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ARTICLES

Inhibition of Phenylephrine-Induced Cardiac Hypertrophy by Docosahexaenoic Acid

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Abstract Many of the cardiovascular benefits of fish oil result from the antiarrhythmic actions of the n-3 polyunsaturated lipids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). The beneficial effects of DHA/EPA in patients with coronary artery disease and myocardial infarction may also result from modulation of the myocardial hypertrophic response. Hypertrophy was assessed in neonatal cardiomyocytes exposed to phenylephrine (PE) by measuring cell surface area, total protein synthesis (^{14}C leucine incorporation), and the organization of sarcomeric α-actinin and by monitoring expression of atrial natriuretic factor (ANF). We report that PE induced a twofold increase in cell surface area and protein synthesis in cardiomyocytes. The hypertrophied cardiomyocytes also exhibited increased expression of ANF in perinuclear regions and organization of sarcomeric α-actinin into classical z-bands. Treatment of cardiomyocytes with 5 μM DHA effectively prevented PE-induced hypertrophy as shown by inhibition of surface area expansion and protein synthesis, inhibition of ANF expression, and prevention of α-actinin organization into z-bands. DHA treatment prevented PE-induced activation of Ras and Raf-1 kinase. The upstream inhibition of Ras → Raf-1 effectively prevented translocation and nuclear localization of phosphorylated extracellularly regulated kinase 1 and 2 (Erk1/2). These effects consequently led to inhibition of nuclear translocation, and hence, activation of the downstream signaling enzyme p90 ribosomal S6 kinase ($p90^{rsk}$). These results indicate that PE-induced cardiac hypertrophy can be minimized by DHA. Our results suggest that inhibition of Ras → Raf-1 → Erk1/2 → $p90^{rsk}$ → hypertrophy is one possible pathway by which DHA can inhibit cardiac hypertrophy. In vivo studies are needed to confirm these in vitro effects of DHA. J. Cell. Biochem. 92: 1141–1159, 2004.

Key words: cardiomyocytes; Ras; Raf; Erk1/2; docosahexaenoic acid; protein synthesis

Congestive heart failure is a major cause of morbidity and mortality. An estimated 4.8 million Americans have heart failure and another 400,000 are diagnosed with heart failure each year. Heart failure is the fourth leading cause of adult hospitalizations in the United States and mortality from heart failure is approximately 50% over 5 years. Despite improvements in the therapy for heart failure, morbidity and mortality remain high. Thus, new therapies aimed at preventing and slowing the progression of heart failure are needed.

A major cause of clinical deterioration in patients with heart failure is progressive hypertrophy of cardiomyocytes [Dhalla et al., 1987]. Although initially compensatory, progressive hypertrophy leads to impaired cardiac contractility and increased susceptibility to cardiac arrhythmias. Cardiac hypertrophy is characterized by an increase in cellular mass caused by an increase in cellular protein content in the absence of cell division [Morgan and Baker,

Abbreviations used: ANF, atrial natriuretic factor; DHA, docosahexaenoic acid; Erk1/2, extracellularly regulated kinase 1 and 2; MEK, MAP/Erk kinase; PE, phenylephrine; $p90^{rsk}$, p90 ribosomal S6 kinase; O3FA, omega-3 fatty acids.

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1991; Rupp et al., 1992]. The concentration increases in a number of mediators, including different growth factors, G-protein-coupled receptor (GPCR) agonists, and cytokines. These agents induce various biochemical alterations in cardiac cells, which result in hypertrophy [Hefti et al., 1997; Molkentin and Dorn, 2001]. Recent studies indicate that activation of GPCRs in cardiomyocytes undergoing hypertrophy induced by phenylephrine (PE), endothelin-1 (ET), and angiotensin-II (ANG-II) leads to activation of the Ras → Raf-1 → Erk1/2 kinase cascade [Yamazaki et al., 1999; Aoki et al., 2000; Yue et al., 2000; Bueno and Molkentin, 2002; Wang and Proud, 2002]. Activation of extracellularly regulated kinase 1 and 2 (Erk1/2) has been linked to the development of cardiac hypertrophy via phosphorylation of p90 ribosomal S6 kinase ($p90^{rsk}$) in cardiac myocytes [Seger and Krebs, 1995; Seko et al., 1996; Chakraborti and Chakraborti, 1998; Takeishi et al., 2001]. $p90^{rsk}$, which catalyzes phosphorylation of the ribosomal subunit protein S6, participates in the regulation of transcriptional factors through phosphorylation of serum response factors and therefore, regulates the expression of genes required for increased protein synthesis [Seko et al., 1996; Chakraborti and Chakraborti, 1998]. Inhibition of the Ras → Raf-1 → Erk1/2 signaling cascade could be an effective therapeutic strategy for preventing the contractile defects associated with cardiac hypertrophy and failure.

Phospholipids containing long-chain fatty acids are one of the constituents of cell membranes. The fatty acid composition of cell membrane phospholipid is known to affect membrane and cell function by modulating ion channels, second messenger generation, receptor coupling to effector molecules, enzyme recruitment, cell deformability, and other functions [Stillwell and Wassall, 2003]. Thus, it is reasonable to postulate that alterations in membrane composition due to dietary fatty acids could modulate important cell responses such as agonist-induced cardiac hypertrophy.

Fish oil enriched in the n-3 long-chain polyunsaturated fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) has been shown to have beneficial effects upon the cardiovascular system. Epidemiological evidence from Greenland Eskimos and inhabitants of Japanese fishing villages has suggested that eating fish oil and marine animals can prevent the development of coronary heart disease [Dyerberg et al., 1975; Bang et al., 1976; Hirai et al., 1984]. Several recent prospective cohort studies have found an inverse association between fish oil consumption and risk of coronary heart disease (CHD) or sudden cardiac death in the general population [Kromhout et al., 1985; Daviglus et al., 1997; Albert et al., 1998, 2002; Hu et al., 2002]. Experiments from various laboratories have similarly indicated that ingestion of fish oil affects several humoral and cellular factors involved in atherogenesis and may prevent atherosclerosis [Leaf and Weber, 1988]. Other studies demonstrate that dietary supplementation of fish oil and n-3 long-chain polyunsaturated fatty acids improves survival in patients with acute myocardial infarction [Burr et al., 1989; Singh et al., 1997; GISSI-Prevenzione, 1999] and that use of fish oil reduces cardiac arrhythmias [Grimsgaard et al., 1998] and angina pectoris [Hallaq et al., 1990; Singh et al., 1997].

Despite the numerous reports linking fish oils to cardiac protection, the effect of omega 3-fatty acids upon cardiac hypertrophy has not been investigated in detail. Since n-3 polyunsaturated fatty acids integrate into cell membranes, altering their composition, and are known to affect membrane-associated receptors and signaling pathways [de Jonge et al., 1996a,b], we hypothesized that n-3 polyunsaturated lipids would modulate the hypertrophic effects of alpha-adrenergic agonists. The investigation presented here was done to determine whether DHA attenuates cardiac hypertrophy induced by α-adrenergic agonists and whether such inhibition could be mediated by inhibiting the α-agonist-induced Ras → Raf → Erk1/2 pathway (Fig. 1).

EXPERIMENTAL PROCEDURES

Materials

The cardiomyocyte isolation kit was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Horse and fetal bovine serum were obtained from Hyclone (Logan, UT). DHA and other fatty acids were obtained from Nu Chek Prep, Inc. (Elysian, MN). [^{3}H]-leucine was purchased from Perkin-Elmer Life Science (Boston, MA). Anti-atrial natriuretic factor (ANF) was obtained from Peninsula Laboratories, Inc. (San Carlos, CA). Anti-α sarcomeric actinin came from Sigma-Aldrich Chemical

