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RESEARCH ARTICLES

# N-3 long-chain polyunsaturated fatty acids inhibit smooth muscle cell migration by modulating urokinase plasminogen activator receptor through MEK/ERK-dependent and -independent mechanisms

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**Abstract**

Smooth muscle cell (SMC) migration is a major and complex feature of atherosclerosis and restenosis. N-3 long-chain polyunsaturated fatty acids (LCPUFAs) affect SMC migration; however, the mechanisms involved are unclear. This study investigated the effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the MEK/ERK pathway and urokinase plasminogen activator receptor (uPAR) in relation to SMC migration.

Transwell migration assays revealed that both EPA and DHA decreased cell migration. Western blotting and real-time reverse transcription polymerase chain reaction showed that n-3 LCPUFAs decreased uPAR expression, but not urokinase plasminogen activator (uPA) expression, without changing plasmin and uPA activity. DHA also inhibited the activation of the MEK/ERK signaling pathway, whereas EPA switched the SMC phenotype from synthetic to contractile. siRNA technology targeting uPAR expression showed that decreased uPAR led to a significant decrease in migration, demonstrating the role of uPAR on SMC migration. We also showed that MEK/ERK pathway activation was involved in the regulation of uPAR gene expression in SMCs.

Our results suggest that n-3 LCPUFAs decrease SMC migration through the inhibition of uPAR expression, with DHA affecting its expression via the modulation of MEK/ERK signaling pathway, while EPA induces a change in SMC phenotype. This could represent another means by which to explain how n-3 LCPUFAs exert their preventive properties against atherosclerosis.


*Keywords:* Docosahexaenoic acid; Eicosapentaenoic acid; Migration; Aortic smooth muscle cells; Urokinase plasminogen activator receptor; MEK/ERK

## 1. Introduction

Accumulation of smooth muscle cells (SMCs) in the intima is a major feature of atherosclerotic plaque development. Build up of SMCs in the intima is seemingly a consequence of their migration from the media to the intima where they proliferate [1]. SMCs present in the intima surround themselves in extracellular connective tissue rich in collagen [2–4]. This onset of fibrosis represents a critical step during atherogenesis when early lesions evolve to irreversible advanced plaques characterized by a well-defined fibrous cap often associated with clinical symptoms.

Cells migrate in response to the binding of various factors [e.g., Platelet-derived growth factor (PDGF), angiotensin II, basic fibroblast growth factor (bFGF), smooth muscle cell-derived migration factor (SDMF) and transforming growth factor-β (TGF-β)] to cell surface receptors, triggering signaling pathways [5–9], particularly the mitogen-activated protein kinase (MAPK) pathway where the activation of extracellular signal-regulated kinases (ERK) seems essential [10]. However, it is still unclear how ERK promotes SMC mobility. Platelet-derived growth factor (PDGF-BB), a stimulator of SMC migration, also increases urokinase plasminogen activator receptor (uPAR) mRNA levels in SMC [11]. Activation of MEK/ERK signaling has been shown to be involved in uPAR expression in human hepatocarcinoma [12].

Inhibition of urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA) activity decreased SMC migration in a baboon aortic explant model, whereas addition of plasminogen restores uPA-inhibited SMC migration [13]. Furthermore, SMCs fail to migrate to the necrotic center during neointima formation in $uPA^{-/-}$ and $tPA^{-/-}/uPA^{-/-}$ mice arteries, unlike in wild-type and $tPA^{-/-}$ mice arteries [14]. Using an *in vitro* model of human umbilical vein SMC migration, Okada et al. (1996) showed that uPA and tPA increase migration, this effect being inhibited by antibodies against uPAR in the presence or absence of added uPA. The authors concluded that uPAR may have a role in SMC migration independent of the mechanism involving uPA [15]. In confluent human vascular SMCs, injury was shown to polarize cell surface uPAR to the leading edge during migration [16]. Binding of the amino-terminal fragment of uPA, which does not contain the catalytic domain, stimulates SMC migration [17], highlighting a possible role for uPAR.

