Eicosapentaenoic acid prevents endothelin-1-induced cardiomyocyte hypertrophy in vitro through the suppression of TGF-β1 and phosphorylated JNK

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Shimojo, Nobutake, Subrina Jesmin, Sohel Zaedi, Seiji Maeda, Masaaki Soma, Kazutaka Aonuma, Iwao Yamaguchi, and Takashi Miyauchi. Eicosapentaenoic acid prevents endothelin-1-induced cardiomyocyte hypertrophy in vitro through the suppression of TGF-β1 and phosphorylated JNK. Am J Physiol Heart Circ Physiol 291: H835–H845, 2006. First published February 24, 2006; doi:10.1152/ajpheart.01365.2005.—The cardiovascular benefit of fish oil in humans and experimental animals has been reported. Endothelin (ET)-1 is a well-known cardiac hypertrophic factor. However, although many studies link a fish oil extract, eicosapentaenoic acid (EPA), to cardiac protection, the effects of EPA on cardiac hypertrophy and underlying mechanism(s) are unclear. The present study investigated whether EPA prevents ET-1-induced cardiomyocyte hypertrophy; the potential pathways likely to underlie such an effect were also investigated. Cardiomyocytes were isolated from neonatal rat hearts, cultured for 3 days, and then treated for 24 h with vehicle only (control), treated with 0.1 nM ET-1 only, or pretreated with 10 μM EPA and then treated with 0.1 nM ET-1. The cells were harvested, and changes in cell surface area, protein synthesis, expression of a cytoskeletal (α-actinin) protein, and cell signaling were analyzed. ET-1 induced a 97% increase in cardiomyocyte surface area, a 72% increase in protein synthesis rate, and an increase in expression of α-actinin and signaling molecule transforming growth factor-β1 (TGF-β1), c-Jun NH2-terminal kinase (JNK), and c-Jun. Development of these ET-1-induced cellular changes was attenuated by EPA. Moreover, the hypertrophied cardiomyocytes showed a 1.5- and a 1.7-fold increase in mRNA expression of atrial and brain natriuretic peptides, the classical molecular markers of cardiac hypertrophy, respectively; these changes were also suppressed by EPA. Here we show that ET-1 induces cardiomyocyte hypertrophy and expression of hypertrophic markers, possibly mediated by JNK and TGF-β1 signaling pathways. These ET-1-induced effects were blocked by EPA, a major fish oil ingredient, suggesting that fish oil may have beneficial protective effects on cardiac hypertrophy.

neonatal cardiomyocyte; transforming growth factor-β1; c-Jun NH2-terminal kinase

CARDIAC HYPERTROPHY, which is defined by an increase in heart size and/or myofibrillar volume without a change in myocyte number, occurs in response to physiological and pathophysiological stimulation (15). Although cardiac hypertrophy is typically viewed as a compensatory response that normalizes ventricular wall stress, sustained hypertension is correlated with an increase in the incidence of and mortality from cardiovascular disease (26). Thus pathological cardiac hypertrophy is different from physiological cardiac hypertrophy. When the myocardial adaptations are unable to satisfy the increased demands or able to meet the increased demands only at the expense of normal function (35), the hypertrophy is considered to be pathological. On the other hand, when the adaptations are sufficient to fulfill the requirements while normal function is maintained, the hypertrophy is physiological (35). Primarily pathological and physiological states of left ventricular hypertrophy (LVH) have been considered on the basis of normal chamber performance and oxygen delivery as well as reversibility of the hypertrophy on removal of the overload (35). Moreover, both states are also characterized by the nature of the imposed load and the resulting myocardial adaptations (35). Only pathological LVH has been shown to be a risk factor for cardiovascular events (24) such as myocardial infarction, ischemia, arrhythmia, and sudden death (47).

