RESEARCH ARTICLE

Docosahexaenoic acid inhibits protein kinase C translocation/activation and cardiac hypertrophy in rat cardiomyocytes

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ABSTRACT

Phenylephrine (PE) induces cardiac hypertrophy through multiple signaling pathways including pathways involving protein kinase C (PKC) activation. Docosahexaenoic acid (DHA), an omega-3 fatty acid, has been shown to reduce the PE-induced hypertrophic responses. However, the effects of DHA on PKC activation and translocation are controversial. The present study investigates the effect of DHA on PE-induced activation of PKC. The results indicate that PE induces PKCα translocation (from cytosol to plasma membranes) and activation in cardiomyocytes during the hypertrophic responses. Although DHA itself has no significant effect on basal PKC translocation and activation, it effectively reduced PE-stimulated PKC translocation and activation. The results of the present study suggest a possible mechanism explaining how dietary fish oil may inhibit development of cardiac hypertrophy and therefore may be an attractive dietary agent for preventing cardiac hypertrophy in patients with heart failure.

KEYWORDS: *Cardiomyocytes, hypertrophy, docosahexaenoic acid, protein kinase C*

INTRODUCTION

Congestive heart failure is a major cause of morbidity and mortality from cardiovascular disease. In the United States, 4.8 million Americans suffer from heart failure and the mortality approaches 50% over 5 years. Despite current therapies for heart failure, mortality remains high. The cost for treating heart failure exceeds 30 billion dollars per year. Progressive hypertrophy of cardiomyocytes can lead to heart failure and increased morbidity. Cardiac hypertrophy, which is commonly characterized by an increase in size of cardiomyocytes via a relative increase in cellular proteins in the absence of cell division (Morgan and Baker, 1991; Rupp et al, 1992), usually occurs as a compensatory mechanism secondary to increased workload in patients with hypertension or decreased muscle mass following myocardial infarction (Dhalla et al, 1987). Since we believe that modification of dietary factors has widespread effects upon the development of cardiovascular diseases, our group has been interested in dietary factors that modulate the development of heart failure. Most dietary factors (ie. lipid intake, folic acid) affect the development of atherosclerosis. Recent data from epidemiological (Bang et al, 1976; Albert, 1998; Kris-Etherton et al, 2002) and prospective randomized clinical trials (Burr et al, 1989; Singh et al, 1997; GISSI-Prevenzione, 1999) suggests that omega-3 polyunsaturated fatty acids may also affect the development of cardiac arrhythmias and prevent sudden death. We have been studying the effects of omega-3 fatty acids upon the cardiac hypertrophic response. In our initial studies (Siddiqui et al, 2004), we found that the omega-3 fatty acid, docosahexaenoic acid (DHA), inhibited the cardiomyocyte hypertrophic response to phenylephrine (PE). This study was aimed at identifying a signalling pathway by which DHA might inhibit cardiac hypertrophy.

Activation of PKC is known to affect multiple cardiovascular functions, including vascular permeability, cell migration, and growth (Lynch et al, 1990; Naruse and King, 2000); extracellular matrix production (Smirnov et

al, 1989; Cagliero et al, 1991; Naito et al, 2002) and expression of various cytokines (Okada et al, 1998; Kondo et al, 2000); ion conductance and transport activity (Aviv, 1994); intracellular calcium homeostasis and properties of contractile proteins (Bowman et al, 1997); ischemic preconditioning of the heart (Strasser et al, 1999); genesis of arrhythmias (Mochly-Rosen et al, 2000); and induction of cardiac hypertrophy (Jalili et al, 1999b; Sabri and Steinberg, 2003). While PKC's importance in cardiovascular functions cannot be doubted, its precise involvement is poorly understood and unquestionably complex.