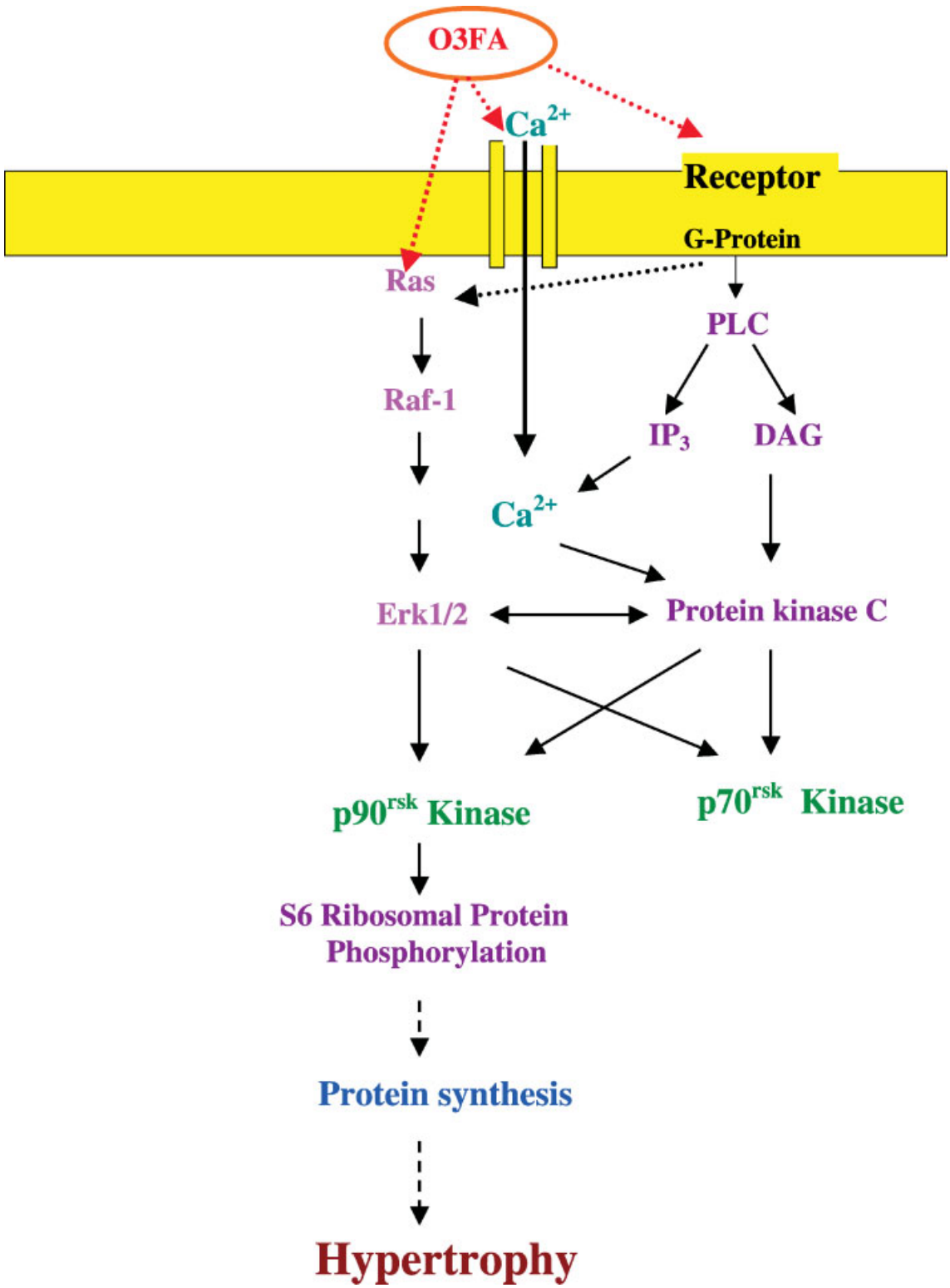

Fig. 1. Potential targets for the effects of n-3 fatty acids on cell signaling pathways for prevention of cardiac hypertrophy. One of the potential targets is membrane translocation and activation of Ras (a small GTP-binding protein), which subsequently affects activation of the Raf-1 → ERK1/2 → $p90^{rsk}$ pathway. Other potential sites for n-3 fatty acid action include intracellular calcium (Ca^{2+}) and GTP-binding protein (G-protein)-mediated activation of phospholipase C (PLC), generation of the second messengers inositol 1,4,5 trisphosphate (IP3), and diacylglycerol (DAG). Ca^{2+} and DAG are potent activators of PKC. Therefore, n-3 fatty acids may prevent activation of PKC, which consequently affects downstream pathways for protein synthesis involving activation of $p70^{rsk}$ and phosphorylation of S6 ribosomal proteins. In this study, we investigated the effects of docosahexaenoic acid (DHA) on the Ras → Raf-1 → Erk1/2 → $p90^{rsk}$ pathway for induction of cardiac hypertrophy. Solid arrows represent the sequence of enzyme-substrate regulatory events. Dashed arrows represent the effect of the end pathways and dotted arrows represent the effects of n-3 fatty acids on signaling mediators.

Co. (St. Louis, MO). Anti-Raf-1 antibody and anti-phospho-Raf-1 (ser338) antibody were obtained from Upstate Biochemicals, Inc. (Lake Placid, NY). Anti-phospho-Erk and anti-phospho $p90^{rsk}$ came from Cell Signaling Technologies (Boston, MA). Anti-β actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse or anti-rabbit Alexa fluor 546 and 488 were purchased from Molecular Probes (Eugene, OR). PE and all other chemicals were obtained from Sigma-Aldrich Chemical Co.

Isolation of Cardiomyocytes

Neonatal cardiomyocytes were obtained using an isolation system from Worthington Biochemical Corporation. Hearts were harvested from 1- to 3-day-old Wistar rats. The isolated hearts were cleared of connective tissue and atria, minced into approximately 1 mm blocks, and then incubated with trypsin for overnight digestion. The next day, trypsin activity was neutralized and the tissues were further digested with collagenase. Single cells were obtained by filtering the cells through a 70 μm filter. Cells were preplated to remove fibroblasts, and cardiomyocytes were isolated using the manufacturer's protocols (Worthington Biochemical Corporation). Dead cells, cellular debris, and contaminating fibroblasts were further removed by centrifugation on a 5 ml layer of an Optiprep density gradient solution (Axis-Shield PoC, Oslo, Norway). This preparation yielded a 95% pure population of cardiomyocytes as analyzed by sarcomeric α-actinin staining [Haq et al., 2000]. Isolated cardiomyocytes were cultured for 24 h in a humidified incubator in the presence of 95% O_2/5% CO_2. The cells were grown on laminin- and collagen-coated plates in F-10 medium containing 10% horse serum, 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM bromodeoxyuridine (to prevent low-level non-myocyte proliferation). Cardiomyocytes were washed twice with serum-free medium (F-10 medium without serum) and then treated with DHA under serum-free conditions for 24 h. Cells were then incubated with PE (100 μM) in a fresh medium with or without a fresh supply of DHA. Cells were incubated for another 48 h in a humidified incubator in the presence of 95% O_2 and 5% CO_2 to induce hypertrophic responses. The DHA solution was made fresh each time from a pure sealed stock solution by dissolving the fatty acid in ethanol so that the final concentration of ethanol added to the culture medium did not exceed 0.05%. Control cells were treated with equal amounts of ethanol in each case.

Characterization of Hypertrophy

Hypertrophy was induced by incubating cardiomyocytes under serum-free conditions in the presence of 100 μM PE for 48 h under the incubation conditions described above. Incubation in the presence of serum-free conditions for a total of 78 h does not result in the detachment of cells from laminin-coated surfaces, and morphological features under the microscope appear to be normal. Hypertrophy was assessed by measuring cell surface area and monitoring protein synthesis via [^{3}H]-leucine incorporation and by expression of ANF and α-sarcomeric actinin.

Cardiac Myocyte Surface Area

The cardiac myocyte surface area was measured as described previously [Nakamura et al., 1998]. Cells were observed under a Leica DMR microscope (Leica Microskopie und Systeme, GmbH, Postfach, Germany), and pictures were taken with a MagnaFire digital camera (Optronics, Goleta, CA) for analysis. All cells were randomly selected for tracing the surface area and at least ten cells were examined in each group.

[^{3}H]-Leucine Incorporation Into Proteins

To quantify the degree of protein synthesis in cardiomyocytes, we incubated cells pretreated with or without fatty acids for 24 h and with PE for 36 h (100 μM) and then pulsed with [^{3}H]-leucine (1 μCi/ml) as described [Nakamura et al., 1998]. The incubation was continued for an additional 12 h. After incubation, the medium was removed and proteins were precipitated with 10% TCA. The proteins were dissolved in 1 ml of 1% sodium dodecyl sulfate (SDS) at 37°C, and an aliquot (0.8 ml) of the solubilized proteins was mixed with scintillation fluid (Scintiverse, Fisher Scientific, Fair Lawn, NJ) and counted in a liquid scintillation counter (Beckman Scintillation Counter LS6000 IC, Beckman Instruments, Inc., Arlington Heights, IL).

Immunohistochemistry

Cardiomyocytes were pretreated with ethanol (control) or DHA (5 μM) under serum-free conditions for 24 h. The cells were further incubated for 48 h with or without PE (100 μM) in fresh serum-free medium containing either

ethanol or DHA. Cardiomyocytes were fixed with 3% paraformaldehyde and then blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Proteins were detected using specific antibodies (anti-ANF, anti-α-sarcomeric actinin, anti-phospho-Erk1/2, or anti-phospho-$p90^{rsk}$ in a 1:200 dilution in blocking buffer) and either Alexa 546- or Alexa 480-labeled anti-mouse or anti-rabbit (1:200 dilution in blocking buffer) antibodies. Cells were examined under a fluorescent microscope and pictures were taken using a Magna-Fire digital camera (Optronics) for analysis.