Pathological cardiac hypertrophy is caused by a prolonged exposure of cardiomyocytes to external stimuli, such as hemodynamic overload and neurohumoral factors (47). One of the most important vascular hormones that contribute to the development of hypertrophy is endothelin (ET)-1. ET-1, which was initially identified and purified from porcine aortic endothelial cell cultures, is a vasoactive peptide that contains 21 amino acids and has 2 intramolecular disulfide bonds (48). We know that a variety of cells, including cardiomyocytes, produce this peptide (22) and that, in addition to its vasoconstrictive effects, it has potent mitogenic effects on a number of cells, including cardiomyocytes (42). In vitro studies have shown that ET-1-induced cardiac hypertrophy involves various hypertrophic signaling cascades mediated by ET type A (ETA) receptors, such as Raf-1 and MAP kinases, in the neonatal rat (46). Other signaling pathways mediating ET-1-induced cardiac hypertrophy include phospholipase C, PKC, ERK1 and ERK2, c-Fos and c-Jun, c-Jun NH2-terminal kinase (JNK), and p38 (22, 41). The role of ET-1 in cardiac hypertrophy has also been well documented in the recent past by in vivo studies (17, 23). ET-1 is markedly increased in the hypertrophied heart and the failing heart (37), conditions that are, interestingly, significantly inhibited by chronic treatment with ETA receptor antagonists (37). Taken together, these data strongly suggest a significant role for ET-1 in the development of cardiac hypertrophy in vitro and in vivo. Thus, whereas the effects of ET-1 on cardiac hypertrophy are well documented, little is known about the possible therapeutic interventions and their underlying signaling pathways.
The transforming growth factor (TGF)-β superfamily comprises a set of regulatory peptides, such as TGF-β1, that have multiple effects on cell growth and differentiation (5). Studies have shown altered expression and biological activity of TGF-β1 in hypertrophic cardiomyocytes (5), an effect that may be mediated by ET-1 (47). However, our knowledge of the relation and interaction between TGF-β1 and ET-1 in the pathophysiology of hypertrophic cardiomyocytes is incomplete. Specifically, the effect of ET-1 on TGF-β1 expression in hypertrophied cardiomyocytes is not known.

Although there is no overwhelming consensus concerning the protective role of dietary fish oil in coronary artery disease (36), a significant number of experimental studies and clinical intervention trials have shown a cardioprotective effect of dietary fish and fish oil intake (19, 43). Among them, ω-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have recently been shown to have potential beneficial effects on cardiovascular diseases (8, 11). Studies demonstrate that dietary supplementation of fish oil and ω-3 long-chain polyunsaturated fatty acids (PUFAs) improves survival in patients with acute myocardial infarction (7, 40) and that use of fish oil reduces cardiac arrhythmias (18) and angina pectoris (40). The cardioprotective effects of fish consumption are thought to be beneficial through modulation of lipid and lipoprotein metabolism (45), regulation of blood pressure (4), improvement of vascular endothelial function (32), reduction of neutrophil and monocyte cytokine production (2), inhibition of thrombogenesis and the inflammatory response (9), and an antiarrhythmic effect (28). Little is known about the role of dietary fish oils in cardioprotection and the effects of fish oils on growth factors associated with cardiac hypertrophy. Siddiqui et al. (39) demonstrated the inhibitory effects of DHA on phenylephrine (PE)-induced cardiac hypertrophy and on Ras, Raf-1, ERK1/2, and p90rsk pathways. More recently, DHA has been shown to modulate activation of extracellular signaling pathways, such as PKC and MAPK (13). Thus it is reasonable to postulate that EPA, another major ω-3 PUFA in fish, may act collectively or singly in cardioprotection through alteration of hypertrophic responses in cardiomyocytes.

The present study was designed to examine whether pretreatment of cardiomyocytes with EPA could prevent ET-1-induced hypertrophy. To unravel potential mechanisms underlying this process, we investigated whether TGF-β1 expression could be induced by ET-1 in neonatal cardiomyocytes and, if so, whether EPA can alter this effect. The effect of EPA on ET-1-induced JNK and c-Jun activation was studied in neonatal cardiomyocytes. Finally, expression levels of atrial and ventricular myocytes. The cells were plated in 24-well dishes at a density of $10^5$ cells/well. After treatment with ET-1 alone or EPA and ET-1 for 24 h, $[^{14}C]$leucine (0.1 μCi/ml) was added, and the cells were incubated for 24 h. The cells were washed twice with cold PBS, and 5% trichloroacetic acid was added for 10 min. The cells were then incubated with 0.25% trypsin at 37°C for 30 min, and cell residues were solubilized in 0.5 N NaOH for 10 min. Aliquots were counted with a scintillation counter (model LS-6500, Beckman Coulter, Fullerton, CA).

