

Published in final edited form as:

*J Nutr Biochem.* 2014 August ; 25(8): 868–874. doi:10.1016/j.jnutbio.2014.03.017.

## Docosahexaenoic acid prevents palmitate-induced activation of proteolytic systems in C2C12 myotubes

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### Abstract

Saturated fatty acids like palmitate contribute to muscle atrophy in a number of conditions (e.g., Type II diabetes) by altering insulin signaling. Akt is a key modulator of protein balance that inhibits the FoxO transcription factors (e.g., FoxO3) which selectively induce the expression of atrophy-inducing genes (atrogenes) in the ubiquitin-proteasome and autophagy-lysosome systems. Conversely, omega-3 polyunsaturated fatty acids have beneficial effects on insulin signaling and may preserve muscle mass. In an earlier report, the omega-3 fatty acid docosahexaenoic acid (DHA) protected myotubes from palmitate-induced atrophy; the mechanisms underlying the alterations in protein metabolism were not identified. This study investigated whether DHA prevents a palmitate-induced increase in proteolysis by restoring Akt/FoxO signaling. Palmitate increased the rate of protein degradation, while co-treatment with DHA prevented the response. Palmitate reduced the activation state of Akt and increased nuclear FoxO3 protein while decreasing its cytosolic level. Palmitate also increased the mRNAs of two FoxO3 atrogene targets, the E3 ubiquitin ligase atrogin-1/MAFbx and the autophagy mediator Bnip3. DHA attenuated the effects of palmitate on Akt activation, FoxO3 localization, and atrogene mRNAs. DHA, alone or in combination with palmitate, decreased the ratio of LC3B-II:LC3B-I protein as well as the rate of autophagosome formation, as indicated by reduced LC3B-II protein in the presence of 10 mmol/L methylamine, suggesting an independent effect of DHA on the macroautophagy pathway. These data indicate that palmitate induces myotube atrophy, at least in part, by activating multiple proteolytic systems and that DHA counters the catabolic effects of palmitate by restoring Akt/FoxO signaling.

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## Keywords

palmitate; docosahexaenoic acid; muscle atrophy; proteolysis; macroautophagy; FoxO3

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## Introduction

Skeletal muscle atrophy contributes to a debilitating loss of functional independence and increases the rate of mortality in individuals with numerous chronic illnesses, including diabetes, renal and heart failure, and cancer [1]. Currently there are few effective treatments that counteract muscle atrophy [2]. Lipotoxicity contributes to muscle atrophy in a number of conditions [3]. In obesity and Type II diabetes, there is an increase in lipid flux and accumulation in skeletal muscle [4]. Recent evidence indicates that both the quantity and saturation of intramyocellular and extramyocellular lipid depots are elevated in skeletal muscle from diabetic patients compared to healthy controls [5]. Rodents that are fed a high-fat diet develop obesity and insulin resistance which have been linked to muscle atrophy [6]. Direct lipotoxicity is among the proposed mechanisms for obesity-related insulin resistance. In support of this hypothesis, treatment of cultured myotubes with the saturated fatty acid palmitate causes similar defects in insulin signaling and reduces myotube diameter [7].

Reduced insulin signaling through the protein kinase Akt can result in the loss of muscle protein through up-regulation of multiple proteolytic systems, including the ubiquitin-proteasome (UbP) and autophagy-lysosome pathways [8, 9]. Akt inactivates the Forkhead box (FoxO3) transcription factors, including FoxO3, which induce the expression of several key atrophy-inducing genes, termed “atrogenes,” including the muscle-specific E3 ubiquitin ligases MuRF1 and atrogin-1/MAFbx [10, 11] and the autophagy mediator Bnip3 [12]. Enhanced expression of MuRF-1 and atrogin-1/MAFbx is linked to muscle atrophy in a variety of disease models and targeted inactivation of either of them reduces muscle loss [13–15]. Bnip3 is important for macroautophagic remodeling of the mitochondrial network [16]. Increasing expression of Bnip3 by constitutively active FoxO3 enhances macroautophagy and decreases muscle mass, while knockdown of Bnip3 reduces autophagosome formation induced by either constitutively active FoxO3 or fasting conditions [12]. LC3 is a small ubiquitin-like molecule required for membrane commitment and growth into autophagosomes [16]. It is enzymatically cleaved to form LC3-II, which allows the protein to localize to autophagosomes and bind phosphatidylethanolamine (i.e. undergo lipidation) [17]. Lipidated LC3-II binds to the cargo adaptor protein p62 which tags cellular targets for macroautophagy [18]. Thus, LC3-II is indicative of autophagosome formation [12].

Unlike saturated fatty acids, plasma levels of the polyunsaturated omega-3 fatty acids ( $\omega$ -3 FA) are positively correlated with insulin sensitivity in individuals with type 2 diabetes [19], and they prevent insulin resistance induced by saturated fatty acids in myotubes [20] and by high-fat feeding in rodents [21]. Apart from the effects on insulin sensitivity, there is other inferential evidence that  $\omega$ -3 FA may affect muscle mass. Transgenic *fat-1* mice have elevated endogenous  $\omega$ -3 FA levels, and their muscle fibers retain their cross-sectional areas following sciatic nerve injury compared to wild-type mice [22]. Supplementing the diets of

rodents with fish oil, but not corn oil, prevents the increase in MuRF1 and atrogin-1 mRNA levels induced by hindlimb mobilization [23]. Fish oil supplementation also has beneficial effects on muscle mass in cancer patients undergoing chemotherapy [24]. Interpretation of the biological effects of  $\omega$ -3 FA on muscle mass in these studies is complicated because commercial fish oil is a highly enriched mixture of  $\omega$ -3 FA, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are reported to exert different physiologic and cellular effects [25, 26]. DHA is the terminal fatty acid in the omega-3 conversion pathway. Studies show that consumption of preformed DHA is the best way to increase its level in the blood while EPA is more readily formed from alpha-linolenic acid which is highly enriched in plant-based foods [25]. It was previously reported that co-treating C2C12 myotubes with DHA and palmitate protects them from atrophy induced by the saturated fatty acid [7]; however, the cellular processes underlying the fatty acid-induced alterations in protein metabolism remain unclear. Since it is well-established that FoxO3 mediates the loss of muscle protein in chronic illness by increasing protein degradation [16, 27], the purpose of this study was two-fold: to investigate whether palmitate increases protein degradation in muscle cells and whether DHA counters the atrophy-inducing effects of palmitate by restoring Akt/FoxO signaling. By using the C2C12 myotube model, we ensure that the observed responses to palmitate and DHA are due to their direct effects on muscle cells.