Immuno-Western Analysis

After incubation with DHA and PE as described above, the cells were treated with 50 μl of lysis buffer [20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 100 mM NaF, 2 mM Na_3VO_4, 10% v/v glycerol, 1% Nonidet P-40, 2 mM phenylmethanesulfonyl fluoride (PMSF), 1 mg/ml leupeptin, 0.15 U/ml aprotinin, and 2.5 mM DIFP] for 10 min on ice. Protein concentrations in each cell lysate were measured using a bicinchoninic acid (BCA) protein assay system (Pierce, Rockford, IL). An equal amount of detergent-solubilized protein extract was loaded onto an 8 or 12% SDS–PAGE and separated by electrophoresis. The separated proteins were transferred electrophoretically onto a PVDF membrane. TTBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20) containing 2% BSA was used to block residual binding sites on the membrane. The membranes were then incubated with primary antibody (anti-ANF antibody, anti-phospho-Erk1/2 antibody, anti-Raf-1 antibody, anti-phospho-Raf-1 antibody, and anti-α-actin, 1:1,000 in 1% BSA in TTBS) for 1 h at room temperature. The membranes were then washed three times with TTBS for 10 min each and incubated with peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G (IgG) (1:1,000 dilution in 1% BSA in TTBS) for 1 h. Blots were developed using an enhanced chemiluminescence (ECL) kit and ECL X-ray film (Amersham Biosciences, Buckinghamshire, UK). Bands were quantified by densitometric analysis using a KODAK Image Station 440CF (Eastman Kodak Company, Rochester, NY) and expressed as relative intensities.

Ras Activation Assay

The Ras activation assay was performed as described in the manufacturer's protocol (Upstate Biotechnology, Inc.). After preincubation with DHA and stimulation with PE as described above, cardiomyocytes were lysed in the lysis buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA 630, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 10 μm/ml aprotonin, 10 μg/ml leupeptin, 25 mM NaF, and 1 mM sodium orthovanadate. Raf-1 immobilized beads were then added to the precleared cell lysate to pull down the GTP-bound Ras (active Ras). The active Ras was eluted from beads by boiling with sample buffer as per manufacturer's protocol. The relative amount of the activated Ras was then determined by immuno-Western analysis using anti-Ras (clone RAS10) antibodies as described above.

Isolation of Detergent-Resistant/Soluble Membranes

After treatment with DHA (5 μM) and PE (100 μM) as described above, cardiomyocytes were scraped and suspended in 1 ml of cold Mes-buffered saline (MBS; 150 mM NaCl, 25 mM Mes, pH 6.5) containing 1% (w/v) Triton X-100. Cells were incubated for 30 min at 4°C and then homogenized on ice. Cold MBS (1.5 ml) was added to the homogenate and 2 ml of this suspension was mixed with 2 ml of 90% (w/v) sucrose in MBS. The mixture was then added onto a sucrose gradient consisting of 8 ml of 5–35% (w/v) sucrose in MBS. All solutions were supplemented with protease inhibitor cocktail (Roche Biochemicals, Indianapolis, IN). Sucrose gradients were then subjected to centrifugation in a Beckman SW40 swinging bucket rotor at 200,000*g* for 20 h at 4°C. One milliliter fractions were collected from the top to the bottom of the tubes and vortexed and stored at −80°C until further use. Fifty microliters of each isolated sucrose gradient fraction were deposited on PVDF membranes using a dot-blot apparatus (BioRad, Hercules, CA) and immunoblotted with peroxidase-labeled α-cholera toxin or anti-Ras antibodies as described above. The amount of GM1 binding and Ras in different fractions was quantified by densitometry analysis as described above.

Statistical Analysis of Data

For each experiment, mean and standard errors were found for each treatment group and plotted accordingly. Analysis of variance (ANOVA) was performed to test for an overall effect across treatments. Individual treatments

were tested against the control using Dunnett's multiple comparison test in order to control the Type I experimental wise error. Analyses were conducted using SAS version 8.2 (SAS Institute, Cary, NC).

RESULTS

The present study was performed in two stages. Initially, we characterized the effect of DHA upon PE-induced cardiomyocyte hypertrophy and then we investigated its effects upon the $\text{Ras} \rightarrow \text{Raf-1} \rightarrow \text{Erk1/2} \rightarrow \text{p90}^{\text{rsk}} \rightarrow$ hypertrophy pathway (Fig. 1). The results are presented below.

DHA- and PE-Induced Hypertrophy

PE induced a significant ($P < 0.05$) twofold increase in protein synthesis (Fig. 2). Pretreatment of cardiomyocytes with different classes of unsaturated fatty acids [oleic acid (OA, n-9); linoleic acid (LOA, n-6); linolenic acid (LOEA, n-3); arachidonic acid (AA, n-6); EPA (n-3); and DHA (n-3)] alone had no effect upon protein synthesis (data not shown). DHA was the only fatty acid tested that was able to inhibit PE-induced protein synthesis (Fig. 2). Induction of hypertrophic responses was also monitored by measuring cell surface, and PE treatment induced a significant ($P < 0.05$) increase in cell surface area by almost twofold (Fig. 3). Similarly, DHA pretreatment inhibited the PE-induced increase in cell size. We further characterized PE-induced hypertrophy by examining sarcomeric α-actinin organization and expression of ANF, a marker for cardiac hypertrophy. Cardiomyocytes stimulated with PE demonstrate organization of α-actinin into classical z-bands (Fig. 4). Alpha-actinin remains unorganized in cells not stimulated with PE. PE also induced expression of ANF in the perinuclear regions of the cell, demonstrated by the appearance of a circular band at the nuclear membrane (Fig. 4). Approximately 70–85% of the PE-stimulated cells under 20× magnification exhibited expression of ANF around the perinuclear regions. DHA alone had no effects upon α-actinin organization or the expression of ANF. However, PE-induced α-actinin organization into classical z-bands and ANF expression in the perinuclear regions were inhibited by DHA (Fig. 4). Because it was difficult to quantify ANF expression using immunohistochemistry, PE-induced ANF expression was also assessed using immuno-Western/densitometric analysis. The data shown in Fig. 5 demonstrate that PE substantially increases ANF expression by approximately threefold

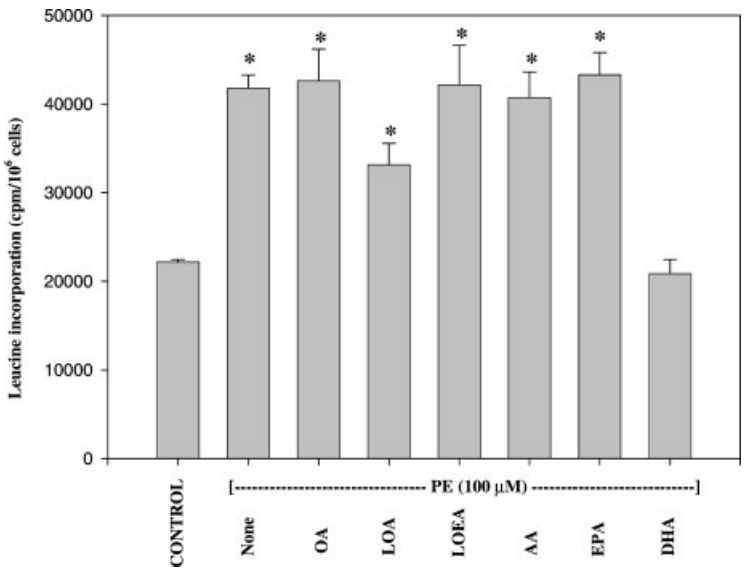

Fig. 2. Effect of phenylephrine (PE) and fatty acids on protein synthesis. Cardiomyocytes were pulsed with [^{3}H]-leucine (1 μCi/ml) for 12 h during incubation with or without fatty acids (5 μM) and PE (100 μM) as described in the text. Incorporation of [^{3}H]-leucine into newly synthesized proteins was measured. Data are expressed as mean ± SEM for three experiments and were analyzed by analysis of variance (ANOVA) and Dunnett's multiple comparison test in order to control the Type I experimental wise error. Significant differences compared to control are reported (*$P < 0.05$).

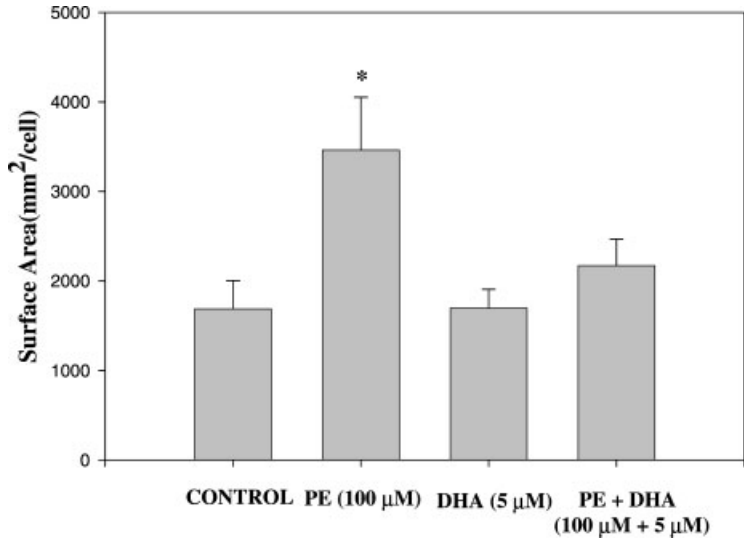

Fig. 3. Effect of PE and DHA on cell surface area. Data are expressed as mean ± SEM for examination of ten cells in each group and were analyzed by ANOVA and Dunnett's multiple comparison test in order to control the Type I experimental wise error. Significant differences compared to control are reported (*$P < 0.05$).