**Immunocytochemistry and Immunofluorescence**

For immunocytochemistry and immunofluorescence staining, the cells were permeabilized with 0.2% Triton X-100 (Wako, Osaka, Japan), fixed with 3% formaldehyde or acetone for 10 min at room temperature in PBS or for 30 min at 40°C, reacted for 8 h with primary antibodies for α-actinin (Sigma), TGF-β1, and phosphorylated JNK (R & D Systems, Minneapolis, MN), and then treated with peroxidase-conjugated secondary antibody (Amersham, Buckinghamshire, UK) and 3,3'-diaminobenzidine tetrahydrochloride for visualization. Hematoxylin was used for counterstaining. For immunofluorescence, after incubation with primary antibodies, the cells were rinsed in PBS and then exposed to the fluorescence secondary antibody Cy3-conjugated AffiniPure goat anti-rabbit IgG or fluorescein-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h according to the manufacturer’s instructions. Sections processed without primary antibodies served as negative controls. For phosphorylated JNK, 4’,6-diamidino-2-phenylindole (Santa Cruz Biotechnology, Santa Cruz, CA) was used for counterstaining. The coverslips were mounted with Immunon (Thermo Shandon, Pittsburgh, PA). Immunofluorescent images were viewed using the laser scanning confocal imaging system (model MRC-1024, Bio-Rad Laboratories).

**Enzyme-Linked Immunosorbent Assay**

Levels of TGF-β1 and phosphorylated JNK in cardiomyocytes were determined using ELISA kits [R & D Systems (TGF-β1) and Sigma (phosphorylated JNK)] according to the manufacturers’ instructions.

**Western Blot Analysis**

Cardiomyocytes were plated at a field density of $2 \times 10^6$ cells/cm² on 60-mm culture dishes with 2 ml of culture medium. Cardiomyocytes of different groups were lyzed on ice with buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% sodium deoxycholate, and 0.1% SDS). Protein concentration of the supernatant was determined using the bicinchoninic acid protein assay.
The PVDF membrane was incubated overnight at 4°C with 5% nonfat milk in PBS containing 0.1% Tween 20 (TPBS). Thereafter, the PVDF membrane was incubated overnight at 4°C with specific antibodies for α-actinin (Sigma), TGF-β1 (R & D Systems), JNK (Cell Signaling), phosphorylated JNK (Cell Signaling), c-Jun (Ser73; Cell Signaling), and phosphorylated c-Jun (Ser73; Cell Signaling) in TPBS; washed three times in TPBS; and then incubated with horseradish peroxidase-conjugated anti-rabbit (Amersham), anti-mouse (Amersham), or anti-goat (Santa Cruz Biotechnology) antibody diluted 1:2,000–10,000 in TPBS at room temperature for 60 min. The blots were visualized with the enhanced chemiluminescence detection system (Amersham, Pharmacia Biotech), exposed to X-ray film, and analyzed using NIH Image software. Antisera against TGF-β1, α-actinin, phosphorylated c-Jun (Ser73), c-Jun, and JNK were purchased from Cell Signaling Technology (Beverly, MA).

RNA Preparation and Real-Time Quantitative PCR

Total RNA from cardiomyocytes was isolated using RNeasy (Qiagen, Tokyo, Japan). After isolation, DNase I treatment, and quantification, RNA was reverse transcribed to cDNA by Omniscript RT using a first-strand cDNA synthesis kit (Qiagen). The gene-specific primers and TaqMan probes were synthesized from Primer Express version 1.5 software (Perkin-Elmer) according to the published cDNA sequences for each gene.

The expression of GAPDH mRNA was used as an internal control. The PCR mixture (25 μl total volume) consisted of forward and reverse primers for each gene (Perkin-Elmer) at 450 nM each, FAM-labeled primer probes (Perkin-Elmer) at 200 nM, and TaqMan Universal PCR Master Mix (Perkin-Elmer). Each PCR amplification was performed in triplicate as follows: 1 cycle at 95°C for 10 min and 40 cycles at 94°C for 15 s and 60°C for 1 min. The quantitative values of target mRNAs were normalized by GAPDH mRNA, because GAPDH mRNA expressions were more stable among all the samples than other internal controls such as β-actin and 18S ribosomal RNA. Primers and probes are as follows: c-Jun [5′-GACCTGCGCCTGACCTT-3′ (forward) and 5′-CCATTGCCTGACCCAGTATCAG-3′ (reverse)], c-Jun probe (5′-CTGCTCAAGCTGCGT-3′), TGF-β1 [5′-CCGGAAGCAGTGCCAGA-3′ (forward) and 5′-TGTCACAGTTGACTGATC-3′ (reverse)], TGF-β1 probe (5′-CAGAGCTGCGGCTGCA-3′), ANP [5′-TGATGGATTTCAAAGTATG-3′ (forward) and 5′-CGCCGATCCTTCC-3′ (reverse)], ANP probe (5′-CGGAGCTTCTCTCAGC-3′), BNP [5′-ACAATCCACGATGCAAGT-3′ (forward) and 5′-CTTCAGCTGCTTATC-3′ (reverse)], BNP probe (5′-CGGAGCTTCTCTCAGC-3′), GAPDH [5′-GATGGCAAGCTGAGACTTCA-3′ (forward) and 5′-GGAGCTGAGACTTCA-3′ (reverse)], and GAPDH probe (5′-CTTGATGAGCTGAGACTTCA-3′). Statistical Analysis