## Methods and Materials

### Cultured myotube model

Mouse C2C12 myoblasts (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose and supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA) plus antibiotics (100 U/mL penicillin, 100  $\mu$ g/mL streptomycin; Invitrogen, Carlsbad, CA, USA). At 90–95% confluence, cells were induced to differentiate into myotubes in DMEM containing 4.5 g/L glucose plus 2% horse serum (Invitrogen) and antibiotics for 3–4 days before treatment with fatty acids.

### Experimental treatments

Palmitic acid and cis-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA) (Sigma Aldrich, St. Louis, MO, USA) were dissolved in ethanol and diluted to 500  $\mu$ mol/L and 100  $\mu$ mol/L, respectively, in DMEM containing 2% Fraction V protease-free bovine serum albumin (BSA; Product number 03117332001, Roche, Indianapolis, IN, USA), 2% FBS (Atlanta Biologicals, Inc., Flowery Branch, GA), 2 mmol/L L-carnitine (Sigma Aldrich), and 1% antibiotics ("treatment media"). Control cells were incubated in treatment media with an equal amount of ethanol substituted for palmitate and DHA. It is reported that 2% BSA and 500  $\mu$ mol/L free fatty acids result in a final molar ratio that closely resembles that of human plasma [28] and that fasting plasma nonesterified fatty acids are around 600  $\mu$ mol/L in obese adults with normal or impaired glucose tolerance or diabetes [29]. Additionally, the circulating level of palmitate is between 3–11 times higher than that of DHA in humans after 6 weeks of DHA supplementation [30]. Lastly, previous dose-response experiments in C2C12 myotubes showed that 100  $\mu$ mol/L was the lowest tested concentration of DHA that could prevent palmitate-induced defects in energy-sensing pathways [7]. Myotubes were

incubated in treatment media for 2–24 h, as indicated in the figure legends. In some experiments to measure converted LC3-II, some myotubes were treated with 10 mmol/L methylamine (Tokyo Chemical Industry Co, Ltd, Tokyo, Japan) to inhibit lysosomal activity for 3 h prior to harvest.

### Protein degradation assay

Protein degradation was measured in differentiated myotubes as previously described [31]. Briefly, cellular proteins were radiolabeled with  $^{14}\text{C}$ -phenylalanine (Phe) for 48 h. After a 2 h chase period to remove labeled Phe released from short-lived proteins, treatment media was applied and the amount of  $^{14}\text{C}$ -Phe released into the media was measured in serial aliquots at intervals up to 28 h. The rate of protein degradation was calculated as described by Gulve and Dice [32].

### RNA isolation and qPCR analysis

RNA was isolated using TRIzol (Invitrogen) and reverse transcribed using the Superscript III First-Strand Synthesis kit (Invitrogen) according to the manufacturer's instructions. mRNA was measured using quantitative real time PCR with the BioRad iCycler and the iQ SYBR Green reagent (BioRad Laboratories, Hercules, CA, USA). Previously published primer sets for MuRF1, atrogin-1, and Bnip3 were used to perform PCR reactions [12, 33];  $\beta$ -actin was used as the normalization control. The data were analyzed for fold change ( $C_t$ ) using the iCycler software, as previously described [10]. Melting curve analyses were performed to analyze and verify the specificity of the reaction.

### Isolation of cytosolic and nuclear cell fractions

Cells were rinsed 3 times and scraped from culture dishes in ice-cold phosphate-buffered saline (PBS), followed by centrifugation at  $1500 \times g$  for 5 min at  $4^\circ\text{C}$ . The pellet was re-suspended in a solution containing 0.01 mol/L HEPES (pH 7.6–7.8), 1.5 mmol/L  $\text{MgCl}_2$ , 2 mmol/L KCl, 0.5 mmol/L DTT, 0.5 mmol/L PMSF, 10  $\mu\text{g}/\text{mL}$  aprotinin, 5  $\mu\text{g}/\text{mL}$  leupeptin, 5  $\mu\text{g}/\text{mL}$  pepstatin A, and 1 mmol/L sodium orthovanadate and incubated on ice for 15 min. After addition of Nonidet P-40 (final concentration 0.5%), samples were mixed vigorously for 10 seconds and centrifuged at  $1500 \times g$  for 30–60 seconds at  $4^\circ\text{C}$ . The supernatant (cytosolic fraction) was transferred to a new tube and stored at  $-80^\circ\text{C}$  until further analysis. The pellet was re-suspended in a solution containing 0.02 mmol/L HEPES (pH 7.6–7.8), 1.5 mmol/L  $\text{MgCl}_2$ , 2 mmol/L KCl, 0.4 mol/L NaCl, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.5 mmol/L PMSF, 10  $\mu\text{g}/\text{mL}$  aprotinin, 5  $\mu\text{g}/\text{mL}$  leupeptin, 5  $\mu\text{g}/\text{mL}$  pepstatin A, 1 mmol/L sodium orthovanadate, and 25% glycerol, and samples were incubated on a shaking platform for 15–60 min at  $4^\circ\text{C}$ . Samples were centrifuged at  $21000 \times g$  for 20 min at  $4^\circ\text{C}$ , and the supernatant (nuclear fraction) was transferred to a new tube and stored at  $-80^\circ\text{C}$  until further analysis.

### Western blot analysis

Whole cell lysates were prepared using the buffers specified by the antibody vendors. Protein concentrations of cleared lysates, including the cytosolic and nuclear fractions described above, were measured using a BioRad DC protein assay kit (BioRad

Laboratories). Western analyses were performed as described [34] using commercial antibodies to phospho-Akt (S473), total Akt, FoxO3a, LC3B,  $\beta$ -actin (Cell Signaling Technology, Beverly, MA, USA), and p62 (Sigma Aldrich) [10]. Antibodies to GAPDH, Histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Na<sup>+</sup>-K<sup>+</sup> ATPase (Cell Signaling Technology) were used to assess the purity of the nuclear and cytosolic samples. Equal loading of total protein in the sample lanes was verified by Ponceau S Red staining [10].