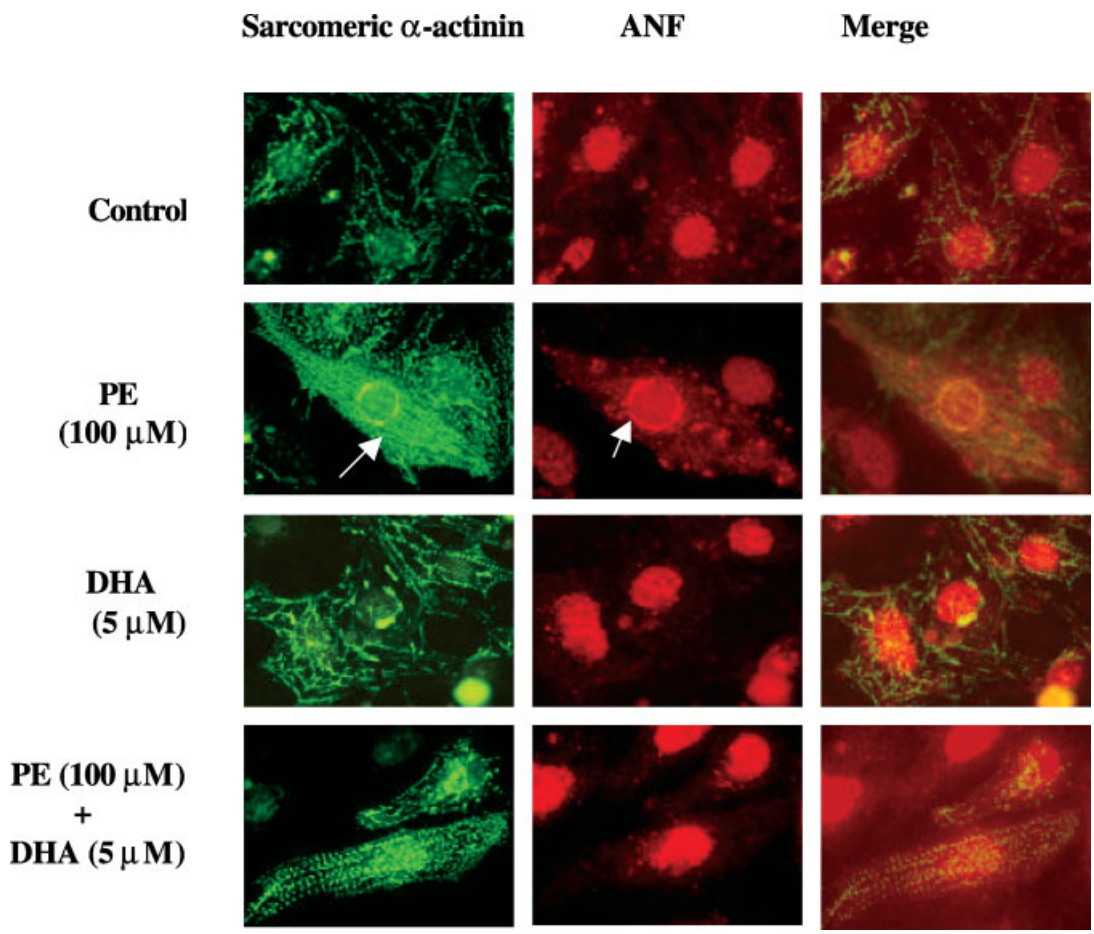

Fig. 4. Effect of PE and DHA upon α-actinin and atrial natriuretic factor (ANF) expression. Cardiomyocytes were treated with either ethanol (control) or DHA (5 μM) in serum-free conditions for 24 h and then stimulated with PE (100 μM) for 48 h. Arrows indicate the expression of α-actinin (green fluorescence) and ANF (red fluorescence). Results are a typical representation of five experiments.

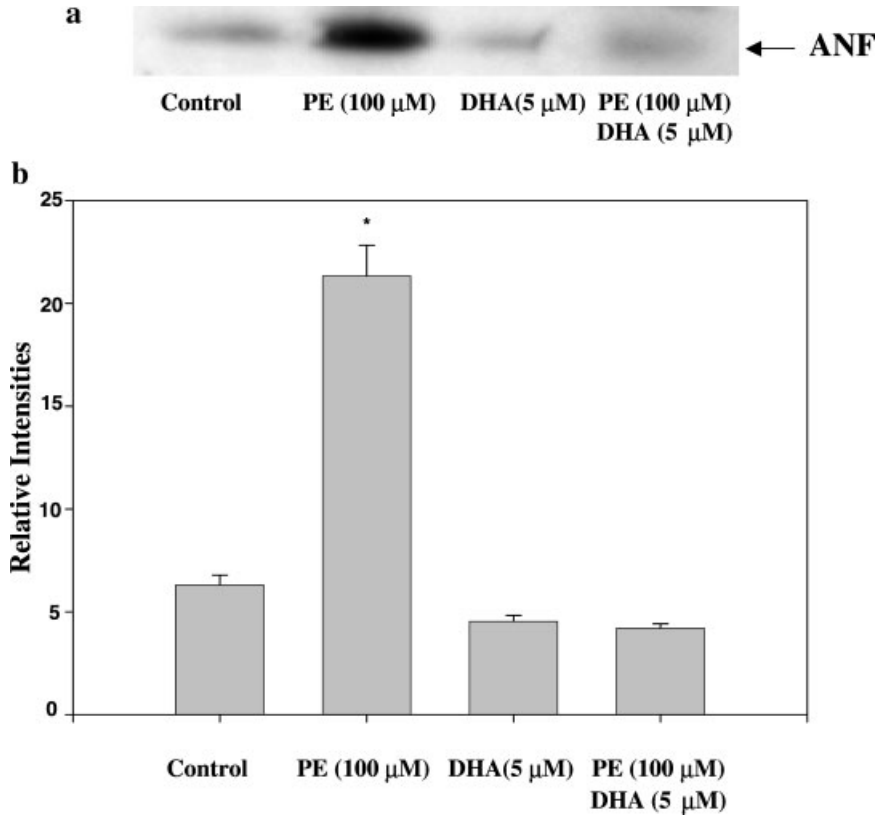

Fig. 5. Effect of PE and DHA on ANF expression. Cardiomyocytes treated with ± DHA (5 μM) and stimulated with PE (100 μM) were lysed and their protein extracts analyzed using immuno-Western analysis. A typical Western blot is shown in (**a**), whereas mean ± SEM for four experiments are represented in (**b**). The densitometry intensities were corrected for loading control using β-actin. Results are analyzed by ANOVA and Dunnett's multiple comparison tests in order to control the Type I experimental wise error. Significant differences compared to control are reported (*$P < 0.05$).

($P < 0.05$), whereas DHA alone slightly inhibits ANF expression. However, DHA treatment of PE-stimulated cells significantly inhibits PE-induced ANF expression ($P < 0.05$).

DHA and Ras Activation

Activation of the Ras → Raf-1 → Erk1/2 cascade shown in Fig. 1 is usually an agonist-induced sequential event. Each step in this pathway was investigated to determine the effect of DHA upon components of the signaling pathway. Results shown in Fig. 6 demonstrate that levels of activated Ras significantly increase over twofold ($P < 0.05$) following PE stimulation. However, levels of activated Ras were significantly lower to almost control levels ($P < 0.05$) in DHA-pretreated cells stimulated with PE. These data demonstrate that activation of Ras by PE is substantially inhibited in DHA-treated cells. This observation was further investigated using another experimental approach by analyzing localization of Ras in membrane fractions. Results shown in Fig. 7 indicate that most of the Ras was present in detergent-insoluble fractions (lipid raft, fractions 4–7), as indicated by the presence of GM1 binding (Fig. 7a). PE stimulation caused accumulation of Ras into lipid rafts (Fig. 7b), whereas DHA pretreatment inhibited PE-stimulated Ras accumulation in these membrane micro domains (Fig. 7c).