Values are means ± SE. Data were compared using one-way ANOVA. Post hoc comparisons were made with Fisher’s protected least significance t-test for multiple comparisons. Differences were considered significant at P < 0.05.

RESULTS

Preliminary Experimental Data

ET-1 dose-response study. Our previous data showed that 10^{-8} M ET-1 induced cardiomyocyte hypertrophy (22). Here, we determined the effects of various ET-1 doses on cardiomyocyte surface area (Fig. 1A). All the ET-1 doses used in the dose-response studies induced significant increases in cardiomyocyte surface area compared with the vehicle-treated (control) cells (Fig. 1A). However, there was no significant difference in cell surface area among the ET-1-treated groups. In addition, when we evaluated the effects of various ET-1 doses on cellular protein synthesis as determined by leucine uptake (Fig. 1B), 10^{-10} M ET-1 was the optimal dose (Fig. 1B). On the basis of these findings, 10^{-10} M ET-1 was used for subsequent experiments.

Cardiomyocyte Response to Varying Doses of EPA After 10^{-10} M ET-1

Inasmuch as 3–300 μM EPA has been shown to upregulate nitric oxide production in endothelial cells (10, 34), we used 1–100 μM EPA to generate a response curve in the present study. In our preliminary experiments, we used 1–100 μM (specifically, 1, 3, 10, and 30 μM) EPA to conduct an EPA dose-response study. EPA at 1, 3, and 10 μM significantly arrested increases in cell surface area induced by 10^{-10} M ET-1 (Fig. 2A). The cell surface area of groups treated with 30 and 100 μM EPA decreased compared with control (Fig. 2A). In addition, to determine the optimal dose of EPA for suppression of ET-1-induced cardiomyocyte hypertrophy, we performed a leucine uptake test (Fig. 2B). EPA at 1 and 3 μM could not exert significant inhibitory effects on the ET-1-induced increase in protein synthesis rate (Fig. 2B). Although 10 and 30 μM EPA prevented augmentation of protein synthesis, 30 μM EPA inhibited protein synthesis compared with...
control (Fig. 2B). On the basis of these findings, 10 μM EPA was used for subsequent experiments.

**Effects of EPA on ET-1-Induced Cardiomyocyte Hypertrophy**

**Cell surface area.** To examine whether EPA treatment could block ET-1-induced cardiac hypertrophy, cardiomyocytes were pretreated with EPA (Fig. 3A). After ET-1 administration, the cardiomyocytes clearly exhibited hypertrophy, which was prevented by pretreatment with EPA (Fig. 3A). Calculation of the cell surface area revealed that administration of ET-1 induced a 1.97-fold increase in cardiomyocyte surface area ($P < 0.0001$), an effect that was reduced to 1.41-fold in EPA-pretreated cells compared with control ($P < 0.0001$; Fig. 3B). When control cells were treated with EPA, there was no change in cell surface area (Fig. 3, A and B).

[^14C]leucine uptake.[^14C]leucine uptake by cardiomyocytes was examined after ET-1 administration. ET-1 induced a 1.72-fold increase ($P < 0.0001$), which was remarkably reduced (1.28-fold) in EPA-pretreated cells compared with control ($P < 0.0001$; Fig. 3C). EPA treatment of control cells did not alter the rate of protein synthesis (Fig. 3C).

**Expression of α-actinin protein.** Immunofluorescence staining showed strong expression of α-actinin in ET-1-treated cardiomyocytes, an effect attenuated by EPA (Fig. 4A). Immunoblotting revealed a 72% increase ($P < 0.001$) in α-actinin

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**Fig. 2.** A: cell surface area of control, ET-1-treated, and eicosapentaenoic acid (EPA)-pretreated ET-1-treated cardiomyocytes. B: [14C]leucine incorporation in control, ET-1-treated, and EPA-pretreated ET-1-treated cardiomyocytes. Values (means ± SE) are expressed relative to control. *$P < 0.05$ vs. control. **$P < 0.0001$ vs. control. ###$P < 0.0001$ vs. ET-1 alone.