### Statistical analyses

Data are presented as mean percentage of control  $\pm$  SE. Differences between 2 treatment groups are compared by two-tailed Student's *t* test. Differences between 3 treatments are compared by one-way ANOVA with post-hoc analysis by Tukey's test for multiple comparisons. Results are considered statistically significant at  $P < 0.05$ .

## Results

### DHA attenuates the effects of palmitate on protein degradation

Previous experiments in C2C12 myotubes indicated that addition of DHA reverses the reduction in myotube size induced by palmitate [7]. To determine if changes in protein degradation contribute to these responses, we measured the rate of protein degradation in myotubes treated with each lipid alone and in combination. Palmitate alone increased proteolysis by 31% whereas DHA alone had no effect on the rate of proteolysis (Figure 1). Co-treatment of DHA with palmitate prevented the proteolytic response to the saturated fatty acid.

### DHA attenuates palmitate-induced inhibition of Akt-FoxO3 signaling

Akt is a key regulator of protein degradation in muscle because it phosphorylates FoxO3, thereby inhibiting its function. We therefore tested whether palmitate and DHA alter the Akt/FoxO3 pathway. Treating myotubes with palmitate consistently suppressed the phospho:total Akt ratio over treatment times between 2–24 h (Figure 2A). DHA alone had no effect on Akt phosphorylation whereas co-treatment of myotubes with DHA and palmitate for 4 h (Figure 2B) and 24 h (Figure 2C) restored Akt phosphorylation to near control levels. The lack of reduction in Akt activation by DHA was not simply due to a lower molarity of fatty acid compared to palmitate because we tested 500  $\mu$ mol/L DHA and it improved Akt phosphorylation compared to control samples (data not shown). This indicates that the type of fatty acid, rather than concentration, is primarily responsible for their disparate effects on Akt signaling in this model.

The level of FoxO3 protein in total cell lysates was unchanged by the fatty acid treatments (Figure 3A). Examination of cytosolic and nuclear cell fractions revealed that palmitate decreased FoxO3 protein in the cytosol (Figure 3B) while simultaneously increasing its level in the nucleus (Figure 3C). Co-treatment with DHA restored the levels of FoxO3 in the cytosol and nucleus to control levels (Figures 3B and 3C). The purity of the cytosolic and nuclear fractions was evaluated by testing for the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, cytosolic), Na<sup>+</sup>-K<sup>+</sup> ATPase (cell membrane) and histone H1

(nuclear) proteins in each of the samples. No obvious contamination between fractions was detected (Figure 3D).

### DHA attenuates the effects of palmitate on proteolytic systems

FoxO3 promotes protein degradation by enhancing expression of key components of multiple proteolytic systems. Therefore, we evaluated whether the fatty acid treatments altered markers of the UbP and autophagy pathways. Palmitate increased the level of E3 ubiquitin ligase atrogin-1/MAFbx mRNA after 4 h compared to control cells, while co-treatment with DHA prevented the response (Figure 4A). Surprisingly, the level of MuRF1 mRNA was unchanged by treatment with the fatty acids (Figure 4B).

Regarding macroautophagy, palmitate increased the level of Bnip3 mRNA whereas co-treatment with DHA reduced the level to that in control cells (Figure 5A). LC3-II protein was unchanged by palmitate but was reduced by DHA, regardless of whether palmitate was present (Figure 5B). To test whether DHA regulates the rate of autophagosome formation, degradation of LC3-II-containing autophagosomes was inhibited by adding methylamine to the media. The level of LC3-II was still reduced in DHA-treated cells after inhibition of lysosomal activity, indicating that DHA significantly reduces autophagic flux while palmitate has no effect (Figure 5C). The levels of p62 were not altered by either fatty acid, regardless of whether lysosomes were active or not (Figures 5D and 5E).

## Discussion

The objective of this study was to determine whether palmitate enhances protein degradation in myotubes and to investigate how DHA protects against palmitate-induced muscle atrophy. We hypothesized that DHA antagonizes the effects of palmitate on the Akt/FoxO3 axis because it is a key determinant of the activity of various proteolytic pathways in muscle cells. Our findings indicate that DHA counteracts the palmitate-induced increase in protein degradation by preventing the upregulation of the UbP and autophagy systems. DHA's effects are mediated, at least in part, through restoration of Akt activation and FoxO3 inhibition (Figure 6). Although several *in vivo* studies have documented that  $\omega$ -3 fatty acids, primarily in the form of fish oil, exert beneficial effects on protein metabolism in muscle [22–24], it remained unclear whether these fatty acids directly target muscle signaling pathways that control protein balance or exert indirect effects on muscle via systemic metabolic alterations. The current experiments establish that  $\omega$ -3 fatty acids, specifically DHA, act directly on myotubes to regulate proteolytic pathways in a high fat environment.

Previous experiments demonstrated that co-treatment with DHA prevents the palmitate-induced decrease in the diameter of C2C12 myotubes [7]. To elucidate the mechanisms underlying the anti-atrophy effect of DHA, we first determined whether the fatty acids altered the rate of protein degradation in myotubes. As hypothesized, palmitate increased proteolysis while co-treatment with DHA inhibited the effect of palmitate. Consistent with an earlier study [35], DHA alone did not alter the rate of protein degradation. Although the present work does not address the possibility that DHA alters protein synthesis, the earlier report indicated that 50  $\mu$ mol/L DHA had no effect on the process in C2C12 myotubes [35]. This finding, however, does not preclude the possibility that DHA prevents a negative effect

of palmitate on protein synthesis. Regardless, our results indicate that the beneficial effects of DHA are more apparent in the presence of an atrophy-inducing signal such as palmitate.