High consumption of n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFAs) from fish and fish oils is beneficial against

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cardiovascular disease, particularly for secondary prevention [18]. However, a postmortem study of arteries from people consuming an oily-fish-rich diet showed significantly less advanced atherosclerotic plaques compared with those who consumed low amount of oily fish [19]. This suggests a possible direct effect of n-3 LCPUFA on SMC migration and atherosclerotic plaque progression due to modification of the arterial connective tissue turnover delaying the progression of atherosclerosis. *In vitro* studies have previously shown that LCPUFAs from marine origin, such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, can inhibit the migration of vascular SMCs [20,21]. However, the mechanisms involved remain to be elucidated.

This study aimed to determine whether EPA and DHA, alone or in combination, could inhibit SMC migration by modulating uPAR expression and MEK/ERK signaling.

## 2. Methods and materials

### 2.1. Materials

Anti-SM-22 antibody, horseradish peroxidase (HRP)-labelled goat anti-mouse antibody and HRP-labelled donkey anti-goat antibody were purchased from Santa Cruz Biotechnology Inc., Heidelberg, Germany. Anti-α-actin antibody was purchased from Biomed Corp., and anti-uPAR antibody was purchased from R&D Systems, Abingdon, UK. Anti-phospho-p44/42 (ERK1/2) (Thr202/Tyr204) (E10) mouse monoclonal antibody (mAb) and anti-p44/42 MAPK (ERK1/2) (137F5) rabbit mAb were all from New England Biolabs. The MEK inhibitor (U0126) was purchased from Cell Signalling Technology. DHA, EPA or oleic acid (OA) (Sigma-Aldrich) were added to the medium bound to delipidated bovine serum albumin (Sigma-Aldrich) as previously described [22] to reach a final concentration of 10 μM (ratio fatty acid:albumin, 2/1). Experiments were also carried out using two different DHA/EPA ratios (3:1 and 1:1), but still using a total final fatty acid concentration of 10 μM. These ratios were chosen to mimic ratios observed in plasma of human volunteers before and after supplementation with fish oil [23]. OA is one of the main fatty acid present in the diet and ubiquitously distributed in human tissues. It was also the most prominent fatty acid in the cells investigated and, for these reasons, was chosen as control fatty acid.

### 2.2. Cell culture

Primary AoSMCs [from three donors: a 36-year-old Hispanic male (Cellmade, lot 1401), a 17-year-old Caucasian male (Lonza, UK lot 3F1243) and a 56-year-old Hispanic female donor (Lonza UK, lot 4F1122)] were cultivated in EGM-2 culture medium (Lonza). Cells were grown up to 50% confluence before treatment. Following treatments, cells intended for RNA isolation were collected in QiaZol (Qiagen), while cells intended for protein analysis were collected in radioimmunoprecipitation assay buffer containing phosphatase inhibitors cocktails I and II (Sigma-Aldrich).

### 2.3. Migration assay

Migration assays were performed using polycarbonate membrane transwell inserts (8 μm pore size) (Corning) coated with Geltrex reduced growth factor basement membrane extract (Invitrogen). Serum-free EGM-2 medium containing 10 ng/ml PDGF-BB (PeproTech EC) was placed in the lower compartment, and 100,000 cells were seeded to the top of the insert. Cells were then incubated for 5 h at 37°C and 5% $CO_2$, before being scraped from the top of the insert and washed out with phosphate-buffered saline (PBS). The medium from the lower compartment was removed, and the cells which had migrated through the insert were fixed with 1% paraformaldehyde before being stored at 4°C. Cells were stained with Nile red (Sigma-Aldrich) prepared from a 1-mg/ml stock solution in acetone and diluted in PBS to 1 μg/ml. Cells were visualized and counted using a QImaging monochrome QIcam on a Zeiss Axiovert 100 microscope and QCapture-Pro software (MediaCybernetics). For each well, the number of cells that had migrated was counted from six fields at 200× magnification.

### 2.4. RNA extraction and reverse transcription real-time polymerase chain reaction (PCR)

RNA was isolated using QiaZol according to Qiagen's protocol and quantified by absorbance at 260 nm (NanoDrop ND-1000 spectrophotometer). RNA samples were reverse transcribed using Moloney-murine leukemia virus reverse transcriptase (Invitrogen) according to Invitrogen's protocol. Real-time PCR analysis was performed using a BIO-RAD iQ5 multicolor real-time detection system. Diluted cDNA samples were mixed with specific primer sets and SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich). The primer sequences used were as follows: 36B4 sense CCATTCTATCATCAACGGGTACAA, anti-sense AGCAAGTGGGAAGGTGTAATCC [24]; uPA sense CACGCAAGGGGAGATGAA, anti-sense ACAGCATTTTGGTGGTGACTT [25]; and uPAR sense ACAACGACACCTTCCACT, anti-sense GGCTACCAGACATTGATTCAT (prepared in-house).

