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Involvement of Cyclooxygenase-2 in Synergistic Effect of Cyclic Stretching and Eicosapentaenoic Acid on Adipocyte Differentiation

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Abstract. The present study examined the combined effects of fish-oil-derived ω-3 poly-unsaturated fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and cyclic stretching on the adipocyte differentiation of 3T3-L1 cells. Treatment with EPA alone did not inhibit the differentiation, although it partially suppressed the expressions of peroxisome proliferator-activated receptor (PPAR)-γ_2 and CCAAT/enhancer-binding protein (C/EBP)α transcripts, which are considered to be indispensable for the determination of adipocyte differentiation. However, the differentiation was significantly reduced when EPA but not DHA was concomitantly applied with cyclic stretching. In addition, EPA, but not DHA, could be a substrate of cyclooxygenase (COX)-2, and we found that the stretching significantly augmented the expression of COX-2 and that a selective COX-2 inhibitor (NS-398) inhibited the combined effect of the stretching and EPA. Taken together, it appears that the stretching and EPA exhibit a synergistic effect for the inhibition of adipocyte differentiation through stretch-induced COX-2.

Keywords: adipocyte differentiation, cyclic stretching, peroxisome proliferator-activated receptor (PPAR)-γ, cyclooxygenase (COX)-2, eicosapentaenoic acid

Introduction

The expression of adipogenic transcription factors, including the γ-isoform of peroxisome proliferator-activated receptor (PPARγ) and the CCAAT/enhancer binding protein (C/EBP) family consisting of C/EBPα, β, and δ, is indispensable to the determination of adipocyte differentiation (1). The cascade-like expression of these transcription factors has been well characterized by adipocyte differentiation of 3T3-L1 cells, which occurs in two discrete steps: first, incubation with an adipogenic cocktail consisting of dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), and insulin (INS) for 2 days (induction period) and then further incubation with INS alone for several days (maturation period).

We have previously reported that cyclic stretching preferentially reduces the expression of PPARγ_2 during the induction period through prolonged activation of extracellular-signal–regulated protein kinase (ERK) (2). This leads to the inefficient acquisition of adipocyte phenotypes such as glycerol-3-phosphate dehydrogenase (GPDH) activities and intracellular triglyceride accumulation in the maturation period (2), suggesting that the stretching perturbs the determination program of adipocyte differentiation in 3T3-L1 cells. These results led us to investigate whether or not agents that down-regulate transcription factors such as C/EBPs would augment the inhibitory effect of the stretching.

It has been widely recognized that a variety of endogenous and exogenous lipids and/or fatty acids play important roles in adipocyte differentiation; at least some of their functions are attributable to their regulation of C/EBPs and PPARγ (3). Eicosapentaenoic acid

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(EPA) and docosahexaenoic acid (DHA) are fish-oil-derived ω-3 polyunsaturated fatty acids (PUFA) and are interesting for their potential to lower the risk of cardiovascular events, especially with regard to their antithrombotic, anti-inflammatory, anti-atherogenic, and anti-arrhythmic actions (4, 5). Furthermore, ω-3 PUFA can modulate gene expression involved in lipid homeostasis in adipocytes (6, 7); however, the administration of either EPA or DHA in a static culture of 3T3-L1 cells only affects adipocyte differentiation slightly (8). In contrast, arachidonic acid (AA), an ω-6 PUFA and a precursor of prostaglandin (PG) synthesis, strongly inhibits adipocyte differentiation via a pathway dependent on the prostaglandin synthesis (8).

The inhibitory effect of AA was associated with sustained expression of cyclooxygenases (COX-1 and COX-2) (8). COX-2 has been implicated in the regulation of body fat, as mice heterozygous for the COX-2 gene develop obesity (9). In this regard, some COX products such as PGE_2 and $PGF_{2\alpha}$ show inhibitory effects on adipocyte differentiation (10 – 12). Although the preferred substrate for COX is AA, EPA can be a substrate for COX-2 both in vitro and in vivo, whereas EPA is only a poor substrate for COX-1 under the specific condition of increased peroxide tone (13). In contrast, DHA is not a direct substrate of COX-1 and COX-2 (14). The elevated EPA/AA ratio significantly increases COX-2–dependent 3-series PGs, that is, PGH_3 and its subsequent metabolites, which may play a primary role in the biological effect of EPA (13). Mechanical stresses such as stretching, have been reported to augment COX-2 expression in a several types of cells (15 – 20). However, as far as we know, there is no report indicating the interactive role between stretching and EPA in the regulation of adipogenesis.

