had no effect on cell density (which may be influenced by changes in proliferation and/or survival) or the rate/extent of myogenic differentiation (%differentiation). Nevertheless, myonuclear accretion (number of nuclei per myotube) and myotube size were greatly enhanced by AA supplementation. These effects specific to later stages of myotube growth/development are consistent with previous reports of exogenous treatment with high doses of the single prostanooids, including PGF$_{2\alpha}$ (15, 17) and PGI$_2$ (2). The fusion events that regulate the formation of multinucleated skeletal muscle cells involve two distinct phases: 1) myoblast-myoblast fusion to form small nascent myotubes containing few myo-

Fig. 5. AA supplementation greatly increases PG release; however, PG-containing conditioned medium fails to mimic effects of direct AA supplementation. A: C2C12 myoblasts were induced to differentiate in the presence or absence of direct AA (25 μM) supplementation (top). Every 24 h, conditioned differentiation medium from these donor cultures was collected and fed to confluent differentiating myoblast cultures grown in parallel (bottom). Cells were immunostained for sarcomeric myosin (green) and DAPI (blue) at day 3 of differentiation. Scale bar, 100 μm. B: nuclear number assays and quantification of myotube width performed to determine percentage of myotubes containing 2–4 vs. ≥5 nuclei and mean myotube diameter. C: PGF$_{2\alpha}$ and PGE$_2$ concentrations in conditioned culture medium as determined by ELISA. PGF$_{2\alpha}$/PGE$_2$ data are from the 24- to 48-h supplementation/collection period. Values are means ± SE of 3 independent experiments. ***P < 0.001 vs. vehicle.
Fig. 6. AA supplementation maintains growth-enhancing effects in preexisting myotubes via a COX-2-sensitive pathway. A: C2C12 myoblasts were differentiated into myotubes for 3 days, treated with supplemental AA in the presence or absence of indomethacin (0.125% ethanol vehicle) or NS-389 (0.1% DMSO + 0.025% ethanol vehicle) for an additional 72 h, and then immunostained for sarcomeric myosin (green) and DAPI (blue) on day 6. Scale bar, 100 μm. B: myotube diameter for postdifferentiation cultures treated with increasing doses of supplemental AA. Different letters (A, B, C, D) indicate significant difference (P < 0.05). C: myotube diameter determined for postdifferentiation cultures treated with 25 μM supplemental AA in the presence or absence of 100–200 μM indomethacin or 50 μM NS-398. D: nuclear and myotube counts for postdifferentiation cultures treated with 25 μM supplemental AA in the presence or absence of indomethacin (200 μM) or NS-398 (50 μM). Respective vehicle (DMSO and ethanol)-treated cells did not significantly differ, so results were pooled as control. ***P < 0.001, **P < 0.01, *P < 0.05 vs. vehicle.
nuclei and 2) subsequent fusion of myoblasts with preexisting multinucleated cells, leading to myonuclear addition and increased myotube/myofiber size (14, 16, 35). The effect of AA appears specific to the later stages of muscle cell fusion. The mechanisms by which skeletal muscle cell fusion occur are incompletely understood, but studies have implicated stable secreted myoblast recruitment factors, which enhance the recruitment of differentiating mononucleated myoblasts by large hypertrophying myotubes (6, 13). Our data from conditioned medium experiments suggest that such mechanisms are unlikely to explain the effects of AA on muscle cell fusion. Although the underlying mechanism involved remains to be determined, our data reveal that AA is among a growing list of stimuli, including creatine (32), PGF2α, (15, 17), and IL-4 (6, 13), as well as intracellular pathways, including mTOR (34) and nuclear factor of activated T cells C2 (12, 35), that appear capable of regulating developing muscle cell size via control of fusion-related myonuclear accretion.

In addition to being the metabolic precursor to bioactive lipid mediators, AA is purported to possess biological activity, independent of its downstream metabolism (5). Provision of exogenous free AA substrate has been reported to directly influence MAPK signaling (1), intracellular Ca2+ concentrations (9, 58), and Na+/K+ currents (8, 11, 18) in other cell types, all of which may be relevant to the control of skeletal muscle cell growth. In these studies the effect of supplemental AA was mimicked by the nonmetabolizable AA analog ETYA, inferring a response to AA independent of the synthesis of bioactive lipid mediators (1, 8, 9, 11, 18, 58). An important consideration in the present study was thus whether the effects were mediated by AA or one or more downstream metabolites. We found that ETYA did not mimic exogenous AA supplementation and, rather, had opposite effects on developing myotube size/myonuclear content. Therefore, the effect of AA supplementation on skeletal muscle cell growth appears secondary to downstream metabolism. As a structural analog, ETYA functions as a nonselective COX/LIBX inhibitor by competing with AA for enzyme-binding sites. Thus the negative effect of ETYA on basal myotube size further suggests that endogenous AA, present under control cell culture conditions, may also contribute to basal cell growth.