Akt is a key regulator of protein degradation in skeletal muscle. Under normal healthy conditions, it inactivates FoxO3 through phosphorylation, thereby inhibiting its nuclear localization and suppressing the expression of key components of the muscle cell's proteolytic systems [36]. Reduced signaling through Akt is a common feature of most cell and animal models of atrophy [8]. In agreement with other studies [37–39], palmitate caused a prolonged suppression of Akt activation, as indicated by decreased phospho:total Akt ratio compared to control cells. Coincident with the change in Akt phosphorylation, the level of nuclear Foxo3 also increased. Importantly, co-treatment with DHA preserved Akt activation as well as restored cytosolic and nuclear FoxO3 protein to control levels. This suggests that the protective effect of DHA against palmitate-induced protein degradation is mediated, at least in part, by reestablishing a normal Akt/FoxO3 axis.

Activation of FoxO3 stimulates multiple proteolytic systems, including the UbP and autophagy pathways. The muscle-specific E3 ubiquitin ligases MuRF1 and atrogin-1/MAFbx are key components of the atrophy program that selectively target specific muscle proteins for ubiquitination and degradation by the 26S proteasome [13, 15]. Their mRNAs are typically elevated in models of atrophy [13, 14]. Overexpression of MAFbx is sufficient to induce atrophy in myotubes, while MAFbx/atrogin-1 or MuRF1 deficiency attenuates muscle loss due to denervation in mice [15]. We hypothesized that the palmitate-induced enhancement in nuclear localization of FoxO3 would result in increased expression of atrophy-related FoxO3 targets. Consistent with this prediction, increased atrogin-1 mRNA expression indicates that the UbP plays a role in palmitate-induced protein degradation. The effects of DHA on atrogin expression have not been investigated previously in a high fat model. Our finding that co-treatment with DHA prevented the palmitate-induced increase in atrogin-1 mRNA further supports the assertion that DHA plays a protective role against the atrophy-inducing effects of palmitate by restoring inhibition of FoxO3 and reducing proteolysis.

Unexpectedly, palmitate increased the level of mRNA encoding atrogin-1 but not MuRF1. Although this is an unusual result, it is not without precedent and may be attributable to the atrophy stimulus in our high fat model. Peterson and colleagues [40] reported that incubating C2C12 myotubes with 200  $\mu\text{mol/L}$  palmitate increased the mRNA for atrogin-1, but not MuRF1; in these experiments, myotubes were co-cultured with either fibroblasts or macrophages. They also noted that 200  $\mu\text{mol/L}$  DHA did not alter the expression of either atrogin (in the absence of palmitate) [40]. Again, this is consistent with our data and further suggests that the disparate effects of palmitate and DHA on atrogin expression are not due to differences in molarity of the fatty acids. We also examined this possibility by incubating myotubes with 500  $\mu\text{mol/L}$  DHA and/or 500  $\mu\text{mol/L}$  palmitate and found no reduction in the phospho-Akt:total Akt ratio due to the higher concentration of DHA (data not shown), confirming that a higher concentration of DHA does not have detrimental effects akin to those of palmitate.

Until recently, the importance of the autophagy pathway in skeletal muscle atrophy has been underappreciated. Several studies have identified autophagy-related atrogene mRNAs, including Bnip3 and LC3, which are increased in some models of atrophy [16, 41]. Mammucari et al. [12] demonstrated that a FoxO3-mediated increase in Bnip3, but not LC3, is necessary and sufficient to induce autophagy and muscle atrophy. In the current study, co-treatment with DHA counteracted the induction of Bnip3 mRNA by palmitate. Surprisingly, palmitate did not significantly increase LC3-II or alter p62 protein; however, DHA did decrease autophagosome formation regardless of whether palmitate was present or not. Therefore, it appears that DHA has an independent effect to inhibit autophagy in muscle cells. This effect alone is not sufficient to significantly reduce the overall rate of protein degradation (Fig. 1), likely because macroautophagy quantitatively represents only a small fraction of total cellular proteolysis in skeletal muscle [42]. Others have suggested that mitochondria may be a critical target for macroautophagy in muscle wasting [16]. This is an interesting corollary to the present work and could be the basis of future experiments to delineate the effects of DHA and palmitate on mitochondrial number and function.

In summary, our data demonstrate that a palmitate-induced increase in protein degradation via suppression of Akt/FoxO3 signaling and induction of the UbP and autophagy pathways contributes to the muscle cell atrophy seen with lipotoxicity. DHA, a major  $\omega$ -3 fatty acid constituent of fish oil, protects against the detrimental effects of palmitate. It acts by reestablishing the Akt-mediated inhibition of FoxO3, thereby suppressing atrogene expression and limiting induction of autophagy. These results underscore the potential for future therapeutic application of the omega-3 fatty acids in counteracting muscle loss due to chronic illness.

## Acknowledgments

The authors would like to thank Sara Zoromsky for her assistance with the protein degradation assay.

This work was supported by NIH RO1DK95610, AHA GRNT7660020 and VA MERIT X01BX001456 awarded to S. Russ Price.