We have demonstrated herein that concomitant use during the induction period of cyclic stretching and EPA, but not DHA, synergistically reduced the adipogenic potential of 3T3-L1 cells. As to the mechanism, stretch-induced augmentation of COX-2 plays a crucial role in the synergistic interaction between the stretching and EPA.

Materials and Methods

Cell culture and adipocyte differentiation

Mouse 3T3-L1 preadipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM, 25 mM glucose) supplemented with newborn calf serum to 10% (v/v) in a humidified 5% CO_2 incubator at 37°C. To induce adipocyte differentiation, confluent 3T3-L1 cells were exposed to induction medium, consisting of 0.25 μM dexamethasone (DEX), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 10 μg/ml insulin (INS), 200 μM ascorbic acid (AA), and 10% fetal bovine serum (FBS), in DMEM for up to 45 h (induction period). Just prior to use, *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) or *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) (each from Sigma, St. Louis, MO, USA) was mixed with fatty acid-free bovine serum albumin (BSA) at a 5:1 molar ratio (6) and further diluted to the concentrations indicated in the figures with the induction medium. The concentration range of EPA or DHA in differentiating 3T3-L1 cells was carefully checked by morphological examination and an adherent assay. No apparent sign of cell damage was observed with EPA or DHA up to concentrations of 300 μM (data not shown). We used 10 – 100 μM of EPA or DHA throughout by referring to published data concerning tissue distribution of orally administered esterified EPA in animals (21). NS-398 (Cayman Chemical, Ann Arbor, MI, USA), a selective COX-2 inhibitor, was dissolved in dimethylsulfoxide at 100 mM and also diluted to a final concentration of 10 μM with the induction medium. The application of uniaxial cyclic stretching to differentiating 3T3-L1 cells was carried out as described previously (2). Briefly, the 3T3-L1 cells were subjected to cyclic stretching up to 120% of the initial length, which our previous study determined to be a sub-maximal condition (2), with a frequency of 1 Hz for the entire induction period of 45 h. The medium was then replaced with maintenance medium consisting of 10% FBS and 5 μg/ml of INS in DMEM, followed by a change of the medium every 2 days during a 9-day maturation period. After this period, Oil-Red-O staining of intracellular lipid droplets and biochemical measurement of triglyceride contents were carried out as described previously (2).

Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using the ISOGEN RNA extraction reagent (Nippon Gene, Toyama) and treated with deoxyribonuclease (DNase) I. Two micrograms of DNase-treated total RNA was reverse transcribed to cDNA using a random deoxyribonucleotide hexamer and M-MLV reverse transcriptase. Quantitative PCR was performed using the SYBR premix Ex Taq kit (Takara Bio, Otsu, Shiga) on the AB 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The oligonucleotide primers used to analyze the various mouse transcripts (Table 1) were designed by PrimerExpress software (Applied Biosystems) with reference to the published nucleotide sequences. The integrity of each amplified product was routinely checked by the dissociation curve protocol of the real-time PCR

Table 1. Oligonucleotide primers used for RT-PCR

Transcript	Forward	Reverse
PPARγ_2	5'-ACTGCCTATGAGCACTTCAC	5'-CAATCGGATGGTTCTTCGGA
C/EBPα	5'-TGGACAAGAACAGCAACGAG	5'-AATCTCCTAGTCCTGGCTTG
C/EBPβ	5'-ACTACGGTTACGTGAGCCTC	5'-CAGCTGCTTGAACAAGTTCC
C/EBPδ	5'-ACCTCTTCAACAGCAACCAC	5'-TTCTGCTGCATCTCCTGGTT
COX-1	5'-AACCAGGGTGTCTGTGTCCG	5'-TTCTTCCCTTTGGTCCCCAT
COX-2	5'-TCAATGAGTACCGCAAACGC	5'-AGGGCTTTCAATTCTGCAGC
18S rRNA	5'-GGTAACCCGTTGAACCCCAT	5'-GGTGTGTACAAAGGGCAGGG

machine and polyacrylamide gel electrophoresis.