### 2.5. Western blot analysis

Protein concentration was determined using the bicinchoninic acid protein assay. Two, 3, 0.5 and 6 μg protein was used for the detection of both α-actin and SM-22α, phospho-p44/42 (ERK1/2) and p44/42 MAPK (ERK1/2), β-actin and uPAR, respectively. Reduced samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 15% polyacrylamide gels and transferred on to Hybond C Extra nitrocellulose (0.45 micron) or Immobilon polyvinylidene fluoride membranes. Primary antibodies in TBS-T containing 4% powdered milk were incubated overnight at 4°C or for 1 h at room temperature. After incubation with appropriate HRP-conjugated secondary antibodies, the signals were visualized using Western chemiluminescent HRP substrate (Immobilon), the images were analyzed (Fujifilm Life Science Luminescent Image Analyzer LAS-3000), and signals were quantified (Bio-Rad Quantity One 4.4.0 software).

### 2.6. Determination of fatty acid incorporation

SMC total lipids were extracted according to Bligh and Dyer [26], and phospholipids were isolated by thin-layer chromatography using a mixture of hexane:diethyl ether:acetic acid (90/30/1 vol/vol/vol). Fatty acid methyl esters (FAME) were prepared by transesterification using boron trifluoride (14% in methanol, Sigma) before analysis by gas chromatography (HP6890, Hewlett Packard, Avondale, PA) using 50-m×20-mm Chrompac CP7488 CP Sil-88 capillary column (film thickness 0.22 μm). Hydrogen was used as carrier gas at a rate of 0.5 ml/min, and the split-splitless injector was used at a split ratio of 20:1. The injector and detector temperatures were 250°C. The column oven temperature was maintained at 80°C for 1 min after sample injection and was programmed to then increase at 25°C/min to 160°C where it was maintained for 3 min. Temperature was then increased to 190°C at 1°C/min and then to 230°C at 10°C/min. The temperature was maintained at 230°C for 30 min. Separation was recorded with HP GC Chemstation software (Hewlett Packard, Avondale, PA). FAME were identified by comparison to standards essayed previously.

### 2.7. Statistical analysis

Results are presented as means±S.E.M., and all experiments were performed in triplicates ($N=3$). The statistical significance of the migration assay, Western blot and fatty acid composition was calculated by one-way analysis of variance, and comparison within groups was performed by least significant difference post hoc test. $P$ values less than .05 were considered significant.

Gene expression results were normalized using 36B4 as a reference gene, before performing pairwise fixed reallocation randomization test with REST-MCS beta software (Gene Quantification). Results were considered significant if $P \leq .05$ determined using 2000 randomizations [27,28].

## 3. Results

### 3.1. Incorporation of n-3 LCPUFAs into AoSMCs phospholipids

After 48-h incubation, DHA content in AoSMC phospholipids was significantly increased with DHA compared with the other treatments (Table 1). EPA incorporation was increased with DHA and EPA treatments compared with the vehicle control and compared with OA treatment. Docosapentaenoic acid (DPA) and DHA were also significantly increased after EPA treatment compared with the vehicle control and OA treatment. The proportion of OA was increased with OA treatment in AoSMCs compared with the vehicle control, while stearic acid increased significantly to the detriment of OA following DHA and EPA treatment.

### 3.2. Effects of n-3 LCPUFAs on uPA and uPAR expression

Treatment with EPA down-regulated uPAR gene expression by a factor of 2.32 and 3.54 compared with the vehicle control and OA treatments, respectively (Fig. 1A). Treatment with DHA also significantly decreased uPAR gene expression by a factor of 2.12 compared with OA treatment. uPA gene expression was not affected by any of the treatments (Fig. 1A).