**Fig. 3.** A: representative charge-coupled device camera images of control (Con), EPA-treated (Con + EPA), ET-1-treated (ET-1), and EPA-pretreated ET-1-treated (EPA + ET-1) cardiomyocytes. Magnification ×400. B and C: surface area and leucine incorporation in control, Con + EPA, ET-1, and EPA + ET-1 cardiomyocytes. Values are means ± SE. *$P < 0.01$ vs. Con. **$P < 0.0001$ vs. Con. #P < 0.0001 vs. ET-1.
expression after ET-1 administration, an effect that was also attenuated or normalized \( (P < 0.05) \) by EPA pretreatment (Fig. 4B). As predicted, EPA treatment of control cardiomyocytes did not cause any change in \( \alpha \)-actinin expression (Fig. 4).

mRNA expression of ANP and BNP. ET-1 treatment caused a 1.54-fold \( (P < 0.001) \) and a 1.65-fold \( (P < 0.001) \) increase in ANP and BNP mRNA expression in cardiomyocytes, respectively, as revealed by quantitative real-time PCR; these increases were remarkably suppressed by pretreatment with EPA \( (P < 0.05); \) Fig. 5). In control cardiomyocytes treated with EPA alone, expression of ANP and BNP was not altered (Fig. 5).

Effects of EPA on TGF-\( \beta \) Protein and mRNA Expression in ET-1-Induced Cardiomyocyte Hypertrophy

Immunohistochemistry of TGF-\( \beta \) showed a strong immunoreactivity of TGF-\( \beta \) in ET-1-treated cardiomyocytes that largely was prevented by pretreatment with EPA (Fig. 6A). TGF-\( \beta \) protein expression was highly upregulated in cardiomyocytes in which hypertrophy had been induced by ET-1, as demonstrated by ELISA (54% increase, \( P < 0.0001 \)) and immunoblotting (2.5-fold increase, \( P < 0.0001 \)), compared with the control group; this upregulation was greatly prevented by pretreatment with EPA \( (P < 0.05); \) Fig. 6, B and C). The protein expression of TGF-\( \beta \) corresponded to the gene expression level. The mRNA expression of TGF-\( \beta \) by real-time PCR was significantly upregulated in neonatal cardiomyocytes after ET-1 administration (1.39-fold, \( P < 0.001 \) vs. control); this upregulation was arrested by pretreatment with EPA \( (P < 0.05 \text{ vs. } \text{ET-1}; \) Fig. 6D). Interestingly, when control cardiomyocytes were treated with EPA only, there was no change in TGF-\( \beta \) expression (Fig. 6).

Effects of EPA on ET-1-Induced Expression of Phosphorylated and Total JNK

The expression level of total JNK, a subfamily of MAPK, activated by ET-1 in cardiomyocytes (22) was significantly enhanced in ET-1-treated cardiomyocytes (59% increase, \( P < 0.0001 \) vs. control) compared with control (Fig. 7A). Results obtained by ELISA and the representative immunofluorescence photomicrographs also revealed an increase in levels of JNK

Fig. 4. Protein expression levels of \( \alpha \)-actinin in Con, Con + EPA, ET-1, and EPA + ET-1 cardiomyocytes. A: immunofluorescence. Magnification \( \times 400 \). B: typical Western blot and intensity of the bands. In each experiment, Con value is normalized as 1.0. Values are means \( \pm \) SE of 5–7 separate experiments. *\( P < 0.001 \) vs. Con. #\( P < 0.05 \) vs. ET-1.

Fig. 5. Gene expression levels of atrial and brain natriuretic peptides (ANP and BNP) by real-time PCR in Con, Con + EPA, ET-1, and EPA + ET-1 cardiomyocytes. Values are means \( \pm \) SE. *\( P < 0.001 \) vs. Con. #\( P < 0.05 \) vs. ET-1.
that is dually phosphorylated at Thr^{183} and Tyr^{185} (Fig. 7, A and B). Nuclear localization of phosphorylated JNK in cardiomyocytes was confirmed by 4′,6-diamidino-2-phenylindole counterstaining (Fig. 7C). Moreover, a slight immunoreactivity of phosphorylated JNK was also seen in cellular cytoplasm.