Western blot analysis

A cell extract containing an equal amount of protein (20 μg) was resolved by 10% SDS-polyacrylamide gel electrophoresis under reducing conditions. Protein was transferred to a PVDF membrane (Millipore, Bedford, MA, USA), and the membrane was blocked with 5% BSA in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20. Western blot analysis was then carried out using either anti-murine COX-2 antiserum (Cayman Chemical, Ann Arbor, MI, USA) or anti–α-tubulin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti-COX-2 antiserum, anti–α-tubulin antibody, species-matched biotinylated secondary antibodies, and streptavidin-horseradish peroxidase complex were diluted with Can Get Signal (Toyobo, Osaka), an immunoreaction enhancer solution, according to the manufacturer's instructions. Immunoreactive signals were visualized using the ECLplus system (GE Healthcare, Buckinghamshire, England). The specificity of the immunoreactive signal was thoroughly checked by a murine COX-2 blocking peptide (Cayman) as well as control normal serum/IgG.

Statistical analyses

Data are expressed as means ± S.E.M. Statistical analyses were performed with the Tukey-Kramer test or Williams' test after analysis of variance (ANOVA). *P* values less than 0.05 (Tukey-Kramer test) or 0.025 (Williams' test) were considered statistically significant.

Results

Effect of EPA and DHA on the expression of adipogenic transcription factors in the determination stage of 3T3-L1 differentiation

The effects of EPA and DHA on the gene expression of C/EBPs and PPARγ_2 during the induction period were examined quantitatively by real-time RT-PCR (Fig. 1). Consistent with published reports (22 – 24), we found that when cells were treated with a standard adipogenic cocktail (DEX + IBMX + INS), C/EBPα and PPARγ_2 transcripts were induced at a late stage (45 h) while C/EBPβ and C/EBPδ transcripts reached a peak level at an earlier stage (3 h) of the induction period. At these time-points, suppression of the expression levels of PPARγ_2 (Fig. 1A) and C/EBPα (Fig. 1B) transcripts by EPA at 100 μM reached statistical significance (60% and 80% of the control, respectively), whereas EPA did not affect the expression of C/EBPβ or C/EBPδ transcripts (Fig. 1: C and D, respectively). Suppression of the expression of PPARγ_2 transcript by DHA at 100 μM also showed a statistical significance (about 80% of the control) (Fig. 1E). However, DHA had no significant effect on the expression of C/EBPα, C/EBPβ, and C/EBPδ transcripts (Fig. 1: F – H).

Synergistic inhibition of adipocyte differentiation by mechanical stretching combined with EPA

Cyclic stretching during the induction period significantly reduces the adipogenic potential of 3T3-L1 cells through downregulation of PPARγ_2, leading to a significant reduction in GPDH activity and intracellular accumulation of triglycerides in the maturation period (2). This inhibitory effect reaches a maximum at the total length of 130% (unstretched length = 100%) with a frequency of 1 Hz. In contrast, as we have already reported (2), submaximal-strength stretching (120% or less at 1 Hz) showed a marginal effect on triglyceride accumulation during the maturation period (Fig. 2A panel ii and Fig. 2B), although the GPDH activity was still reduced significantly (2). Likewise, treatment with EPA alone during the induction period also showed a marginal effect on the triglyceride accumulation (Fig. 2A panel iii and Fig. 2B). Nevertheless, concomitant treatment by subthreshold stimuli, such as stretching to 120% at 1 Hz and 30 μM EPA, efficiently downregulated both C/EBPα and PPARγ_2 transcripts (Fig. 2: C and D), led to enhanced suppression of triglyceride accumulation (Fig. 2A panel iv and Fig. 2B). In contrast, DHA combined with cyclic stretching showed no