Two proteins with molecular weights around 70-kDa and 55-kDa corresponding to two forms of uPAR [glycosylated and bound to the amino-terminal fragment (ATF) of uPA] were detected by Western blot analysis (Fig. 1B). The relative quantity of the 70-kDa protein was significantly decreased by 26.2%, 30.9% and 25.9% in the presence of

Table 1
Fatty acid composition of phospholipids in AoSMCs after 48-h treatment with n-3 LCPUFAs

| Fatty acids | Vehicle control | OA | DHA | EPA |
|---|---|---|---|---|
| Palmitic acid C16:0 | 17.97±0.92 | 18.31±0.50 | 18.81±0.60 | 18.77±0.48 |
| Palmitoleic acid C16:1 | 2.53±0.04$^{\dagger\dagger}$ | 1.88±0.04** | 2.40±0.13$^{\dagger\dagger}$ | 2.37±0.25$^{\dagger\dagger}$ |
| Stearic acid C18:0 | 19.14±0.91 | 17.94±0.26 | 20.75±0.31$^{*\dagger}$ | 20.35±1.16$^{\dagger}$ |
| Oleic acid C18:1 | 40.39±0.37 | 41.74±0.27 | 31.12±0.72$^{**\dagger\dagger}$ | 33.00±1.17$^{*\dagger\dagger}$ |
| Linoleic acid C18:2 | 1.97±0.01 | 1.93±0.05 | 1.91±0.08 | 1.86±0.06 |
| Alpha-linolenic acid C18:3 n3 | 0.07±0.00 | 0.06±0.00** | 0.06±0.00** | 0.06±0.01* |
| *cis*-11,14,17-Eicosatrienoic acid C20:3 n3 | 1.83±0.05 | 1.82±0.03 | 1.56±0.09$^{**\dagger\dagger}$ | 1.58±0.07$^{**\dagger\dagger}$ |
| Arachidonic acid C20:4 | 5.81±0.08 | 5.78±0.16 | 4.85±0.20$^{**\dagger\dagger}$ | 5.07±0.17$^{**\dagger\dagger}$ |
| Eicosapentaenoic acid C20:5 | 1.74±0.04 | 1.61±0.06 | 3.09±0.07$^{**\dagger\dagger}$ | 4.86±1.35$^{***\dagger\dagger\dagger a}$ |
| DPA C22:5n3 | 2.10±0.03 | 2.20±0.05 | 2.52±0.09 | 5.32±1.88$^{**\dagger\dagger a}$ |
| Docosahexaenoic acid C22:6 | 2.88±0.04 | 2.93±0.08 | 10.26±0.13$^{***\dagger\dagger\dagger}$ | 3.86±0.73$^{*\dagger b}$ |
| Others | 1.77±0.00 | 1.78±0.04 | 1.58±0.28 | 1.66±0.09 |
| Total | 100 | 100 | 100 | 100 |

Values are % means of total±standard deviation ($n$=3). *$P$≤.05, **$P$≤.01, ***$P$≤.001 vs. vehicle control; $^{\dagger}P$≤.05, $^{\dagger\dagger}P$≤.01, $^{\dagger\dagger\dagger}P$≤.001 vs. OA; $^{a}P$≤.01, $^{b}P$≤.001 vs. DHA.

EPA alone or combined with DHA at a ratio of 3:1 and 1:1, respectively, compared with the vehicle control (Fig. 1C). Compared with OA, DHA and EPA alone induced a 12.5% and 31.7% decrease in the 70-kDa protein, respectively, while their combination reduced the protein relative amount by 36.3% and by 31.4% with DHA:EPA ratios of 3:1 and 1:1, respectively. EPA alone or combined with DHA was more effective than DHA alone in reducing glycosylated uPAR.

The expression of the 55-kDa protein was decreased by the n-3 LCPUFA alone or combined. Significant reductions of 10.7%, 18.6%, 24.5% and 22.6% were observed when cells were treated with DHA, EPA and DHA:EPA at ratios of 3:1 and 1:1, respectively, compared with the vehicle control (Fig. 1D). Similar results were observed when compared with OA treatment (Fig. 1D). EPA tended to be more efficient in reducing the 55-kDa protein expression compared with DHA ($P$≤.094). Both ratios of DHA:EPA, 3:1 and 1:1, also significantly reduced the 55-kDa protein expression.