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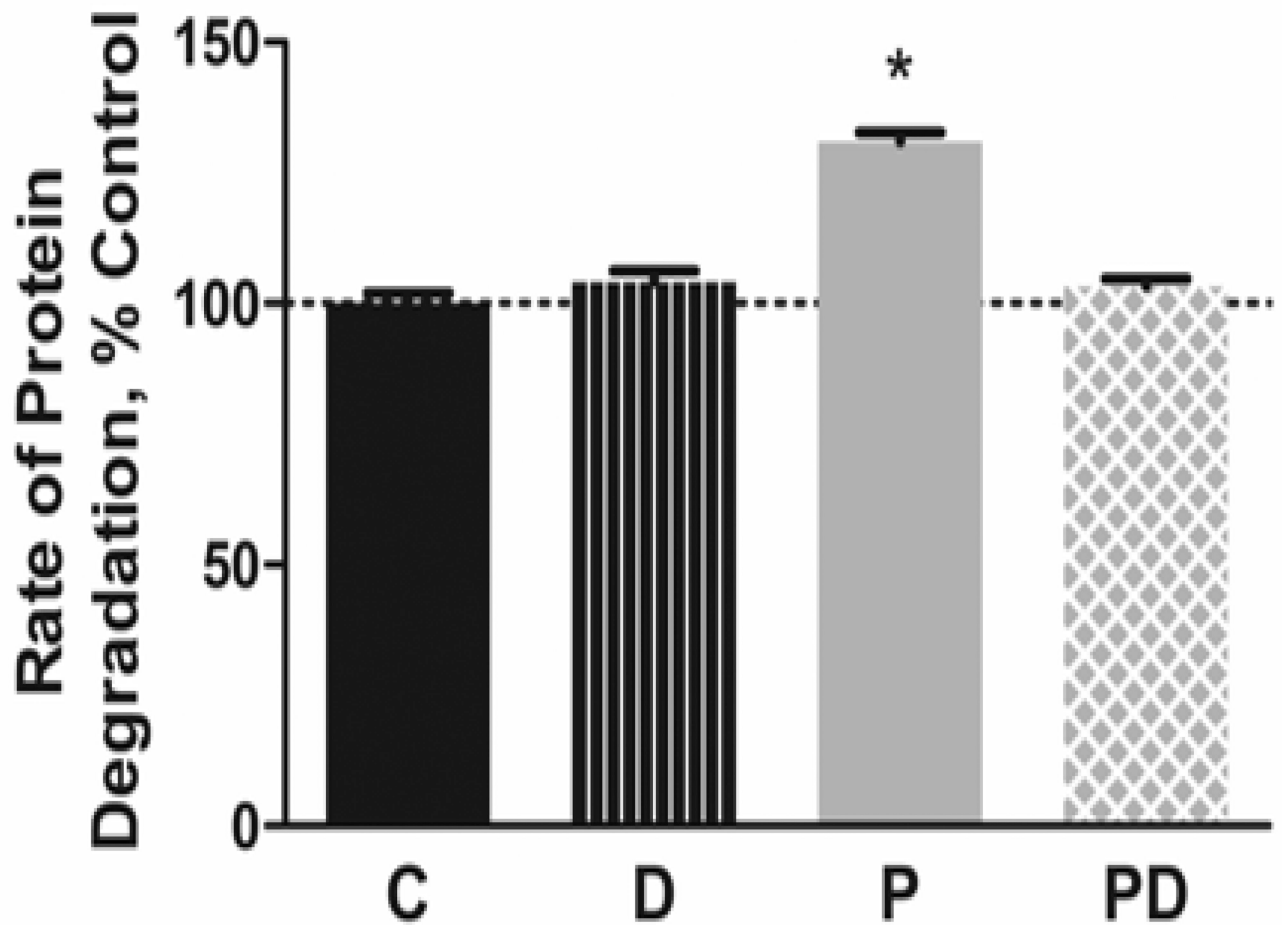
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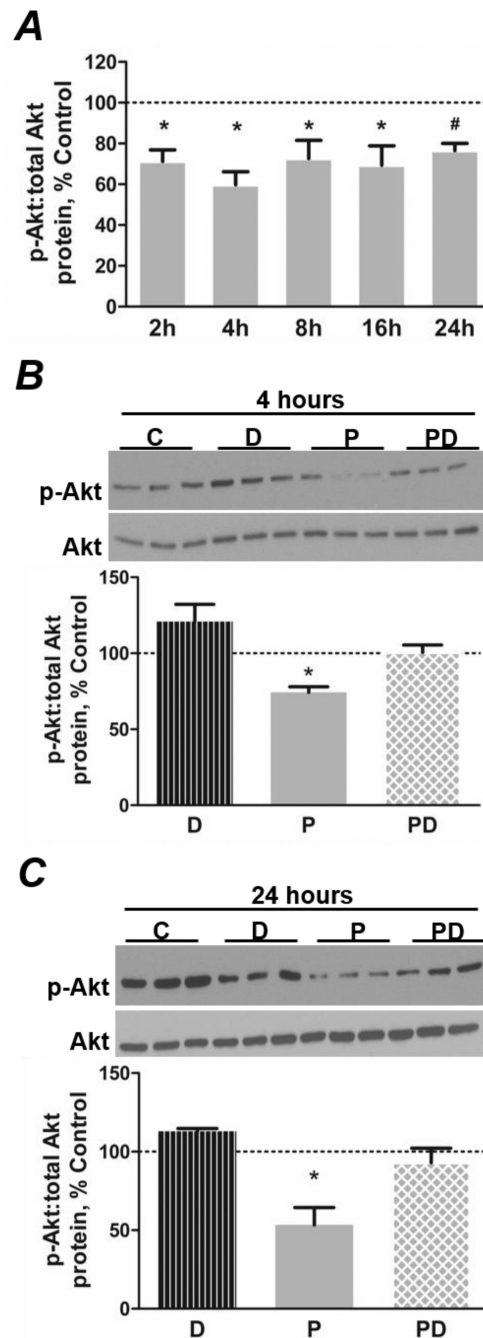
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**Figure 1.**