The PKC family of serine-threonine kinases functions downstream of nearly all membrane-associated signal transduction pathways involved in myocardial hypertrophy (Molkentin and Dorn, 2001). The three groups of the PKC family of kinases comprise approximately 13 different isozymes [Conventional (PKCα, -β I, -β II, -γ), novel (PKCδ, -ε, -θ, -η, -μ), and atypical (PKCζ, -ι, -ν, -λ)]. Once activated, PKC isozymes translocate from cytoplasm to discrete subcellular membrane sites (Mochly-Rosen, 1995). Many observations suggest that different isoforms of PKC are recruited to membranes with different stimuli, phosphorylate different sets of cellular substrates, and may regulate different cellular functions. PKC activation/translocation is initiated during hypertrophic responses by a variety of stimuli including pressure overload (Gu and Bishop, 1994; Jalili et al., 1999a; Takeishi et al., 1999; De Windt et al., 2000), bradykinin (Clerk et al, 1996), hypoxia (Goldberg et al, 1997), myotropin (Sil et al, 1998), norepinephrine (Rohde et al, 2000), angiotensin II (Rouet-Benzineb et al, 2000), endothelin-1 (Ito et al, 1997), and mechanical stress (Yamazaki et al, 1995). Although PKCα, -βII, -δ, and -ε are broadly distributed in the cytoplasm of non-stimulated cardiomyocytes (Braz et al, 2002), recent studies implicate PKCα as a critical regulator of the cardiomyocyte hypertrophic response, in part via ERK1/2–MAPK activation (Braz et al, 2002).

Conflicting reports about the role of DHA in regulating PKC activities have appeared in the literature. DHA has been shown to both activate and inhibit PKC activity and its translocation to membranes. For example, free DHA, DHA containing phospholipids or diacylglycerol caused translocation and activation of PKC in several cellular systems (Hrelia et al, 1992; Hardy et al, 1994; Giorgione et al, 1995; Goldberg and Zidovetzki, 1997; Huang et al, 1997). In contrast, a number of studies have suggested that DHA actually inhibits PKC activation (Mirnikjoo et al, 2001). For example, studies have shown that DHA was a highly potent inhibitor of phosphatidylserine (PS)- and diolein (DO)-stimulated PKC in rat colon cells (Holian and Nelson, 1992). Furthermore, DHA also reduced activation of membrane-bound PKC in isolated cardiomyocytes (Bordoni et al, 1992) and suppressed PKC activity in thioglycollate-induced rat peritoneal macrophages (Tappia et al, 1995). The present investigation was therefore undertaken to clarify the effects of DHA on PKCα activation during hypertrophic responses in neonatal cardiomyocytes.

MATERIALS AND METHODS

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). Anti-ANF antibody was obtained from Peninsula Laboratories, Inc. (San Carlos, CA). Anti-sarcomeric α-actinin came from Sigma-Aldrich Chemical Co. (St. Louis, MO) and anti-PKCα antibody was from Upstate Biotechnologies, Inc. (Lake Placid, NY). Anti-mouse or anti-rabbit Alexa Fluor 546 and 480 antibodies were purchased from Molecular Probes (Eugene, OR). Phenylephrine 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 by the trypsin inhibitor and the tissues were further digested with collagenase. Single cells were obtained by filtering the digest through a 70 μm filter. Cells were pre-plated to remove fibroblasts, and cardiomyocytes were isolated using the Worthington's protocols. 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 hr 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% (v/v) fetal bovine serum, 100-units/ml penicillin, 100μg/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 (5 μM) under serum-free conditions for 24 hr. Cells were then incubated with PE (100 μM) in fresh medium with or without a fresh supply of DHA (5 μM). Cells were incubated for another 48 hr 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 hr under the incubation conditions described above. Incubation in the presence of serum-free conditions for a total of 78 hr 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 monitoring cell surface area, expression of sarcomeric α-actinin, and ANF.

Cardiac myocyte surface area

The cardiac myocyte surface area was measured as described previously (Simpson, 1983). 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 by a blinded operator for tracing the surface area of 10 cells in each group. Result represents the mean±SE of three experiments and analyzed by ANOVA and Tukey's multiple comparison tests. Significant differences within groups are reported. ***P<0.05.