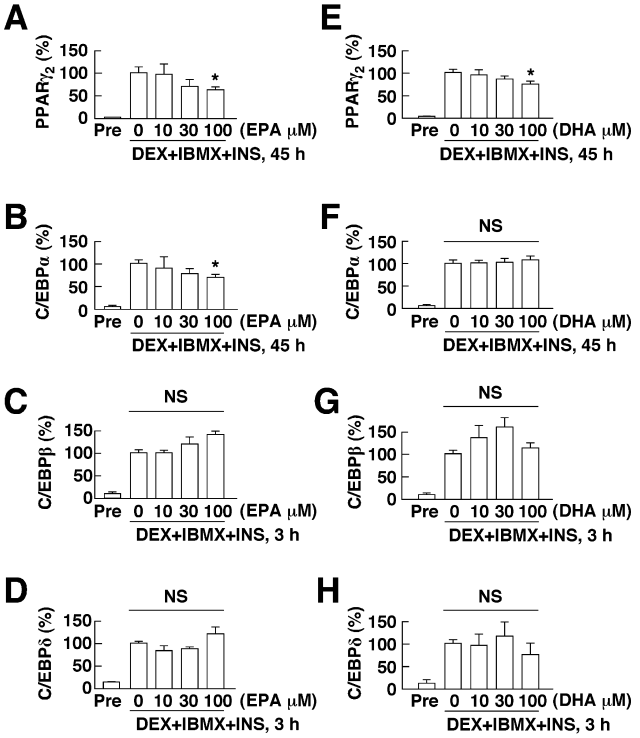

Fig. 1. Effects of EPA and DHA on the expression of adipogenic transcription factors during the induction period. 3T3-L1 cells were treated with an induction medium (DEX + IBMX + INS) containing 10, 30, or 100 μM of either EPA (A – D) or DHA (E – H) for the indicated periods of time. The total RNA of the cells was subjected to real-time RT-PCR to detect the following transcripts: A and E, PPARγ_2 (n = 10); B and F, C/EBPα (n = 10); C and G, C/EBPβ (n = 3); D and H, C/EBPδ (n = 3). Values are expressed as means ± S.E.M. of the relative levels in control cells (DEX + IBMX + INS = 100%) normalized by the level of 18S rRNA. *P<0.025 *vs* control by Williams' test. Pre, uninduced 3T3-L1 preadipocytes. NS, not significant.