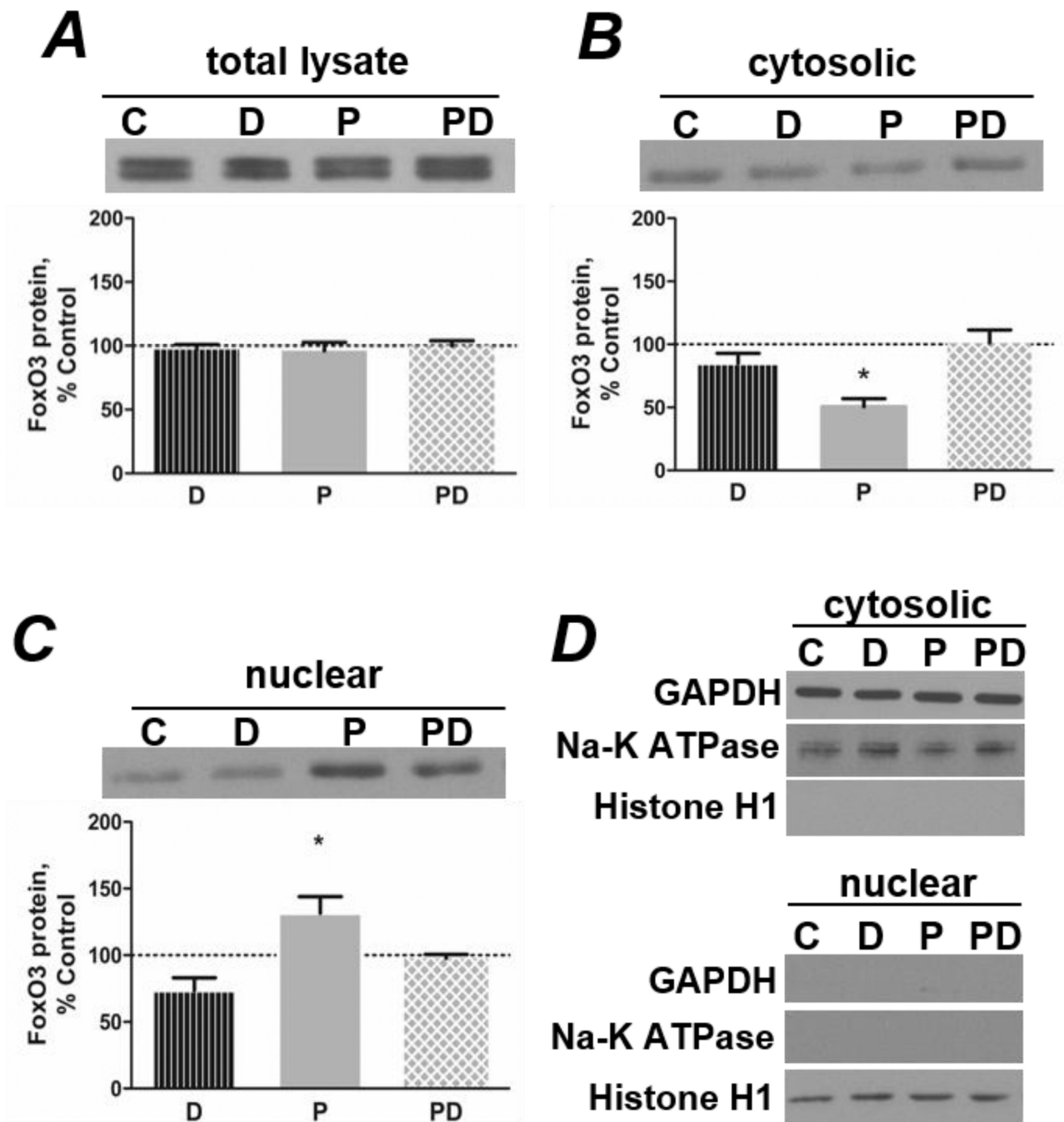
Docosahexaenoic acid prevents palmitate-induced protein degradation. C2C12 myotubes were pre-labeled with  $^{14}\text{C}$ -phenylalanine then treated with 500  $\mu\text{mol/L}$  palmitate (PA) and/or 100  $\mu\text{mol/L}$  docosahexaenoic acid (DHA) for 28 h. The rate of protein degradation was calculated by measuring the release of  $^{14}\text{C}$ -phenylalanine into the culture media. PA increased the rate of protein degradation, while co-treatment with DHA prevented the response. Representative results from 1 of 3 independent experiments are shown (\* $p < 0.01$  versus other groups,  $n = 6$  per group per experiment)



**Figure 2.**

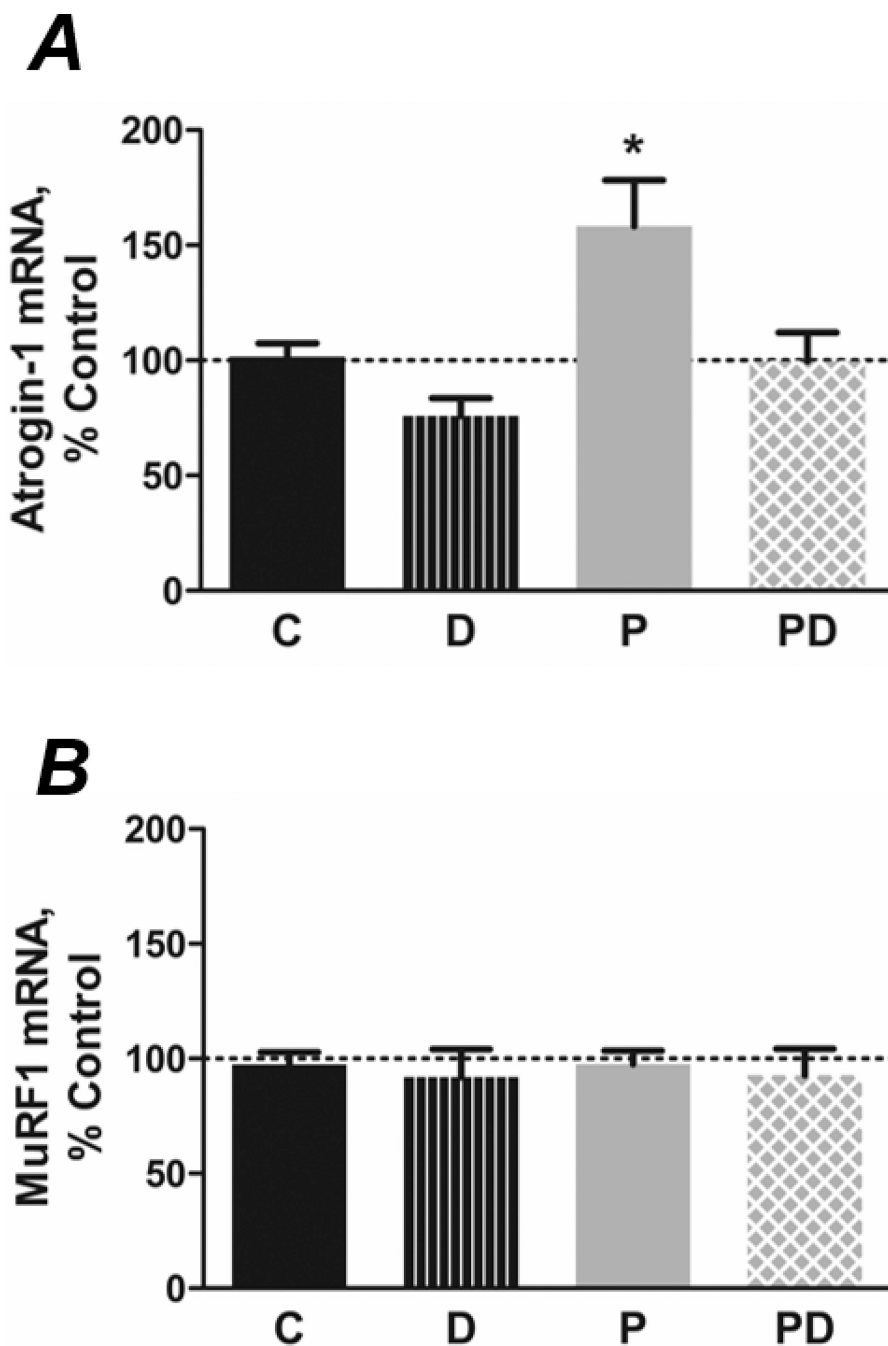
DHA prevents palmitate-induced suppression of Akt activation. C2C12 myotubes were treated with 500  $\mu\text{mol/L}$  PA (P) for 2–24 h and the protein levels of phospho(S473)-Akt and total Akt were evaluated by western blot analysis. **A)** PA reduced the ratio of phospho(S473):total Akt at all timepoints, indicating a continuous suppression of Akt activation by the saturated fatty acid (\* $p < 0.05$  versus control,  $n = 3-5$ /timepoints; # $p < 0.01$  versus control,  $n = 5$ /timepoint). **B/C)** Co-treatment with 100  $\mu\text{mol/L}$  DHA (PD) attenuates