PKC activity

The total PKC activity in membrane fractions was assayed using a PKC assay kit containing a specific substrate peptide for PKC in the presence of [γ-^{32}P] ATP and an inhibitor mixture that blocks protein kinase A and calmodulin kinase activities (Upstate Biotechnologies, Inc, Lake Placid, NY). The ^{32}P-substrate from each treatment was separated from residual [^{32}P] ATP using p81 phosphocellulose paper, and the radioactivity incorporated into the substrate was measured by scintillation counting as described previously (Siddiqui and Exton, 1992).

Subcellular localization and activation of PKC

Following treatment with DHA and subsequent stimulation with PE, the cells were homogenized and the cytosolic and membrane fractions isolated by centrifugation at 100,000 x g for one hr at 4^{o}C. The membrane proteins were separated on 8% SDS-PAGE as described (Siddiqui et al, 2004). The relative distribution of PKCα was determined by densitometric analysis using a KODAK imaging system (Eastman Kodak company, Rochester, NY).

Translocation of PKC in cardiomyocytes

Following treatment with PE and 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-PKCα antibodies) in a 1:200 dilution in blocking buffer and either Alexa 546- or Alexa 480-labelled anti-mouse or anti-rabbit (1:200 dilution in blocking buffer) antibodies. Cells were examined under a fluorescence microscope and pictures were taken using a MagnaFire digital camera for analysis.

Statistical analysis

Results typically represent three experiments in each group and were analyzed by ANOVA and Tukey's multiple comparison tests. Significant differences within groups are reported. ***P<0.05.

RESULTS AND DISCUSSION

We previously reported that DHA reduces PE-hypertrophy through inhibition of the Ras→Raf-1→Erk1/2→p90rsk pathway (Siddiqui et al, 2004). However, there is evidence that multiple signaling pathways are involved in the progression of cardiac hypertrophy (Hefti et al, 1997; Aoki et al, 2000; Molkentin and Dorn, 2001; Bueno and Molkentin, 2002), including those involving PKC (Gu and Bishop, 1994; Clerk et al, 1996; Goldberg et al, 1997; Ito et al, 1997; Sil et al, 1998; Jalili et al, 1999a; Takeishi et al, 1999; De Windt et al, 2000; Rohde et al, 2000; Rouet-Benzineb et al, 2000). However, involvement of DHA in the activation of PKC remains controversial (Holian and Nelson, 1992; Hrelia et al, 1992; Hardy et al, 1994; Giorgione et al, 1995; Goldberg and Zidovetzki, 1997; Huang et al, 1997; Mirnikjoo et al, 2001). For this reason, we investigated the effect of DHA on PKC activation during PE-induced cardiac hypertrophy.

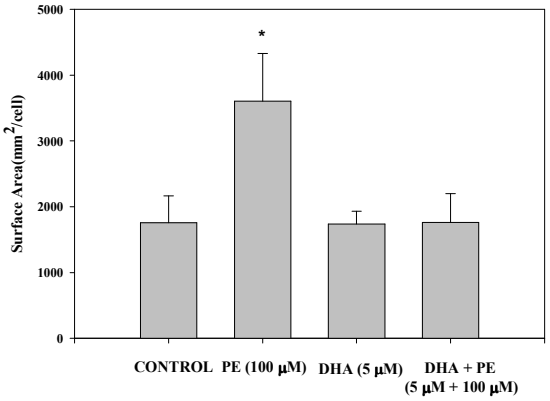

Figure 1. Effect of PE and DHA on cell surface area. Cardiomyocytes were treated with either ethanol (control), serum free media or DHA (5 µM in serum-free conditions) for 24 hr and then incubated with/without PE (100 µM) for 48 hr. Data are expressed as mean ± SEM for examination of 10 cells in each group from three separate experiments and were analyzed by ANOVA and Tukey's multiple comparison tests. Significant differences within groups are reported. ***P<0.05.