The increases in expression of total JNK and phosphorylated JNK in ET-1-treated cardiomyocytes were greatly reversed by EPA pretreatment (Fig. 7). When control cardiomyocytes were treated with EPA only, there was no change in JNK expression or JNK activity (Fig. 7).
Fig. 7. Protein expression and activity of JNK in Con, Con + EPA, ET-1, and EPA + ET-1 cardiomyocytes. A: total JNK protein expression by immunoblot analysis (typical Western blot and intensity of the bands). In each experiment, the band obtained with control is normalized as 1.0. B: phosphorylated JNK quantitation by ELISA. Values are means ± SE of 5–7 separate experiments. C: phosphorylated JNK expression by immunofluorescence. Red, phosphorylated JNK (a); blue, 4′,6-diamidino-2-phenylindole (b). Images are merged in c. Magnification ×200. *P < 0.0001 vs. Con; #P < 0.0001 vs. ET-1 (in A). *P < 0.01 vs. Con; #P < 0.01 vs. ET-1 (in B).
EPA in cardiomyocytes in which hypertrophy had been induced by ET-1. Treatment with EPA alone did not induce any alteration in c-Jun expression in control cardiomyocytes (Fig. 8).

Effect of EPA on Prepro-ET-1 mRNA Expression Induced by ET-1

ET-1 (10^{-10} M) increased the ET-1 mRNA level in cardiomyocytes (153.0 ± 15.7% vs. control, P < 0.01); this increase was inhibited by pretreatment with 10 μM EPA (99.0 ± 9.3% vs. ET-1, P < 0.01), whereas treatment of cardiomyocyte with EPA alone did not affect ET-1 mRNA level (Table 1).

DISCUSSION

The findings of the present study are important, in that they demonstrate for the first time that EPA pretreatment suppresses ET-1-induced neonatal ventricular cardiomyocyte hypertrophy in vitro. We show that pretreatment with EPA prevents ET-1-induced cardiac hypertrophy at the morphological and signal levels (ANP and BNP mRNA expression). Moreover, we also showed that ET-1 caused an upregulation of TGF-β1 and JNK, one of the important components of the MAPK system, in neonatal ventricular cardiomyocytes. EPA pretreatment repressed TGF-β1 and JNK upregulations and, importantly, also attenuated ET-1-induced hypertrophic changes in neonatal ventricular cardiomyocytes.

The cardiovascular system undergoes structural and functional adaptations, most notably cardiac hypertrophy, induced by various elements, such as mechanical factors and neurohormones (47). Initially, cardiac hypertrophy plays a beneficial physiological role that leads to improved cardiac contractile force (26, 47). However, if left unchecked, it can lead to contractile dysfunction and, subsequently, heart failure (26). ET-1 has been well documented in different studies in vivo and in vitro as one of the crucial factors for the development of cardiomyocyte hypertrophy. Consistent with other reports, in the present study, ET-1 also caused cardiomyocyte hypertrophy. In this study, we characterized hypertrophy of cardiomyocytes by monitoring cell size, protein synthesis, expression of ANP and BNP, and cytoskeletal organization of α-actinin. Our data indicate that cardiomyocyte surface area and protein synthesis were significantly increased 1.7- to 2.0-fold after stimulation with ET-1. In these cells, α-actinin organization was also increased. A growing body of evidence suggests ANP and BNP upregulation in cardiac hypertrophy, as well as in heart failure, as an adaptive mechanism. In the present investigation, we also found that ET-1 induced cardiomyocyte hypertrophy.

Table 1. Effect of EPA on prepro-ET-1 mRNA expression in ET-1-induced hypertrophied cardiomyocytes