### 3.3. Effects of n-3 LCPUFAs on SMC migration

All fatty acids tested significantly decreased AoSMC migration compared with the vehicle control (Fig. 2), DHA and EPA being the most effective (−24.3%, and −22.2%, respectively). The investigation on the effect of DHA and EPA treatments was enlarged to two different ratios both with a total n-3 LCPUFA content of 10 μM: DHA: EPA at a ratio of 3:1 (7.5 μM:2.5 μM) and DHA:EPA at a ratio of 1:1 (5 μM:5 μM). These ratios were chosen to mimic ratios observed in plasma of human volunteers supplemented with fish oil as described by Thies et al. [23]. The authors found a 3:1 ratio of DHA:EPA in the

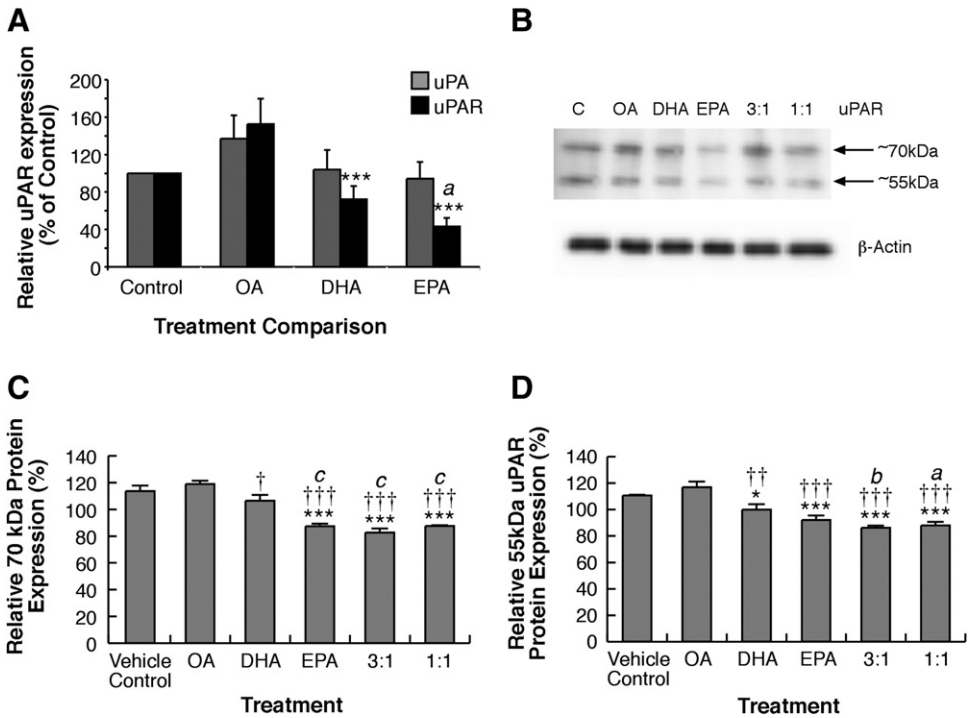


Fig. 1. N-3 LCPUFAs and uPAR expression. AoSMCs were treated for 48 h with vehicle control or 10 μM OA, DHA, EPA or 10 μM total fatty acid DHA:EPA at a ratio of 3:1 or 1:1. (A) uPA and uPAR gene expressions were analyzed using real-time reverse transcriptase PCR following treatment of AoSMCs with OA, DHA and EPA. $^{a}P$≤.05 compared with control; **$P$≤.01, ***$P$≤.001 compared with OA. (B) Representative uPAR, with bands at 70 kDa and 55 kDa, and β-actin (loading control) Western blots. C=vehicle control; OA, DHA & EPA=10 μM; 3:1=DHA:EPA at a ratio of 3:1 (7.5 μM:2.5 μM); 1:1=DHA:EPA at a ratio of 1:1 (5 μM:5 μM). The percentage of the mean density of the Western blot signal measured using BioRad Quantity One software is presented in (C) for the 70-kDa uPAR and (D) for the 55-kDa uPAR. *$P$≤.05, ***$P$≤.001 compared with the vehicle control; $^{\dagger}P$≤.05, $^{\dagger\dagger}P$≤.01, $^{\dagger\dagger\dagger}P$≤.001 compared with OA treatment; $^{a}P$≤.05, $^{b}P$≤.01, $^{c}P$≤.001 compared with DHA treatment.