Our results demonstrated that cardiomyocytes undergo hypertrophy upon PE exposure (Figure 1). PE induced an increase in cell surface area by two fold (P<0.05). DHA itself had no effect on cardiac cell size; however, DHA reduced the PE-stimulated increases in cell surface area. Results presented in Figure 2 demonstrated that PE treatment of cells caused extensive synthesis of sarcomeric α-actinin with well-organized z-band structure (as indicated by solid arrows). The PE-stimulated cells also exhibited expression of atrial natriuretic factor (ANF) (as indicated by dotted arrows), in characteristic perinuclear rings. Approximately 70-85% of the PE-stimulated cells under 20x magnification exhibit these rings. DHA treatment itself had no effect on cardiomyocytes; however, pretreatment of cells with DHA reduced the expression of

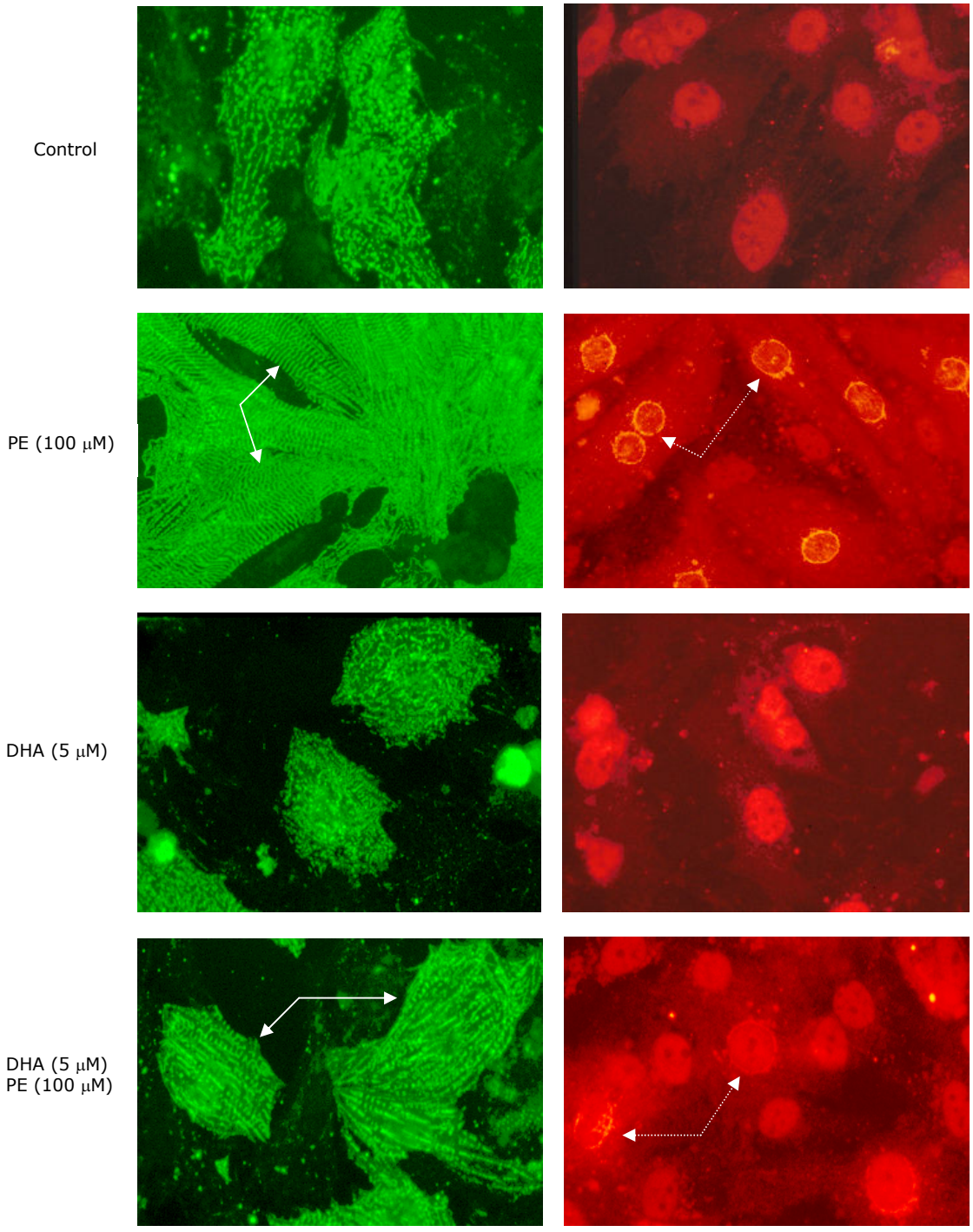

Figure 2. Effect of PE and DHA on α-actinin and ANF expression. Cardiomyocytes were treated with PE and DHA as described in the legend of Figure1. Solid arrows indicate the expression of sarcomeric α-actinin in z-bands (green fluorescence) whereas dotted arrows indicate expression of ANF (red fluorescence). Results are a typical representation of five experiments.

sarcomeric α-actinin and ANF upon PE stimulation. ANF, a universal and specific marker of cardiac hypertrophy (Decker et al, 1995; Knowlton et al, 1995), was particularly affected. However, its role in the development of hypertrophy is not yet clear (Ito et al, 1993; Cao and Gardner, 1995; Calderone et al, 1998; Silberbach et al, 1999).

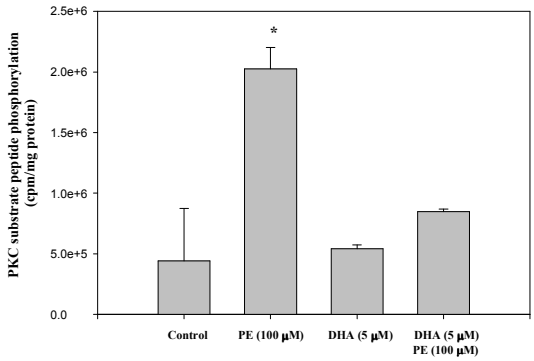

Figure 3. Effect of PE and DHA on PKC activity. Cardiomyocytes were treated with PE and DHA as described in the legend of Figure1. The total PKC activity in membrane fractions of cardiomyocytes was assayed as described in the Material and Methods section. Results are expressed as the mean±SE for three experiments and analyzed by ANOVA and Tukey's multiple comparison tests. Significant differences within groups are reported. ***P<0.05.