DHA and Raf-1 Phosphorylation

Raf-1 is a direct downstream effector of Ras activation. Because activity of Raf-1 is regulated by phosphorylation, we monitored its phosphorylation using specific anti-phospho Raf-1 antibodies to determine whether DHA inhibits Raf-1 activation. DHA alone slightly decreases Raf-1 phosphorylation ($P < 0.03$) (Fig. 8). Stimulation of cardiomyocytes with PE causes a significant threefold increase in Raf-1 phosphorylation ($P < 0.05$). Phosphorylation of Raf-1 by PE is reduced to control levels ($P < 0.05$) in DHA-pretreated cardiomyocytes (Fig. 8b). Changes in phosphorylation of Raf-1 are not due

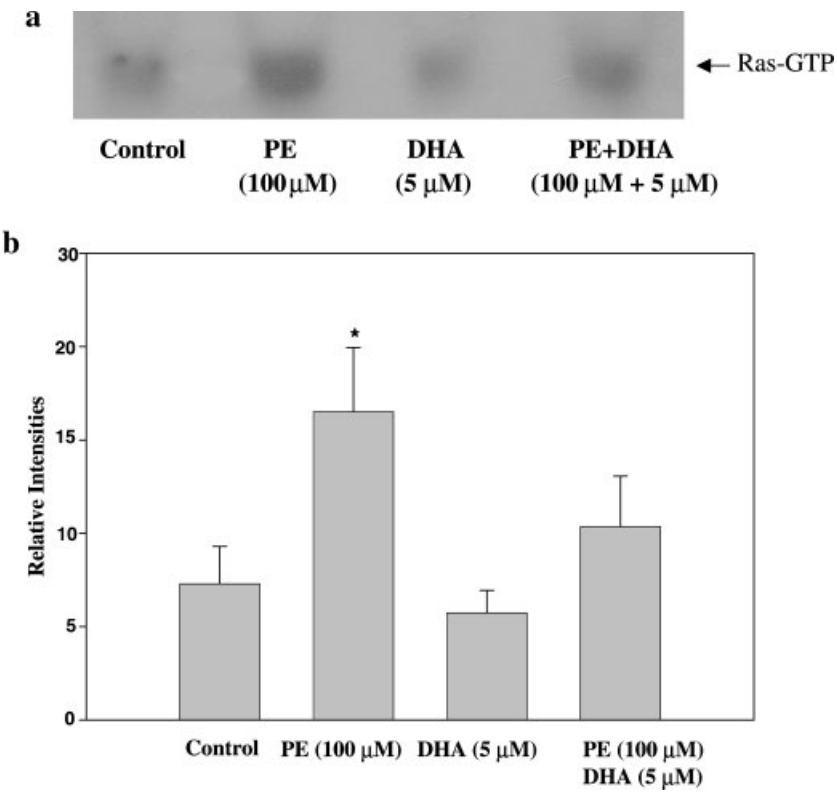

Fig. 6. Effect of PE and DHA upon Ras activation. Cardiomyocytes treated with ± DHA (5 μM) and stimulated with PE (100 μM) were analyzed by immuno-Western analysis using anti-Ras (clone RAS10) antibodies (**a**) and quantified by densitometric analysis (**b**). Results are the mean ± SEM for four experiments. Results were analyzed by ANOVA and Dunnett's multiple comparison tests in order to control the Type I experimental wise error. Significant differences compared to control are reported (*$P < 0.05$).

to alterations in the expression of Raf-1 because staining of the same blots with anti-Raf-1 antibodies indicates similar levels of total Raf-1 proteins (Fig. 8a).

DHA and Erk1/2 Phosphorylation and Nuclear Translocation

We next investigated the effects of DHA upon another downstream signaling molecule in the Ras-mediated hypertrophic pathway. Activation of Ras → Raf-1 leads to downstream activation of Erk1/2 (Fig. 1). Upon activation, Erk1/2 (p42 and p44) undergoes phosphorylation and translocation to the nucleus. Phospho-Erk1/2-specific antibodies (red fluorescence) were used to visualize Erk1/2 activation. In control and DHA-treated cells, only a small amount of phosphorylated Erk1/2 is present (Fig. 9), and it is diffusely distributed in the cytosolic region. Upon PE stimulation, there is an increase in Erk1/2 phosphorylation and translocation to the nuclear region. However, pretreatment of the cells with DHA inhibits PE-induced Erk1/2 phosophorylation and its translocation (Fig. 9). These results were further confirmed by immuno-Western analysis (Fig. 10). PE stimulation significantly increased levels of phosphorylated Erk1/2 threefold to fourfold. This increase was attenuated by pretreating the cells with DHA (Fig. 10). These data indicate that DHA pretreatment reduces PE stimulation of Erk1/2 phosphorylation.

DHA and $p90^{rsk}$ Activation and Translocation to the Nucleus

Activation of $p90^{rsk}$ is a downstream event involved in the regulation of hypertrophy; its activation is regulated by the Ras → Raf-1 → Erk1/2 signaling pathway (Fig. 1). Activation of $p90^{rsk}$ involves phosphorylation of the molecule [Seko et al., 1996]. Phosphorylated $p90^{rsk}$ was present in control cells in a diffuse pattern (red staining) over the cytoplasmic region (Fig. 11). Induction of the hypertrophic response by PE resulted in increased phosphorylation and translocation of $p90^{rsk}$ to the nuclear region.

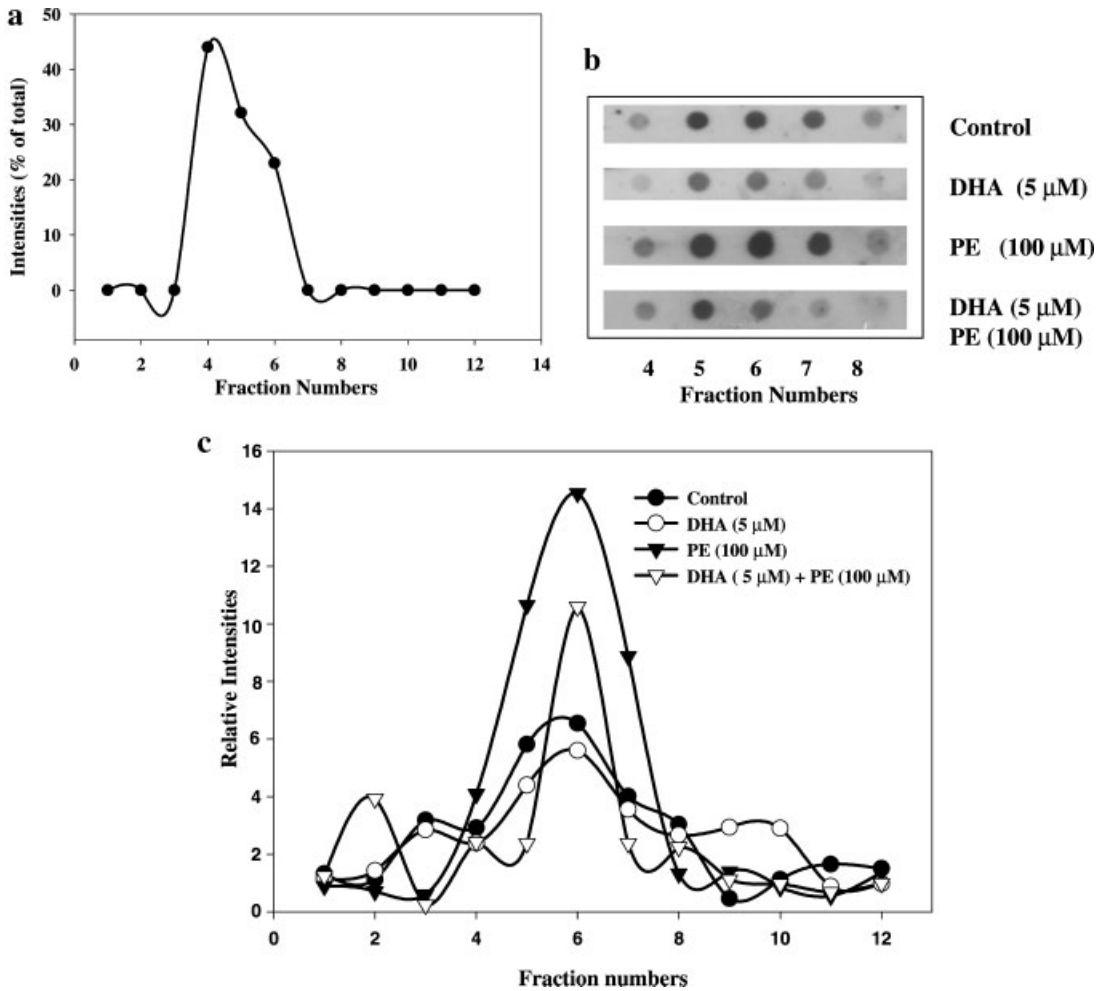

Fig. 7. Effect of PE and DHA upon Ras localization in the membrane fractions. Cardiomyocytes were subjected to detergent-resistant and -soluble fractionation using sucrose density gradients as described in the text. **a**: The lipid rafts (detergent-resistant) regions (fractions 4–7) were characterized by analyzing the GM1 binding. **b**: Ras in the detergent-resistant and soluble-fractions from cardiomyocytes after DHA and PE treatment was detected using anti-Ras antibody and (**c**) analyzed by densitometry analysis as described in the text. The results shown are representative of three experiments.

While DHA treatment itself had little effect upon phosphorylation of $p90^{rsk}$, DHA pretreatment inhibited PE-induced phosphorylation and nuclear translocation of phosphorylated $p90^{rsk}$.