the effects of PA on the Akt ratio after **B**) 4 h (\* $p < 0.01$  versus all other groups,  $n = 3-4$ /group) and **C**) 24 h (\* $p < 0.001$  versus all other groups,  $n = 5-6$ /group).



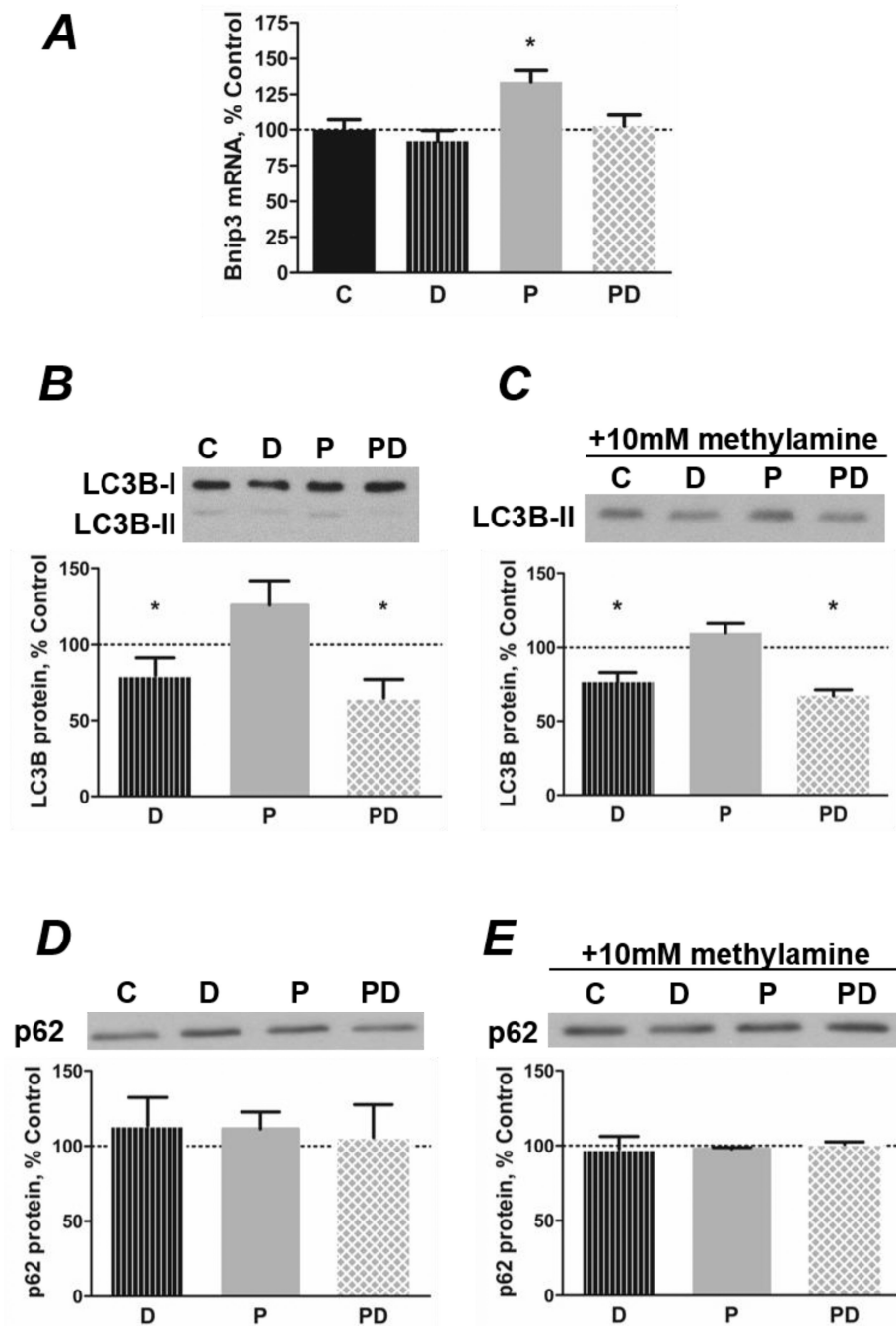
**Figure 3.**

Co-treatment with DHA prevents palmitate-induced alterations in FoxO3 localization. **A)** Treatment (4 h) with 500  $\mu\text{mol/L}$  PA (P) and/or 100  $\mu\text{mol/L}$  DHA (D) does not alter FoxO3 protein in total cell lysates ( $n=3/\text{group}$ ). PA decreases cytosolic (**B**) and increases nuclear FoxO3 protein (**C**), while co-treatment with DHA (PD) prevents the responses ( $*p<0.01$  versus all other groups,  $n=4/\text{group}$ ). **D)** Representative western blots show markers of cytosolic (GAPDH), nuclear (histone H1), and cell membrane ( $\text{Na}^+\text{-K}^+$  ATPase) proteins to confirm the purity of cell fractions.