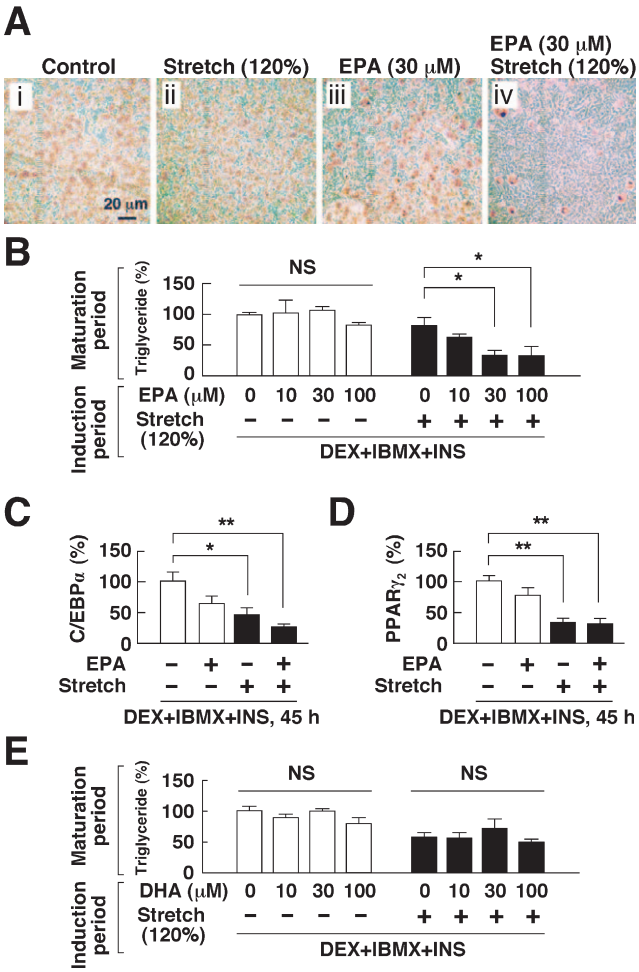

Fig. 2. Effect of concomitant use of cyclic stretching and EPA or DHA on differentiation of 3T3-L1 cells. A: Phase-contrast microscopic images of Oil-Red-O stained 3T3-L1 cells after a maturation period of 9 days; cells were subjected to the following conditions during the induction period: i) unstretched cells (control); ii) stretched (120%, 1 Hz) cells; iii) unstretched cells with 30 μM EPA; iv) stretched (120%, 1 Hz) cells with 30 μM EPA. Scale bar, 20 μm. B: Concentration-response effect of EPA with (solid column) or without (open column) submaximal-strength cyclic stretching (120%, 1 Hz) on accumulation of triglycerides in 3T3-L1 cells after a maturation period of 9 days. Values are expressed as means ± S.E.M. of relative triglyceride levels compared with those of the control (DEX + IBMX + INS = 100%). *P<0.025 *vs* stretching without EPA by Williams' test. N = 3 to 6. NS, not significant. C and D: Summarized data showing the expression of C/EBPα (C) and PPARγ_2 (D) transcripts at hour 45 of the induction period with concomitant use of EPA (30 μM) and stretching (120%, 1 Hz). Values are expressed as means ± S.E.M. of the relative levels in control cells (DEX + IBMX + INS = 100%) normalized by the level of 18S rRNA. *P<0.05, **P<0.01 *vs* corresponding value by Tukey-Kramer test. N = 4 to 6. E: Concentration-response effect of DHA with (solid column) or without (open column) submaxial-strength of cyclic stretching (120%, 1 Hz) on accumulation of triglyceride in 3T3-L1 cells after a maturation period of 9 days. Values are expressed as means ± S.E.M. of relative triglycerides levels compared with those of the control (DEX + IBMX + INS = 100%). N = 7 to 8. NS, not significant.

apparent effect on triglyceride accumulation in the maturation period (Fig. 2E). These results indicated that cyclic stretching and EPA, but not DHA, synergistically inhibited adipocyte differentiation of 3T3-L1 cells.

Involvement of stretch-induced COX-2 expression in synergistic effect of cyclic stretching and EPA

It has been demonstrated that EPA can be a substrate for COX-2 both in vitro and in vivo, whereas this fatty acid could be a substrate for COX-1 only under specific circumstances such as increased peroxide concentration (13). Thus, we tested whether or not COX-2 was upregulated in response to cyclic stretching during the induction period. The expression of COX-2 mRNA was induced transiently during the induction period, and the peak levels of COX-2 transcript at 3 h after the onset of induction were significantly augmented by stretching (Fig. 3A), while there was no measurable change in the level of COX-1 transcripts (Fig. 3B). In addition,