DISCUSSION

Cardiomyocytes are terminally differentiated cells that under stress exhibit adaptive responses that help compensate for functional impairment. Cardiac hypertrophy is regarded as a functional consequence of many diseases of the heart [Colucci and Braunwald, 1997], including hypertension, aortic and mitral valve disease, and heart failure. While hypertrophy initially plays a beneficial role and may improve cardiac contractile force, progressive and especially uncontrolled hypertrophy can lead to contractile dysfunction and subsequent heart failure [Levy et al., 1990]. Initially, hypertrophy may result in only diastolic dysfunction. However, continued hypertrophy can lead to both systolic and diastolic dysfunction. Hypertrophy that results in cardiac contractile dysfunction is termed pathologic hypertrophy. Current drug therapy to treat heart failure is aimed at antagonizing growth factors that induce the hypertrophic response (i.e., α-agonists such as PE, ANG-II, and ET) [Molkentin and Dorn, 2001]. However, the effect of dietary substances upon the hypertrophic response is unknown.

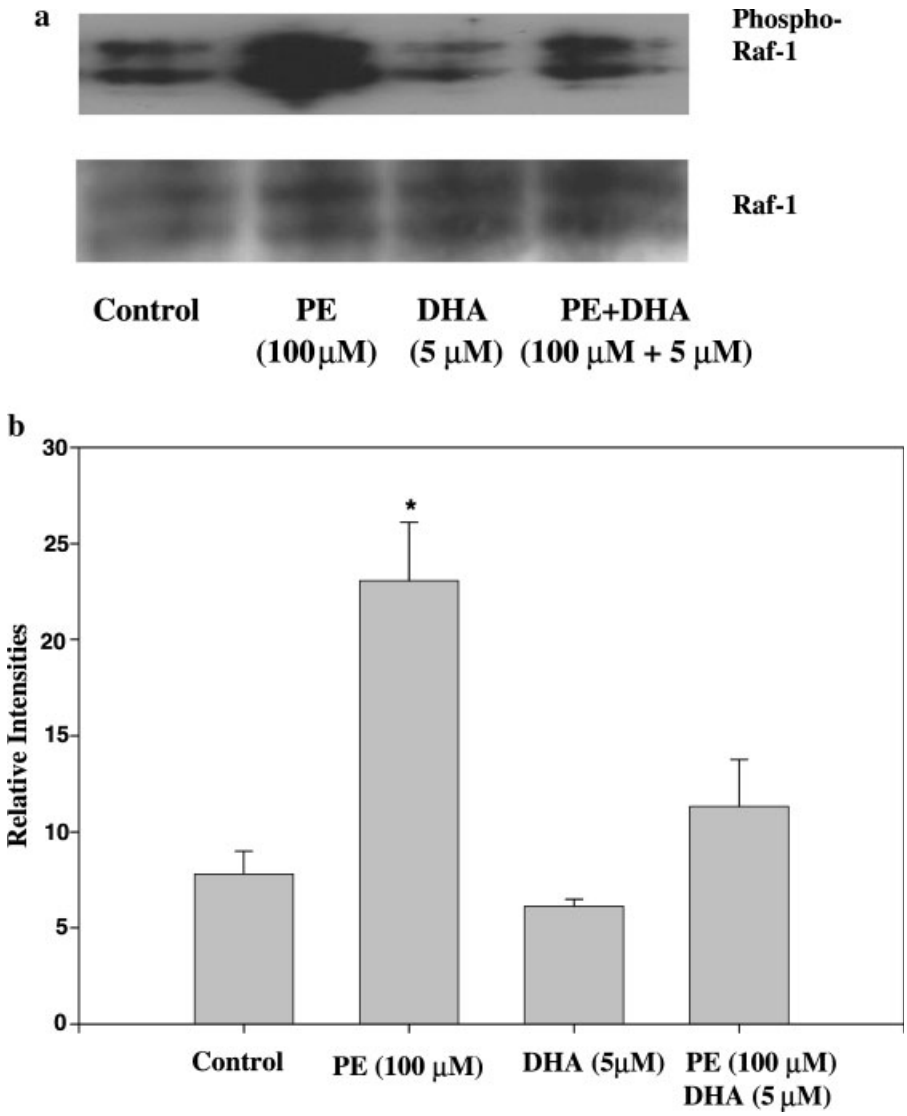

Fig. 8. Effect of PE and DHA upon Raf-1 phosphorylation. Cardiomyocytes treated with or without DHA (5 μM) and stimulated with PE (100 μM) were lysed and their protein extracts were analyzed using immuno-Western analysis. Phosphorylated Raf-1 (**a**) was quantified using densitometric analysis (Kodak Image station) and corrected for total Raf-1 amounts. The results represent the mean ± SEM of four experiments (**b**). Results were analyzed by ANOVA and Dunnett's multiple comparison tests in order to control the Type I experimental wise error. Significant differences compared to control are reported (*$P < 0.05$).

Recent prospective randomized studies indicate improved survival in patients administered n-3 polyunsaturated fatty acids following myocardial infarction [Marchioli et al., 2002]. The exact mechanisms for this effect remain unknown. The goal of this study was to determine whether the n-3 polyunsaturated fatty acid DHA alters the hypertrophic response to the α-agonist PE. Hypertrophy involves an increase in protein content, cell size, myofibrillar organization, and gene expression [Sugden, 1999; Swynghedauw, 1999; Miyata et al., 2000]. ANF expression is particularly affected and is considered to be a universal and specific marker of cardiac hypertrophy [Shubeita et al., 1990; Knowlton et al., 1991, 1995; Ito et al., 1993; Decker et al., 1995; Ohta et al., 1996]. However, its role in the development of hypertrophy is not yet clear [Itoh et al., 1990; Cao and Gardner, 1995; Calderone et al., 1998; Silberbach et al., 1999].

In this study, we characterized hypertrophy of cardiomyocytes by monitoring cell size, protein synthesis, expression of ANF, and cytoskeletal organization of α-actinin. Our data indicate that cardiomyocyte cell surface area and protein synthesis were significantly

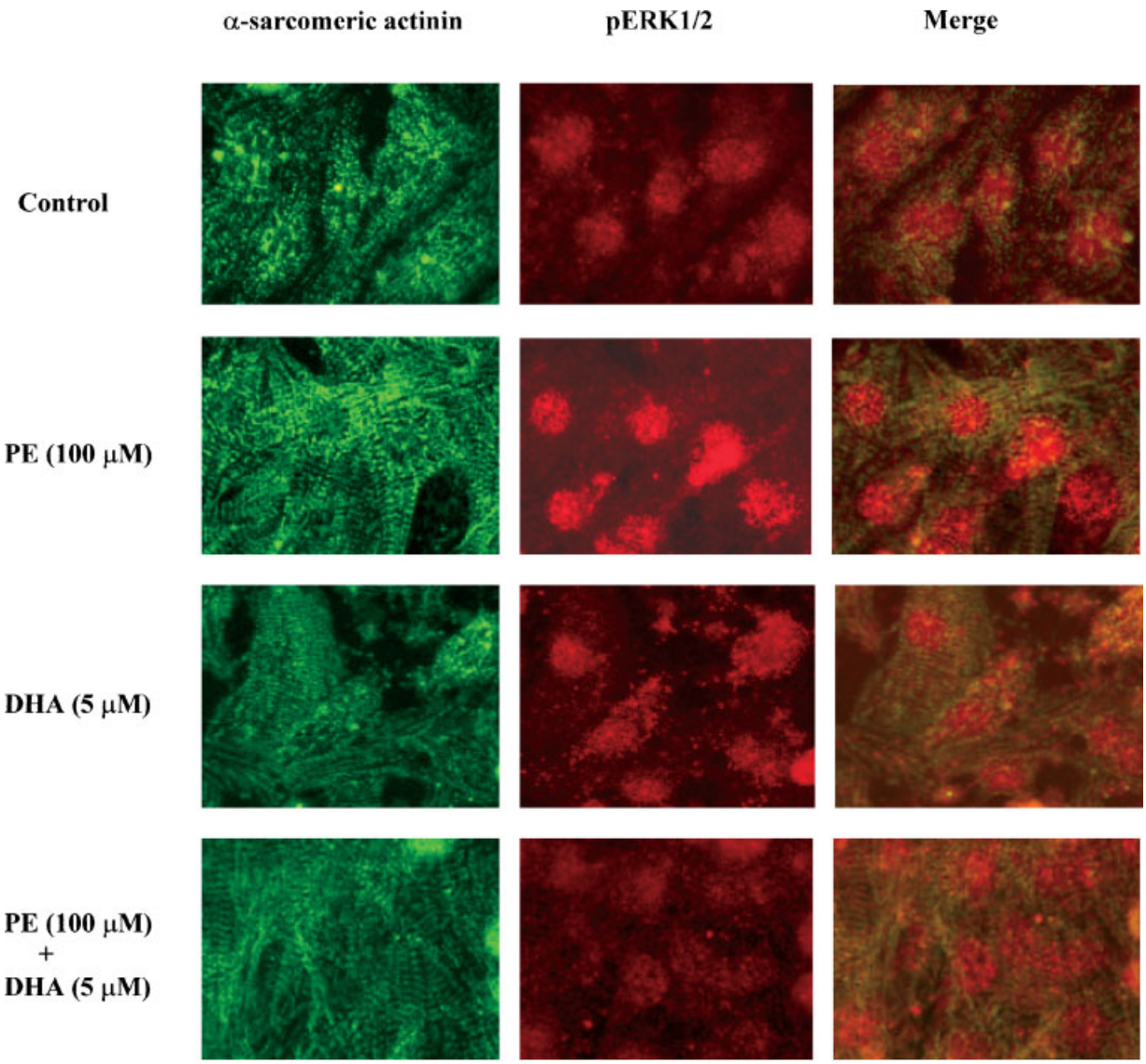

Fig. 9. Effect of PE and DHA upon Erk1/2 phosphorylation and nuclear translocation. Cardiomyocytes were treated with either ethanol (control) or DHA (5 μM) in serum-free conditions for 24 h and then stimulated with PE (100 μM) for 48 h. Sarcomeric α-actinin was detected using sarcomeric α-actinin and Alexa 546-conjugated secondary antibodies (green fluorescence), whereas phosphorylation of Erk1/2 was detected using phosphospecific Erk1/2 and Alexa 488-conjugated secondary antibodies (red fluorescence). The results are a typical representation of three experiments.