We next evaluated the effect of DHA upon PKC enzymatic activity. Results presented in Figure 3 indicated that PE increased PKC activity in membrane fractions four fold (P<0.05), as determined by phosphorylation of exogenous PKC specific peptide. DHA had no effect upon basal PKC activity, while DHA substantially reduced PE-induced activation of PKC. Since this *in vitro* PKC kinase assay did not distinguish between PKC isoenzymes, we next investigated DHA effects upon PKCα, the primary PKC isoenzyme in cardiac tissue. Using specific anti-PKCα antibodies, results of western analysis indicated that PE treatment indeed caused a 2.8-3.2 fold (P<0.05) increased accumulation of PKCα in the membrane fractions (Figure 4). DHA itself had no significant effect on PKCα translocation but effectively reduced PE-stimulated increases in PKC translocation to membranes to a non-significant 1.5-2.2 fold. Translocation of PKC to the membranes is well recognized as a mechanism for activation of classical PKC isozymes including PKCα (Parker and Murray-Rust, 2004; Spitaler and Cantrell, 2004). Next, we evaluated translocation of PKCα using immunohistochemical analysis. Results shown in Figure 5 demonstrated that PKCα is diffusely present throughout the cytoplasm in non-treated cells. PE treatment of cardiomyocytes caused translocation and accumulation of PKCα in membranes as evidenced by the disappearance of most of the diffuse staining from cytoplasm and appearance of intense staining along the cytoskeletal structure. In DHA treated cells, most of the PKCα remained present throughout the cytoplasm. However, DHA partially inhibited PE-induced PKCα translocation to membrane sites.