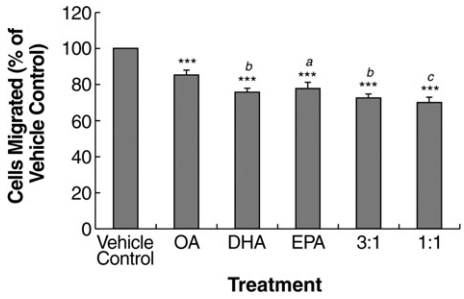


Fig. 2. Effect of n-3 LCPUFA on AoSMC migration. AoSMCs were treated for 48 h with vehicle control or 10 μM OA, DHA, EPA or 10 μM total fatty acid DHA:EPA at a ratio of 3:1 or 1:1. Cells were collected, counted and seeded on transwell inserts at 100,000 cells/insert. Number of cells having migrated after 5-h incubation was counted. ***$P \leq .001$ compared with the vehicle control; $^{a}P \leq .05$, $^{b}P \leq .01$, $^{c}P \leq .001$ compared with the OA treatment.

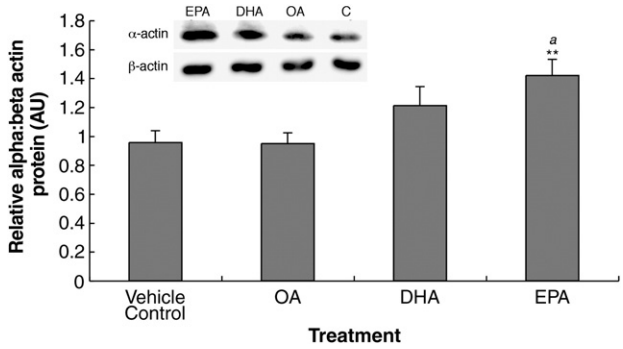


Fig. 3. Effect of n-3 LCPUFAs on AoSMC phenotype. AoSMC were treated for 48 h with vehicle control (C), OA, DHA, EPA (10 μM). AoSMC phenotype was estimated by calculating the ratio of α-actin:β-actin. Relative expression of both proteins was calculated as the percentage of the mean density of the Western blot signal measured using BioRad Quantity One software. **$P \leq .01$ compared with the vehicle control; $^{a}P \leq .05$ compared with OA.

serum of control volunteers not receiving fish oil supplementation, while DHA:EPA ratio was close to 1:1 in volunteers after supplementation with fish oil (720 mg EPA+280 mg DHA/day). The two ratios of DHA:EPA induced a similar inhibition of migration, 27.5% and 30.1% for 3:1 and 1:1 ratios, respectively. DHA, EPA, DHA:EPA at 3:1 and DHA:EPA at 1:1 ratios were also significantly more effective than OA. Migration was found to be decreased by 53% compared to control when uPAR was knocked down by 93% using siRNA (data not shown). This decrease in migration was not associated with changes in uPA, plasmin or MMPs (gelatinases) (data not shown).

### 3.4. N-3 LCPUFAs and phenotype of SMCs

The ratio of α-actin:β-actin increased by 48% after EPA treatment compared with both the vehicle control and OA (Fig. 3), suggesting a shift towards a more contractile phenotype. DHA increased the ratio compared with the vehicle control and OA, but this effect was not significant (Fig. 3).

### 3.5. N-3 LCPUFAs and MEK/ERK signaling pathway

The effect of n-3 LCPUFAs treatments on the activation of the MEK-ERK signaling pathway was assessed by measuring the ratio of phosphorylated/total ERK (both ERK1 and 2) following Western blotting detection (Fig. 4A and B). OA and EPA had no effect on MEK/ERK activation compared with the vehicle control, apart from p-ERK2 which significantly decreased after OA treatment (−37.7%,). DHA decreased both pERK1 and pERK2 phosphorylation compared with the vehicle control and EPA, while only pERK1 was significantly affected compared with OA. Both DHA and EPA mixtures significantly decreased the phosphorylation of both pERK1 and 2 compared with the vehicle control. Phosphorylation of both pERK1 and pERK2 was decreased by DHA:EPA at a ratio of 1:1 compared with OA and EPA (Fig. 4C).

MEK/ERK pathway activation was found to be associated with a PDGF-induced increase of uPAR expression (gene and protein levels) in AoSMCs (data not shown). The effect of the MEK inhibitor (U0126) on AoSMCs was similar to what was observed with N-3 LCPUFAs, with a significant reduction of PDGF-induced uPAR expression and significant inhibition of AoSMC migration (data not shown).