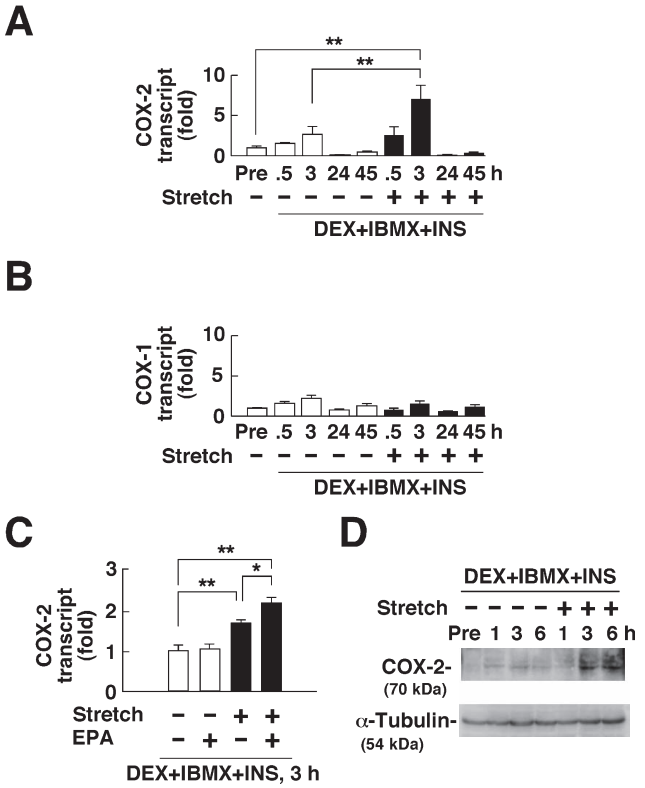

Fig. 3. Augmentation of COX-2 expression by cylic stretching. COX-2 (A) and COX-1 (B) transcripts during the induction period with (solid column) or without (open column) cyclic stretching (120%, 1 Hz) by real-time RT-PCR are shown. Values are expressed as means ± S.E.M. of the relative amounts of transcripts normalized by the level of 18S rRNA (Pre = 1). **P<0.01 *vs* corresponding value by Tukey-Kramer test. N = 10 to 11. C: Summarized data showing the augmented expression of COX-2 transcript at hour 3 of the induction period with concomitant use of EPA (30 μM) and stretching (120%, 1 Hz). Values are expressed as means ± S.E.M. of the relative amounts of COX-2 transcripts at hour 3 of induction, normalized by the level of 18S rRNA (DEX + IBMX + INS = 1). *P<0.05, **P<0.01 *vs* corresponding value by Tukey-Kramer test. N = 7. D: Western blot analysis of COX-2 and α-tubulin proteins. Representative data from 3 independent experiments with similar results are shown.

concomitant treatment of EPA (30 μM) and cyclic stretching (120%, 1 Hz) further augmented COX-2 expression, while EPA alone did not affect the COX-2 expression (Fig. 3C). The stretch-induced expression of COX-2 was also confirmed at the protein level; the COX-2 protein was significantly augmented in response to stretching in hours 3 to 6 of the induction period (Fig. 3D).

Involvement of stretch-induced COX-2 in the synergistic inhibition of adipocyte differentiation by concomitant use of stretching and EPA was functionally examined. Administration of NS-398 (10 μM), a selective inhibitor of COX-2 (25, 26), in the induction medium with the concomitant use of EPA (30 μM)

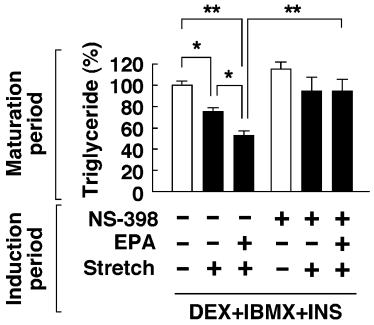

Fig. 4. Effect of NS-398, a COX-2 inhibitor, on the synergistic inhibition of cyclic stretching and EPA on adipocyte differentiation. 3T3-L1 cells were treated with or without cyclic stretching (120% 1 Hz), EPA (30 μM), and NS-398 (10 μM) during the induction period. Afterwards, the intracellular triglyceride accumulation after a maturation period of 9 days was measured. Values are expressed as means ± S.E.M. of relative triglyceride levels (DEX + IBMX + INS = 100%). *P<0.05, **P<0.01 *vs* corresponding values by Tukey-Kramer test. N = 6 to 20.

and cyclic stretching (120%, 1 Hz) completely reversed the synergistic inhibition of triglyceride accumulation in the cells (Fig. 4). In addition, the inhibitory effect of cyclic stretching alone on triglyceride accumulation was also partially reversed by NS-398 (Fig. 4). These results suggest that COX-2 was involved in the stretch-induced inhibitory mechanism for adipocyte differentiation, probably mediated by endogenous AA and PGs; this inhibitory mechanism would be further enhanced by administration of an exogenous substrate for COX-2 such as EPA. It has been reported that several COX inhibitors also act as PPARγ agonists (27, 28). However, the concentration of NS-398 (10 μM) used in this study has been used before (for example, 5 – 50 μM) for specific inhibition of COX-2 (8, 19, 29). In fact, we did not observe any apparent effect of NS-398, when used during the induction period, on the triglyceride accumulation in the maturation period (Fig. 4). In contrast, troglitazone, a PPARγ agonist, significantly enhanced adipocyte differentiation (2).