increased 2- to 2.5-fold following stimulation with PE. These cells also exhibited increased ANF expression in the perinuclear region and increased organization of α -actinin into classical z-bands. Importantly, DHA treatment inhibited these PE-induced responses. Because we did not find effects from other long-chain fatty acids, the antihypertrophic effect appears specific to DHA (Fig. 2).

A number of signaling pathways are affected by DHA treatment of cardiomyocytes, and each may offer some explanation for DHA's mechanisms of action. For example, DHA has been shown to inhibit calcium mobilization, one of the major mediators of heart contractility, by directly acting on plasma membrane calcium channels [Hallaq et al., 1992; Pepe et al., 1994]. Similarly, DHA has also been shown to regulate several protein kinases, including protein kinase C (PKC), that are involved in a number of very diverse cardiac functions, including hypertrophy [Speizer et al., 1991; Moore et al., 2001; Seung Kim et al., 2001; Denys et al., 2002]. In this study, we investigated the effect of DHA

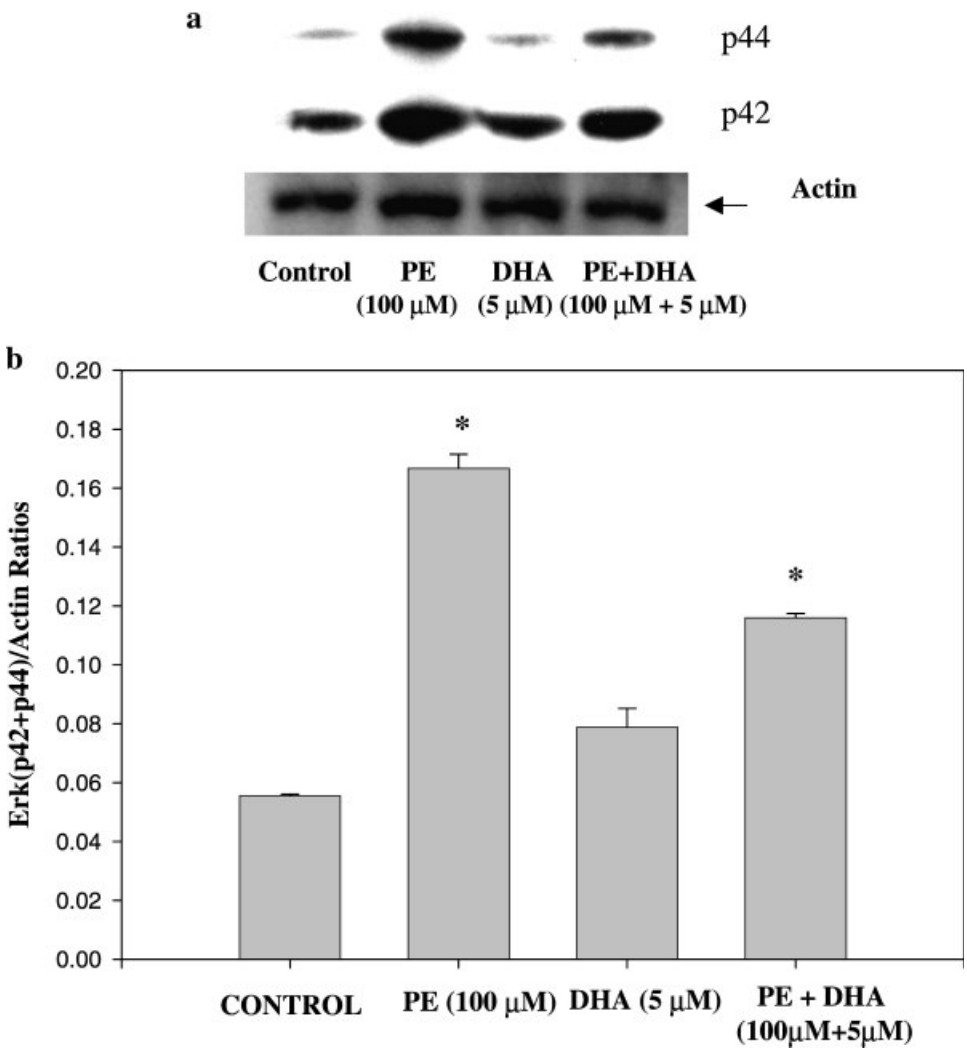

Fig. 10. Effect of PE and DHA upon Erk1/2 phosphorylation. Cardiomyocytes treated with ± DHA (5 μM) and stimulated with PE (100 μM) were lysed and the protein extracts were analyzed using immuno-Western analysis. A typical Western blot of phosphorylated Erk1/2 (p42 and p44) and β-actin are shown in (**a**). Erk1/2 and β-actin from each lane were quantified using densitometric analysis (Kodak Image Station) and the ratios of Erk1/2(p42 + p44):actin were calculated. The results in (**b**) are the mean ± SEM for four experiments. The results are analyzed by ANOVA and Dunnett's multiple comparison test in order to control the Type I experimental wise error. Significant differences compared to control are reported (*$P < 0.05$).

on a Ras-mediated pathway that has also been implicated in the regulation of hypertrophic responses (Fig. 1) [Clerk and Sugden, 2000]. In support of the role of a Ras signaling pathway in the development of cardiac hypertrophy, microinjection of H-RAS into cardiac myocytes elicits a strong growth response and hypertrophic gene expression [Thorburn et al., 1993]. In addition, expression of H-RAS in transgenic mice increases heart size concomitant with an increase in myofibrillar organization [Hunter et al., 1995; Gottshall et al., 1997]. Ras activation in cardiomyocytes leads to activation of MAP kinases [Ho et al., 1998]. Studies have shown that the coordinated activation of the Ras-MAP kinase pathway is involved in the expression of a number of genes that encode atrial natriuretic peptide (ANP), α and β myosin heavy chains, and sarcomeric calcium ATPase 2 (SERCA2) and in increased protein synthesis without DNA synthesis [Komuro et al., 1990, 1991; Sadoshima and Izumo, 1993; Yamazaki et al., 1993; Thorburn et al., 1994]. Our data are consistent with other studies [Yue et al., 2000; Bueno and Molkentin, 2002; Wang and Proud, 2002] showing that Ras activation is involved in PE-induced hypertrophic responses. We report for the first time that DHA inhibits PE-