## 4. Discussion

N-3 LCPUFAs have previously been reported to affect SMCs migration [20,21]. Some suggested mechanisms included Notch regulation of MMP-2 and MMP-9 activity [29], modification of calcium mobilization by EPA treatment [30] and changes to voltage-gated sodium channel activity [21].

This study investigated more specifically the effects of n-3 LCPUFAs on the MEK/ERK pathway and uPA/uPAR plasminogen system, both known to be involved in SMC migration.

N-3 LCPUFAs were well incorporated into cellular phospholipids, as previously shown in other cell types [30,31]. The increase of EPA in AoSMCs following DHA treatment suggests that part of DHA taken up by the cells was retroconverted into EPA [32], while part of EPA delivered to the cells was effectively converted into DPA and, to a lesser extent, into DHA [32–34].

Both EPA and DHA inhibited AoSMC migration, which confirmed previous findings [20,21]. EPA inhibition of cell migration was associated with changes in cell phenotype, shifting from synthetic to contractile, which is less prone to migrate compared with synthetic SMC [35–38]. SMCs in the intima of atherosclerotic plaques tend to be in a synthetic phenotype, whereas SMCs from the media tend to be more contractile [2,39,40]. Reducing synthetic SMC in the intima could prevent or reduce the onset of fibrosis as synthetic cells produce more collagen than contractile cells [41].

This study shows for the first time that DHA can inhibit both uPAR expression and AoSMC migration *via* mechanisms involving the modulation of MEK/ERK signaling pathway. Using PDGF to treat the cells in the presence or absence of MEK inhibitor (U0126), we demonstrated that the activation of MEK/ERK signaling pathway was involved in the regulation of uPAR gene expression in AoSMCs. This indicates that the decrease of uPAR following treatment with DHA alone or combined with EPA was at least in part related to the inhibition of the MEK/ERK pathway. This inhibitory effect of DHA on the MEK/ERK signaling pathway was still observed after 24 h of treatment, while MEK/ERK activation happens usually 10 to 30 min following the stimulus [42], suggesting that DHA could have a long-term repressive effect on MEK/ERK.

Interestingly, EPA modulated uPAR expression and AoSMC migration similarly to DHA but without affecting MEK/ERK signaling pathway. However, EPA modified SMC phenotype. Whilst MEK/ERK activation seems to be involved in the switch of SMC phenotype from contractile to synthetic [43], our results with EPA show that MEK/ERK activation is not essential for this process or for the modulation of uPAR expression and SMC migration. A decrease in uPAR expression associated with a contractile phenotype in vascular SMCs [44] suggests that the effect of EPA on migration may be associated with changes in AoSMC phenotype involving MEK/ERK-independent signaling pathways. Differences in mechanisms by which EPA and DHA modulate SMC migration could be related to the production of