Discussion

Regarding the potential usefulness of mechanical stresses on the regulation of adipocyte functions, we have previously found that cyclic stretching counteracts the stimulating effects of troglitazone, a synthetic ligand for PPARγ, on adipocyte differentiation (2). Therefore, it may be possible that stretching exerts a fine-tuning action on drugs that affects both the differentiation and post-maturation functions of adipocytes. In the present study, we demonstrated that cyclic stretching and EPA, but not DHA, synergistically inhibit adipocyte differen-

tiation of 3T3-L1 cells.

Ingestion of ω-3 long-chain PUFA, including EPA and DHA, has been shown to help ameliorate some pathophysiological conditions (4, 5) such as arrhythmia, atherosclerosis, and thrombosis by decreasing plasma triglyceride levels or by protecting against insulin resistance (30). Although the majority of these effects have been attributed to changes in lipid metabolism in hepatic tissues (30, 31), ω-3 PUFA can also directly modulate adipocyte functions such as lipogenic gene expression (6, 7). However, a previous observation indicated that the effect of EPA or DHA on adipocyte differentiation in a static culture of 3T3-L1 was quite small (8).

During the induction period, which is initiated by an adipogenic cocktail (DEX + IBMX + INS) for 2 days, 3T3-L1 cells are irreversibly committed to differentiate through a transcription factor cascade, especially PPARγ_2 and C/EBPα (32, 33). In this study, we examined the effect of EPA and DHA on the commitment stage of differentiation and found that EPA per se at 100 μM produced a slight but significant downregulation of mRNAs for PPARγ_2 (Fig. 1A) and C/EBPα (Fig. 1B) without affecting the transcripts for other adipogenic factors such as C/EBPβ and C/EBPδ (Fig. 1: C and D). Nevertheless, EPA alone did not affect the acquisition of differentiation potential in 3T3-L1 cells (Fig. 2: A and B). We therefore expected that EPA would synergistically interact with cyclic stretching, which preferentially downregulates PPARγ_2 (2), in the determination of 3T3-L1 adipogenesis. In fact, the combination of cyclic stretching with EPA in each submaximal strength /subthreshold concentration efficiently downregulated the expression of C/EBPα and PPARγ_2 (Fig. 2: C and D), and this combination also significantly disturbed the differentiation of 3T3-L1 (Fig. 2: A and B). DHA tended to downregulate PPARγ_2 transcript (Fig. 1E), but it did not affect the expression of C/EBPα (Fig. 1F), C/EBPβ (Fig. 1G), and C/EBPδ transcripts (Fig. 1H). Furthermore, no synergistic effect between DHA and the stretching was observed on adipocyte differentiation (Fig. 2E).

Part of the action of EPA has been attributed to its increased incorporation into the *sn*2 position of membrane phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, which may lead to compelling replacement and/or competition with AA. Thus, the elevated EPA/AA ratio significantly increases production of 3-series prostanoids, namely PGH_3 and its metabolites, resulting in altered biological activities (13). DHA also could be incorporated into the membrane, but it could not enter the eicosanoid pathway efficiently (13) and so could not be a direct substrate for COXs (14). The present study clearly showed that mechanical stretching alone significantly upregulates COX-2 expression (Fig. 3: A, C, and D) and concomitant treatment with EPA and cyclic stretching synergistically augments the expression of COX-2 (Fig. 3C). Furthermore, NS-398, a selective inhibitor of COX-2, apparently reversed the combined effect of EPA and the stretching on determination of adipocyte differentiation (Fig. 4). A variety of prostaglandins, especially PGE_2, $PGF_{2\alpha}$, and PGI_2, are produced in 3T3-L1 cells throughout their differentiation stages (6). It has been indicated that PGE_2 and $PGF_{2\alpha}$ show inhibitory effects on adipogenesis (10 – 12), whereas PGI_2 (prostacyclin) has a promoting effect (34, 35). At present, the exact metabolite or metabolites of EPA that inhibit adipocyte differentiation remain to be determined.

In conclusion, our results suggest that the application of stretching combined with EPA but not DHA could act synergistically to inhibit adipocyte differentiation. Cyclic stretching not only downregulates PPARγ_2 but also upregulates COX-2, which could metabolize EPA probably into 3-series PGs (13). Mechanical stresses, including cyclic stretching, increase the efficacy of pharmacological and nutritional interventions in differentiation and cell renewal of adipocytes.

Acknowledgments

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