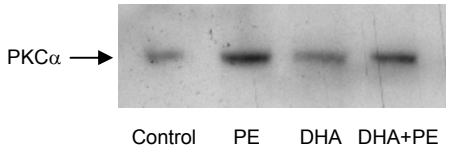

Figure 4. Effect of PE and DHA on PKCα distribution in membranes. Cardiomyocytes were treated with PE and DHA as described in the legend of Figure 1. Membranes were isolated and separated on 8% SDS-PAGE as described in the Material and Methods section. PKCα was detected by Western blotting using anti-PKCα antibodies and the relative distribution of PKCα was determined by densitometric analysis. Results are a typical representation of three experiments.

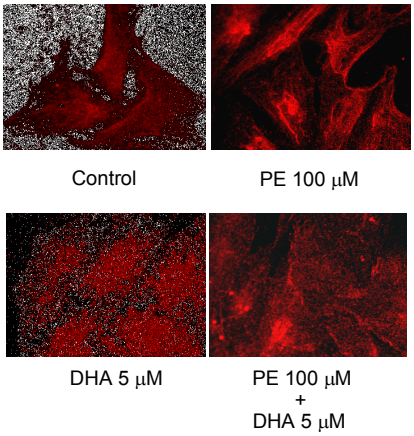

Figure 5. Effect of PE and DHA on PKCα translocation in cardiomyocytes. Cardiomyocytes were treated with PE and DHA as described in the legend of Figure 1. Proteins were detected using anti-PKCα specific antibody and visualized with Alexa 546- (red fluorescence) labelled anti-mouse as described in the Material and Methods section. Cells were examined under a fluorescence microscope and images were captured using a MagnaFire digital camera (Optronics) for analysis. Results are a typical representation of three experiments.

Results shown in Figures 3-5 indicated that DHA itself had no significant effects upon PKCα translocation and activation, but DHA was very effective at reducing PE-induced PKC translocation to membranes. The hypertrophy inhibitory effect of DHA is unique to this fatty acid, since we have not observed inhibition of cardiac hypertrophy by other long chain fatty acids such as oleic acid, linoleic acid, linolenic acid, arachidonic acid, and eicosapentaenoic acid (Siddiqui et al, 2004). We have found in another study (data not Shown) that feeding diets rich in omega-3 fatty acids with equal amounts of DHA

and EPA (10-12%) resulted in greater proportion of DHA incorporation in cardiac tissues (24%) than that of EPA (3%). It appears that a greater proportion of DHA accumulation in cardiac tissues may be responsible for some of the unique effects of DHA in the cardiovascular system. However, our data does not imply that DHA is unique in effecting cardiac hypertrophy through PKC activation or translocation since these effects were not evaluated with different fatty acids.

In this study, we evaluated the effects of DHA upon the hypertrophic response induced by the alpha-adrenergic agonist, PE. Although other important hypertrophic growth factors for the heart (i.e., angiotensin II, endothelin) exist, the effect of DHA upon the hypertrophic response to these growth factors will require additional study. Similarly, there are also other signaling pathways that may contribute to PE-stimulated cardiac hypertrophy. Further studies are required to investigate if DHA has any effect on other signaling pathways. Moreover, the molecular mode of action by which DHA affects PKC activity remains unknown. We hypothesize that DHA incorporates into the cell membrane where it alters structure and physical properties of the cell membrane. These alterations inhibit interaction (translocation) and activation of PKC within the cell membrane. The net effect is diminished activation of the enzyme. It is also possible that DHA inhibits phospholipase C and generation of inositol triphosphate and diacylglycerol activators of PKC.

CONCLUSIONS

Our studies demonstrate that DHA alone has no significant effects upon PKCα translocation and activation. Conversely, this fatty acid effectively prevents the activation and translocation of PE-induced PKCα. We conclude that DHA inhibition of PE-induced activation of PKCα contributes to the anti-hypertrophic actions of DHA. The results of this *in vitro* study need to be confirmed *in vivo*. If confirmed, DHA in fish oil may be an important dietary agent that modifies the development of cardiac hypertrophy.

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STATEMENT OF COMPETING INTERESTS

The authors declared no competing interests.

LIST OF ABBREVIATIONS

ANF: anti-atrial natriuretic factor
DHA: docosahexaenoic acid
PE: phenylephrine
PKC: protein kinase C

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