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<tr>
<th>Condition</th>
<th>Prepro-ET-1 mRNA Expression, % of control</th>
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<tr>
<td>Control</td>
<td>100±5.6</td>
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<tr>
<td>EPA (10 μM)</td>
<td>95±7.4</td>
</tr>
<tr>
<td>ET-1 (10^{-10} M)</td>
<td>153±15.7*</td>
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<tr>
<td>ET-1 (10^{-10} M) + EPA (10 μM)</td>
<td>99±9.3†</td>
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Values are means ± SE of 7 independent experiments performed in triplicate. ET-1, endothelin-1; EPA, eicosapentaenoic acid. *P < 0.01 vs. control. †P < 0.01 vs. ET-1.
hypertrophy. Expression levels of ANP and BNP as revealed by real-time PCR were upregulated. Whereas the role of ET-1 in cardiomyocyte hypertrophy is well established and the present data, showing ET-induced cardiac hypertrophy with the upregulation of ANP, BNP, and α-actinin, are consistent with these earlier studies, less is known about the possible inhibitory action of the fish oil extract EPA on ET-1-induced cardiac hypertrophy. The present study is the first to provide such evidence. This finding is important, in that it may potentially provide insights that may lead to the development of alternative approaches to heart failure therapies that are largely aimed at antagonizing factors that induce hypertrophic responses (i.e., angiotensin II and ET) (47). Indeed, recent prospective randomized studies indicate improved survival in patients treated with ω-3 PUFAs after myocardial infarction (40) and beneficial effects of DHA/EPA in patients with coronary artery disease and myocardial infarction (1, 12). The exact mechanisms underlying these effects are unknown. It is possible, on the basis of the present data, that EPA, among other factors, may be one of the active ingredients responsible for the improved prognosis. This speculation is supported by several human and animal model studies and by a recent study showing that fish oil rich in the ω-3 fatty acids EPA and DHA not only has an anti-hypertensive effect in spontaneously hypertensive rats, but it also decreases LVH (6, 40, 44).

The exact mechanism(s) underlying the prevention of cardiac hypertrophy by dietary substances has not been well clarified. It is possible that EPA and DHA, among other factors, may be involved, in that they regulate expression of some gene clusters likely to be implicated in the pathogenesis of cardiac hypertrophy, such as cytokines, signal transduction, transcription, cell cycle defense and repair, apoptosis, cell adhesion, the cytoskeleton, and hormones. Li et al. (27) reported an important modification of the lipid composition of membrane lipid rafts and T cell function by EPA supplementation, which suggests that EPA can enter directly into the cell membrane and directly affect the cell signal pathway and expression of various genes. Moreover, in human breast cancer cells, ω-3 PUFAs decrease cell proliferation, possibly by decreasing signal transduction through the Akt-nuclear factor-κB cell survival pathway (38). Recently, it was reported that DHA inhibited PE-induced cardiomyocyte hypertrophy via the ERK pathway (39). Collectively, these data suggest that EPA may diffuse through the cell membrane and affect the cell signal pathway and gene expression.

TGF-β1, a locally generated cytokine, plays a pivotal role in the development of cardiac hypertrophy and heart failure, and its influence on ET-1 expression in nonmyocyte heart cells (fibroblasts) and rat liver stellate cells has been demonstrated (16, 20). However, the effect of ET-1 on TGF-β1 expression and hypertrophy in cardiomyocytes is unknown. Because ET-1 is known to regulate TGF-β1 in extracardiac cells, such as fetal skin-derived cultured mast cells (30), and ET-1 and TGF-β1 are implicated in cardiac hypertrophy, it is likely that ET-1 regulates TGF-β1 expression in cardiac cells. Consistent with our hypothesis, the present study found increased levels of TGF-β1 mRNA and protein in ET-1-induced cardiomyocyte hypertrophy, suggesting a role for ET-1 in the regulation of TGF-β1 expression. The effects of ET-1 on TGF-β1 are likely to be regulated by the ET_α receptor, the levels (mRNA and protein) of which are upregulated in cardiomyocytes (data not shown). This observation is consistent with studies showing that ET_α receptor antagonism prevents elevation of cardiac TGF-β1 mRNA and protein levels in doxycorticosterone acetate-salt hypertensive rats (3). Importantly, the present study demonstrates that 10 μM EPA significantly suppresses the overexpressed ET-1-induced TGF-β1 at protein and mRNA levels; these findings are consistent with earlier studies showing significant inhibition of TGF-β1 expression (33, 49).

In vascular smooth muscle cells from spontaneously hypertensive rats, 20 μM EPA significantly inhibited expression of TGF-β1 mRNA (25). Moreover, EPA, which inhibits procarcinogenic PKC-βI activity and colon carcinogenesis, inhibits the TGF-β signaling axis and induces a hyperproliferative state within the colonic epithelium (49). Although the present study does not explain the mechanism by which EPA inhibited ET-1-induced TGF-β1 upregulation in hypertrophied cardiomyocytes, EPA pretreatment of ET-1-treated cardiomyocytes could downregulate the increase in ET_α receptor expression (unpublished observation). Thus, on the basis of the present findings, one might speculate that the inhibitory action of EPA on ET-1-induced upregulation of TGF-β1 in cardiomyocytes may be partly mediated through the ET_α receptor.