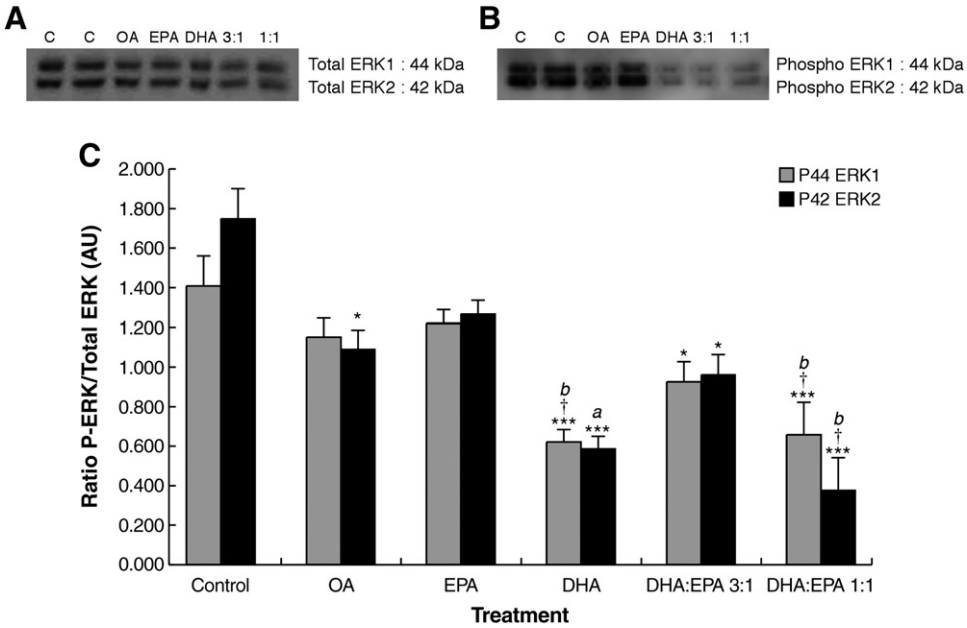


Fig. 4. Effect of n-3 LCPUFAs on MEK/ERK pathway. AoSMCs were treated for 24 h with vehicle control (C), OA, DHA, EPA (10 μM) or DHA:EPA mixture at ratio of 3:1 (7.5 μM:2.5 μM) and 1:1 (5 μM:5 μM). (A) Representative Western blot with anti-total ERK antibody and (B) anti-phospho-ERK antibody. (C) Representation of the ratio of phospho-ERK and total ERK signals. *$P \leq .05$, **$P \leq .01$, ***$P \leq .001$ when compared with vehicle control; $^{\dagger}P \leq .05$, $^{\dagger\dagger}P \leq .01$, $^{\dagger\dagger\dagger}P \leq .001$ when compared with OA; $^{a}P \leq .05$, $^{b}P \leq .01$ when compared with EPA.

derived oxygenated product from cyclooxygenases and/or lipoxygenases. PPAR agonists, such as 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$, can modulate MEK/ERK pathway in SMC [45]. Enriching cell membranes with EPA could lead to the production of prostaglandins and leukotrienes which have different properties compared to their related compounds synthesized from arachidonic acid. Furthermore, EPA and DHA are the precursors of resolvins and protectins [46], seemingly potent immunoregulators but whose biological properties and activities remain to be fully elucidated.

PDGF-induced migration is associated with an up-regulation of uPAR expression [47], and cell migration could be induced by the binding of uPA to its receptor uPAR [17]. In atherosclerotic plaques from both rabbits and humans, uPAR was associated with macrophages and SMCs of the neointima and, to a lesser extent, the media and adventitia, whereas none was detected in normal arterial tissue [47]. This effect of uPA binding to uPAR on migration is thought to be independent of its proteolytic activity [17,48–50]. Okada et al. found that anti-uPAR antibodies blocking binding of uPA to uPAR inhibited SMC migration in the presence and absence of single-chain uPA or the ATF of uPA despite low endogenous uPA levels. Thus, the inhibition of migration by anti-uPAR antibodies, despite the low level of endogenous uPA, suggested a role for uPAR in migration independent of uPA [15]. Glycosylated uPAR has a molecular weight of 55–60 kDa [51,52], suggesting that the 55-kDa protein detected by Western blotting in our experiment is uPAR. Full-length uPA bound to its receptor correspond to a band at around 110 kDa. However, the 70-kDa band observed in our experiment could correspond to uPAR bound to the ATF of uPA, which contains the uPAR binding site [51,53,54]. In our results, this 70-kDa protein showed the greatest decrease out of the two proteins after treatment with n-3 LCPUFA.

Moreover, both DHA and EPA significantly affected uPAR expression but not uPA expression in AoSMCs. The inhibition of uPAR expression in AoSMC induced a significant decrease in cell migration. The main function of uPAR is to bind uPA and to catalyze plasminogen activation into plasmin. Both activities remained unchanged following n-3 LCPUFA treatments (data not shown). This suggests that the decrease in AoSMC migration was not associated to the role of uPAR associated to uPA proteolytic activity. However, the binding of the A chain of uPA could be involved in AoSMC migration, as previously suggested [48–50]. These results reveal a direct role for uPAR in AoSMC migration.

For all the experiments, adding EPA and DHA together, independently of the ratio considered, tended to increase the particular effects observed with the individual fatty acids, indicating that they could act synergistically as both fatty acids do not modulate exactly the same signalization pathways. The ability of n-3 LCPUFAs to decrease SMC migration and alter their phenotype through various mechanisms adds to the potential benefit of their consumption with regards to atherosclerosis.

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