Consistent with previous reports that NSAIDs may negatively influence skeletal muscle growth via deleterious effects on satellite cell myogenesis (22, 26, 28, 29, 31, 39), we found basal C2C12 myogenesis to be impaired by nonselective and COX-2-selective NSAIDs. These findings support an important role of the COX-2 pathway in basal early myogenesis (4, 26, 39, 41). Despite the lack of a stimulatory effect of AA supplementation on this stage of muscle cell development, surprisingly, AA supplementation potentiated the blockade of myogenesis induced by a given NSAID dose. This was unexpected, given that the negative effect of NSAIDs on myogenesis has been attributable to a decrease in the basal release of downstream effectors, including PGF2α or PGE2 (41). While secreted PGF2α and PGE2 were greatly reduced by cotreatment with AA and NSAID in the present study, their release was not entirely abolished and remained above basal levels. Other factors, such as accumulation of excess intracellular free AA substrate and/or diversion of surplus AA via parallel COX-independent pathways, may contribute to the negative influence of NSAIDs on myogenesis under certain circumstances.

As NSAIDs inhibit the early stages of basal myogenesis, they may indirectly prevent AA-induced myotube growth by reducing cell density and/or preventing normal myogenic differentiation. To assess the requirement of COX enzyme activity in AA-stimulated myotube growth without the influence of the potentially confounding effects of NSAIDs on early myogenesis, AA supplementation was additionally tested in post-fusion myotube cultures. Our findings suggest that COX activity plays little role in the maintenance of healthy mature myotube/myofiber mass, as NSAID treatment had no effect on the basal size of preexisting myotubes. This is in contrast to reports that NSAID treatment increased the mass of atrophied skeletal muscle in states of chronic inflammatory disease (10, 21, 24, 36, 44). The positive effects of AA on myotube size persisted in preexisting myotubes, and these effects were entirely prevented by nonselective or COX-2-selective NSAID cotreatment. Thus COX-2 appears to play a positive role in mediating growth of preexisting myotubes in response to AA, independent of its established role in early myogenesis. The hypertrophic effect of supplemental AA in preexisting myotubes appears at odds with an earlier report of a net catabolic effect in terms of short-term rates of protein turnover in response to acute free exogenous AA exposure in skeletal muscle tissue isolated ex vivo (37). Variations in experimental conditions (ex vivo vs. in vitro) and AA treatment regimens (acute vs. chronic) may explain these differences. Collectively, our data provide evidence to suggest that the net effect of supplemental AA in actively growing myotubes is anabolic and mediated by a COX-2-dependent pathway.

The primary biologically active COX-derived PGs (PGD2, PGE2, PGF2α, PGI2, and thromboxane A2) are short-lived molecules in vivo, undergoing rapid enzyme-dependent degradation to form a complex array of PG metabolite products. In vitro, exogenous AA exposure induces PGE2 and PGF2α synthesis/release of PGs within the cell culture medium to easily detectable levels (2, 30, 40, 41, 54, 56). AA supplementation greatly increased the synthesis/release of PGE2 and PGF2α by skeletal muscle cells in the presence of supplemental AA. These PGs accumulated in the culture medium to relatively high concentrations (30–40 nM) in the presence of supplemental AA. Nevertheless, when fresh cells grown in parallel were treated with PG-containing conditioned medium, no significant effects on cell morphology were apparent. Therefore, in contrast to previous reports with exogenous PG, the levels of eicosanoids secreted endogenously by skeletal muscle cells during AA incubation appear to have little effect on muscle cell growth. Such disparity between the requirement for COX activity and the role of downstream PGs in mediating COX-dependent effects has been observed previously. For example, mechanical stretch-induced increases in skeletal muscle protein turnover are NSAID-sensitive (COX-dependent), but much higher concentrations of exogenous PGF2α and PGE2 than that produced physiologically during mechanical stimulation are required to influence basal muscle protein turnover (54). In another study, ≥1 μM exogenous PGF2α was reported to be required to significantly influence skeletal muscle cell growth (15). Such concentrations are substantially higher than that produced in cell culture endogenously, even in the presence of high doses of supplemental AA substrate employed in the present investigation. On the other hand, low-nanomolar to micromolar
doses of exogenous PGF2α were sufficient to stimulate dose-dependent changes in growth-related ERK/mTOR signaling in C2C12 myotubes (23). A recent study investigating the stability of eicosanoid species in cell culture medium showed that the primary PGs undergo gradual degradation over time under typical cell culture conditions (22a). On this basis, growing cells may be exposed to significantly higher concentrations of PGs than remain in conditioned medium following 24 h of incubation (and subsequent conditioned medium incubation). A further possibility is that, over the treatment period, AA may exert additional effects that heighten the capacity of cells to respond to secreted PGs. Future studies should focus on delineating the downstream COX-2-dependent events involved in AA-stimulated skeletal muscle cell hypertrophy.

In conclusion, the findings of the present study show that an increased availability of free AA and subsequent metabolism by the COX-2 pathway have a net stimulatory effect on in vitro skeletal muscle cell growth. This effect appears distinct from the role of COX-2 in early myogenesis, as AA supplementation of postdifferentiation myogenic cells also promotes hypertrophy of preexisting myotubes by a NSAID-sensitive pathway. The specific downstream COX-2-dependent events responsible to be determined but do not appear to exclusively involve endogenously secreted eicosanoids (or other stable secreted factors) at the concentrations present in conditioned medium following AA treatment.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.F.M. and D.C.-S. are responsible for conception and design of the research; J.F.M. performed the experiments; J.F.M. and D.C.-S. analyzed the data; J.F.M. and D.C.-S. interpreted the results of the experiments; J.F.M. prepared the figures; J.F.M. and D.C.-S. drafted the manuscript; J.F.M. and D.C.-S. edited and revised the manuscript; J.F.M. and D.C.-S. approved the final version of the manuscript.

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