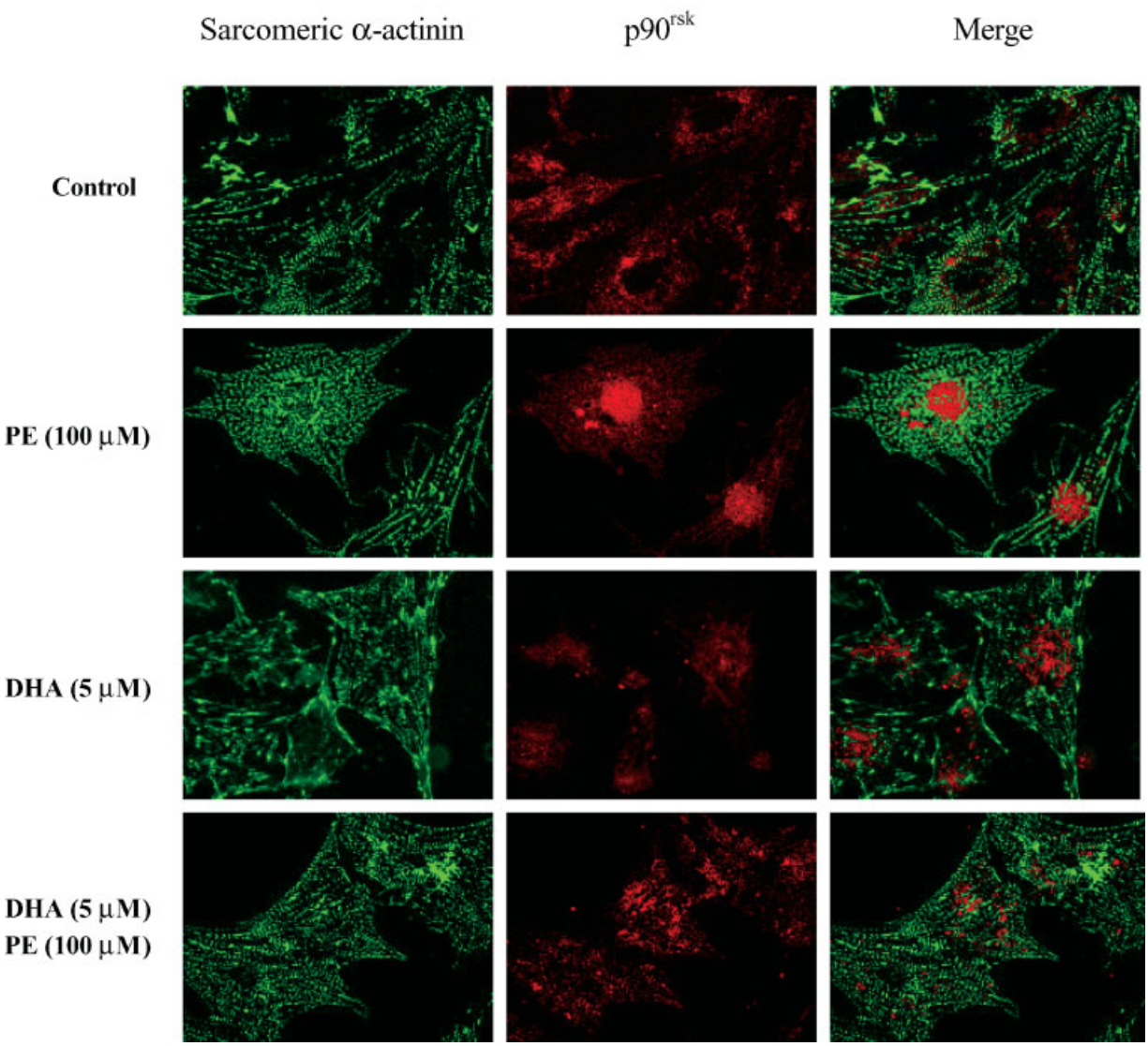

Fig. 11. Effect of PE and DHA upon phosphorylation of $p90^{rsk}$. Cardiomyocytes were treated with either ethanol (control) or DHA (5 μM) in serum-free conditions for 24 h and were then stimulated with PE (100 μM) for 48 h. Expression of sarcomeric α-actinin (green fluorescence) and phosphorylated $p90^{rsk}$ (red fluorescence) was detected by immunohistochemistry. The results are a typical representation of three experiments.

induced activation of the Ras → Raf-1 → Erk1/2 → $p90^{rsk}$ signaling pathway in cardiomyocytes and inhibits myocardial hypertrophy. In support of our observation of an effect of DHA upon cardiomyocyte Ras activation, DHA reduces Ras localization to the plasma membrane and lowers GTP binding and Erk-dependent signaling in colonocytes [Collett et al., 2001].

Activation of the Erk pathway has been implicated in the development of cardiac hypertrophy. A diverse set of signals, including those initiated by PE, converge at the level of MAP kinases [Gillespie-Brown et al., 1995; Yamazaki et al., 1996; Foncea et al., 1997]. Each of the MAP kinase family members, Erk [Gillespie-Brown et al., 1995; Glennon et al., 1996], JNK [Ramirez et al., 1997], and p38 [Wang et al., 1998] has been implicated in the hypertrophic response. Here, we only examined PE-induced activation of Erk1/2, and our data indicate that DHA treatment inhibits its activation, as assessed by phosphorylation and nuclear translocation. Results of Western analysis indicate that DHA is able to significantly inhibit PE-induced phosphorylation of Erk1/2 but does not affect unstimulated levels. The role of Erk1/

2 kinase in initiating and maintaining cardiac hypertrophy is controversial. A number of studies suggest that activation of Erk is essential for the development of hypertrophy. For example, Erk activation has been reported in myocytes undergoing hypertrophy in response to stretching, pressure overload, and stimulation with hormones and cytokines [Yamazaki et al., 1993; Bogoyevitch et al., 1994; Yue et al., 2000; Tanaka et al., 2001; Bueno and Molkentin, 2002; Wang and Proud, 2002]. Furthermore, transfection of constitutively active MAP/Erk kinase (MEK)1 (an immediate upstream activator of Erk1/2) stimulates ANF promotor activity, whereas transfection of dominant-negative MEK1 results in the inhibition of its activity [Glennon et al., 1996]. Use of the selective MEK1 inhibitors SB386023, PD98059, and U0126 has also been reported to reduce β-type natriuretic promotor activity [Liang et al., 2000] and the hypertrophic response [Kodama et al., 2000; Liang et al., 2000; Yue et al., 2000]. Depletion of Erk by treating cultured cells with antisense nucleotides for Erk1/2 downregulates PE-induced hypertrophy [Glennon et al., 1996]. In contrast to these reports, other studies suggest that the initiation and maintenance of cardiac hypertrophy is Erk independent. For example, Erk inhibition does not downregulate ANF expression, a biochemical marker of hypertrophic activity, in PE-treated cardiac myocytes [Post et al., 1996]. More recent studies with the MEK1 inhibitor PD98059 suggest a minimal role for Erk in cardiac hypertrophy [Zechner et al., 1997; Choukroun et al., 1998]. Similarly, some stimulants, including ATP and carbachol, are known to activate Erk but do not stimulate hypertrophy [Post et al., 1996]. The discrepancy over the role of Erk in the development of hypertrophy could be due to the fact that activation of Erk alone under some conditions is not sufficient and may require activation of other pathways to induce hypertrophic responses. In the present investigation, our data indicate that DHA treatment causes inhibition of Erk phosphorylation and its nuclear translocation and are consistent with a role for Erk in the hypertrophic response to PE. Clearly, more investigation into the role of Erk1/2 in the modulation of cardiac hypertrophy is required.

We also investigated the effect of DHA on $p90^{rsk}$ activity in PE-stimulated cardiomyocytes. Erk is known to directly phosphorylate $p90^{rsk}$ in cardiac myocytes [Blenis, 1993; Seko et al., 1996; Takeishi et al., 1999]. $P90^{rsk}$, which then catalyzes phosphorylation of the ribosomal subunit protein S6, participates in the transcriptional regulation of c-fos through phosphorylation of serum response factors [Seko et al., 1996; Abe et al., 2000] and results in increased protein synthesis [Angenstein et al., 1998; Takeishi et al., 2002]. Results presented in Fig. 11 indicate that DHA treatment also inhibits PE-stimulated $p90^{rsk}$ activation by phosphorylation.

The data presented in this study indicate that DHA treatment inhibits activation of the Ras → Raf-1 → Erk1/2 → $p90^{rsk}$ signaling pathway and results in the inhibition of protein synthesis and cardiac hypertrophy. However, the exact mechanism by which DHA inhibits these responses and subsequent PE-induced hypertrophy is not entirely clear. We have observed that 10% of the total DHA incorporated into cardiomyocytes is present in phospholipids of detergent-resistant membrane micro domains (lipid rafts) (data not shown). It is also interesting to note that Ras is localized in these raft fractions and that DHA treatment inhibits PE stimulation of Ras accumulation in these fractions (Fig. 7). We have shown previously that DHA incorporation into cell membrane phospholipids results in changes in raft structure/composition [Shaikh et al., 2003] and it is possible that this effect of DHA leads to prevention of Ras localization into rafts inhibiting the Ras-mediated downstream signaling pathways.

It is also not clear how PE, which activates G-protein-coupled receptors, leads to activation of Ras-mediated signaling events that are usually initiated by activation of growth factor receptors. Reports suggest that Gβγ subunits of the G-protein can directly activate Ras signaling in hearts [Crespo et al., 1994; Pumiglia et al., 1995; Naga Prasad et al., 2000]. However, it is also possible that PE stimulation of cardiomyocytes results in the release of paracrine/autocrine growth factors, which in turn bind to a growth factor receptor, activating Ras-mediated signaling events. Additional studies are needed to improve our understanding of these alternate mechanisms. In this study, we evaluated the effects of DHA upon the hypertrophic response induced by PE. There are other important hypertrophic growth factors for the heart (i.e., ANG-II and ET). The effect of DHA on the

hypertrophic response to these growth factors will require additional study.

In conclusion, our investigation demonstrates that DHA treatment results in the inhibition of PE-induced cardiac hypertrophic responses, and one of the mechanisms appears to be mediated by inhibition of Ras activation. DHA treatment appears to inhibit the $Ras \rightarrow Raf\text{-}1 \rightarrow Erk1/2 \rightarrow p90^{rsk}$ pathway, leading to a reduction in PE-induced hypertrophy in cardiac myocytes. The results of this in vitro study need to be confirmed in vivo. If confirmed, DHA in fish oil may be a dietary agent that modifies the development of cardiac hypertrophy and therefore, may also be an attractive therapeutic strategy for treating cardiac hypertrophy in patients with heart failure.

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