We previously demonstrated an upregulation of JNK in ET-1-induced hypertrophied cardiomyocytes and a regression of this upregulated JNK by peroxisome proliferator-activated receptor-α agonist administration (22). Here, we further show the upregulation of JNK and c-Jun in ET-1-induced hypertrophied cardiomyocytes, in agreement with earlier studies that showed ET-1-induced activation of the JNK pathway in smooth muscle cells and cardiomyocytes (22) and others that showed a signaling pathway linking the ET_α receptor to JNK (14, 22). JNK, which is thought to be the principal factor involved in c-Jun upregulation (22), is activated by its dual phosphorylation motif, which results in phosphorylation of serine residues in the NH2-terminal region of c-Jun and, thereby, an increase in the transcriptional-activating activity of c-Jun (22). Moreover, it has been shown that activation of the JNK pathway contributes to transcriptional and morphological responses to Gα receptor-coupled hypertrophic agonists, such as ET-1 and PE (14). Collectively, these data, along with the present findings, suggest that upregulation of the JNK signaling pathway could further amplify the ET-1-induced cellular response. EPA may arrest the ET-1-induced cardiomyocyte hypertrophic progression through the inhibition of hypertrophic factors other than TGF-β1, and MAPK may be one of the factors involved. Accordingly, in this study, we demonstrated that EPA interrupted the earliest ET-1-induced events, i.e., JNK activation, c-Jun phosphorylation, and c-Jun induction in cardiomyocytes. A growing body of evidence has focused on the effect of EPA on different MAPK signalings. EPA can inhibit ultraviolet-induced matrix metalloproteinase-1 expression by inhibiting the MEK1-ERK-c-Fos and SEK1-JNK-c-Jun pathways in human dermal fibroblasts (25) and by decreasing LPS-induced c-Jun phosphorylation, protein levels, and JNK activation in human monocyte THP-1 cells (50). Treatment of hippocampal neurons with EPA abrogated the LPS-induced increases in phosphorylation of JNK and c-Jun (29). EPA and DHA, other PUFAs, significantly suppressed IL-6 superinduction by deoxyxylavenol and impaired deoxyxylavenol-induced ERK1/2 and JNK1/2 phosphorylation in spleens of mice (31). In our preliminary experiment, EPA could not
suppress the ET-1-induced upregulation of ERK in cardiomyocytes. Therefore, the inhibitory effect of EPA on ET-1-related hypertrophic responses might be through interference in the JNK, rather than the ERK, pathway. Moreover, as shown in Table 1, ET-1-induced ET-1 mRNA (prepro-ET-1) expression in cardiomyocytes was suppressed by pretreatment with EPA, which further suggests that EPA may suppress ET-1-induced cardiomyocyte hypertrophy through the inhibition of JNK signaling.

To gain more insights into the antihypertrophic action of EPA on cardiomyocytes, one should look at the effects of EPA on angiotensin II-induced cardiomyocyte hypertrophy. In addition, the effects of another important fish oil, DHA, on the ET-1-induced cardiomyocyte hypertrophy should be studied. Although DHA was able to inhibit PE-induced cardiomyocyte hypertrophy, EPA could not suppress the increased protein synthesis in PE-induced cardiomyocyte hypertrophy (39). The differential protective role of different fish oils, such as EPA and DHA, should be investigated at a more molecular level to clarify the mechanism of antihypertrophic action of EPA and DHA on cardiomyocyte hypertrophy caused by various hypertrophy-inducing factors.

In the present study, we have investigated the effects of EPA pretreatment on ET-1-induced hypertrophy of neonatal ventricular cardiomyocytes in vitro and deciphered their possible signaling pathways. Here, we report that EPA limits ET-1-induced cardiomyocyte hypertrophy. In isolated cardiomyocytes, EPA inhibits the important hypertrophic pathway involving TGF-β as induced by the vascular peptide ET-1. We cytoblasts, EPA inhibits the important hypertrophic pathway induced cardiomyocyte hypertrophy. In isolated cardiomyocytes in vitro and deciphered their possible differential protective role of different fish oils, such as EPA and DHA, should be investigated at a more molecular level to clarify the mechanism of antihypertrophic action of EPA and DHA on cardiomyocyte hypertrophy caused by various hypertrophy-inducing factors.

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