**Figure 4.** Co-treatment with DHA prevents the PA-induced increase in atrogin-1 mRNA levels. **A)** PA (P; 500  $\mu\text{mol/L}$ , 4 h) increases mRNA encoding the muscle-specific E3 ubiquitin ligase atrogin-1 (\* $p < 0.01$  versus all other groups,  $n = 8-12/\text{group}$ ), while co-treatment with 100  $\mu\text{mol/L}$  DHA (PD) prevents these responses. **B)** mRNA encoding the MuRF-1 E3 ligase is unchanged by fatty acids (4 h,  $n = 7/\text{group}$ ).

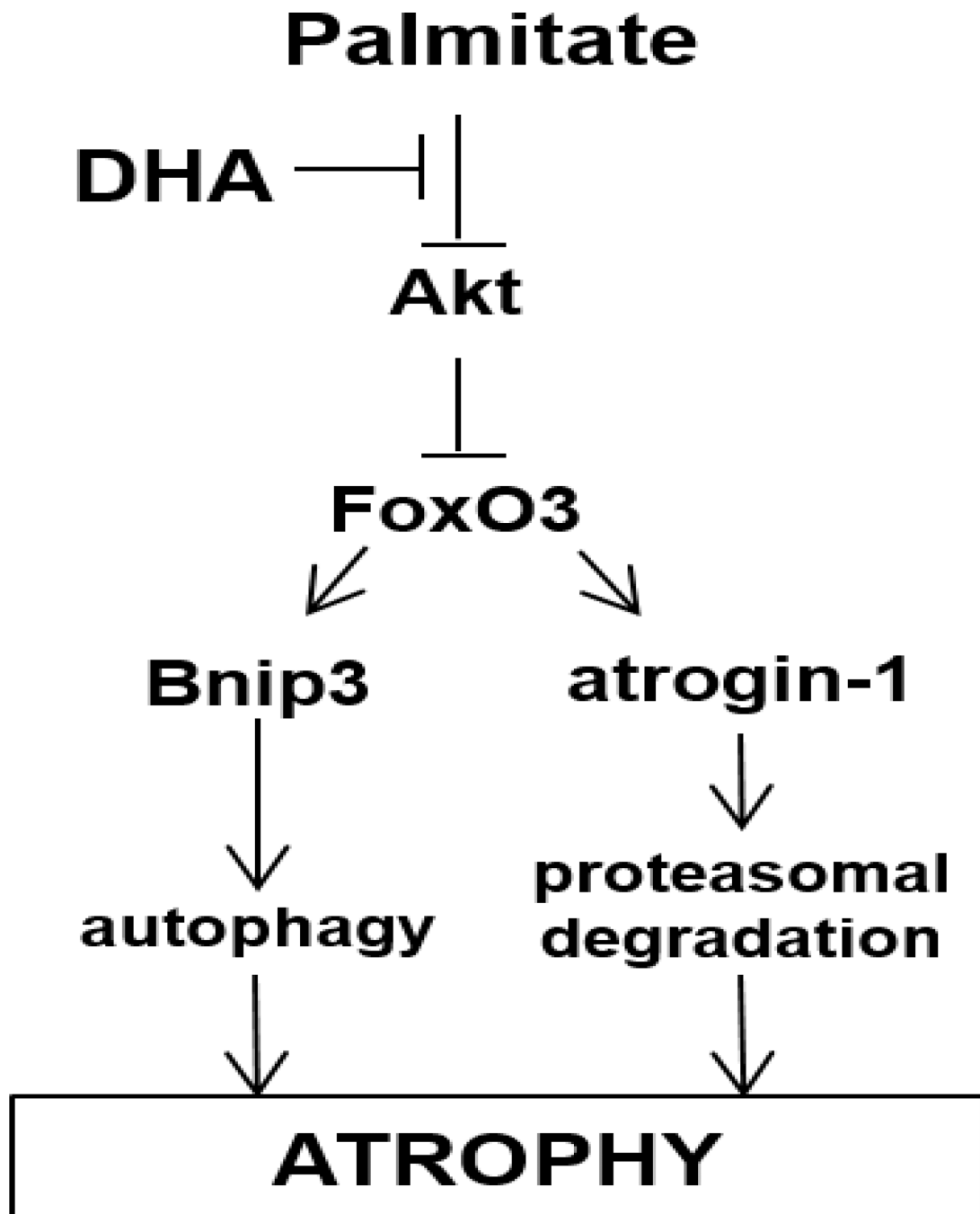




**Figure 5.**

Co-treatment with DHA prevents the PA-induced increase in macroautophagy. **A)** PA (P; 500  $\mu\text{mol/L}$ , 24 h) increases mRNA levels of the macroautophagy mediator Bnip3, while cotreatment with 100  $\mu\text{mol/L}$  DHA (PD) prevents the response (\* $p < 0.01$  versus all other groups,  $n = 5-6$ ). **B/C)** DHA inhibits autophagosome formation. **B)** DHA decreases the ratio of LC3BII: LC3B-I protein in the absence (D) or presence (PD) of PA (24 h, \* $p < 0.05$  versus PA,  $n = 4$ ). **C)** Myotubes were treated with 10 mmol/L methylamine to inhibit lysosomal degradation of autophagosome contents for 3 h before lysis. The level of LC3B-II protein

remained decreased, indicating an independent effect of the omega-3 fatty acid to inhibit macroautophagy (24 h, \* $p < 0.001$  versus control and PA,  $n = 3/\text{group}$ ). *D/E*) p62 protein levels remain unchanged by treatment with PA or DHA for 24 h in the *D*) absence ( $n = 4/\text{group}$ ) or *E*) presence ( $n = 3/\text{group}$ ) of 10 mmol/L methylamine.



**Figure 6.**

The Akt/FoxO3 axis is a key regulator of the activity of various proteolytic pathways in muscle cells, including the ubiquitin-proteasome and macroautophagy systems. Palmitate induces the activity of multiple proteolytic systems, resulting in myotube atrophy. DHA counteracts the effects of palmitate by restoring Akt activation and FoxO3 inhibition, thus preventing the upregulation of protein degradation.