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(54) **Title**: METHOD OF TREATING OR LIMITING DEVELOPMENT OF HEART FAILURE WITH PRESERVED EJECTION FRACTION AND TISSUE FIBROSIS

(57) **Abstract**:

METHOD OF TREATING OR LIMITING DEVELOPMENT OF HEART FAILURE WITH PRESERVED EJECTION FRACTION AND TISSUE FIBROSIS

CROSS-REFERENCE

[001] This application claims priority to U.S. Provisional Patent Application Serial Number 61/592124 filed January 30, 2012, incorporated by reference herein in its entirety.

STATEMENT OF GOVERNMENT RIGHTS

[002] This invention is supported by the National Institutes of Health under Grant Number 1P20RR017662-06A1. Thus, the U.S. Government has certain right in the invention.

BACKGROUND OF THE INVENTION

[003] Heart failure (HF) is the leading cause of cardiovascular morbidity and mortality worldwide. About half of heart failure patients have heart failure with preserved ejection fraction (HFPEF). Distinct from traditional HF, *i.e.*, heart failure with reduced ejection fraction in which the ventricle cannot contract, patients with HFPEF show declined performance of heart ventricle, not at the time of contraction, but during the phase of diastole. HFPEF patients show normal ejection fraction of blood pumped out of the ventricle, but the heart muscle does not quickly relax to allow efficient filling of blood returning from the body. Morbidity and mortality of HFPEF are similar to traditional HF; however, therapies that benefit traditional HF are not effective in treating or preventing HFPEF. See From *et al.*, Cardiovascular Therapeutics, 2011, 29:e6-21.

[004] One of the causes of diastolic dysfunction is cardiac fibrosis. Cardiac fibrosis is characterized by pathological accumulation of fibrillar collagen throughout the myocardium, which results in reduced cardiac muscle compliance, impaired filling, and ultimately heart failure. In the heart, collagen is produced primarily by cardiac fibroblasts. Stress in the heart can lead to profibrotic cytokine-induced proliferation of cardiac fibroblast and transformation of cardiac fibroblasts into myofibroblasts. Myofibroblasts are responsible for excessive accumulation of extracellular matrix under pathological conditions.

[005] Myofibroblasts secrete extracellular matrix to strengthen the tissue and are important in wound healing. Normally, myofibroblasts are eliminated by apoptosis after the healing is complete. Under pathological conditions, persistent proliferation of myofibroblasts and consequently expansion of the extracellular matrix can lead to tissue fibrosis, often seen in tissues such as liver, kidney, lung and heart.

[006] Omega-3 polyunsaturated fatty acids were shown to be beneficial in coronary artery disease and heart failure. See, *e.g.*, Levitan *et al.*, Eur Heart J. 2009, 30:1495-1500, Mozaffarian *et al.*, J Am Coll Cardiol. 2005, 45:2015-202, Yamagishi *et al.*, Am Coll Cardiol. 2008, 52:988-996, and Tavazzi *et al.*, Lancet, 2008, 372:1223-1230. However, currently there is no effective therapy for treating or preventing tissue fibrosis, especially cardiac ventricular fibrosis. Thus, there exists a need for a therapy effective for treating or limiting development of tissue fibrosis, especially cardiac fibrosis, and HFPEF.

SUMMARY OF THE INVENTION

[007] The invention thus provides methods and reagents directed toward treating or limiting development of heart failure with preserved ejection fraction (HFPEF) and fibrosis that are not hampered by the limitations found in conventional treatments. Without being bound by any particular mechanism of action, the methods and reagents provided in the instant application are capable of reversing or limiting development of HFPEF by inhibiting or preventing cardiac fibrosis.

[008] In accordance with one aspect of the present invention, method are provided for treating or limiting development of heart failure with preserved ejection fraction (HFPEF), comprising administering to a patient having or at risk of developing HFPEF an amount effective of a pharmaceutical composition comprising docosahexaenoic acid (DHA) or pharmaceutically acceptable salts, esters, amides, epoxides, and prodrugs thereof, to treat or limit development of HFPEF. In certain preferred embodiments, the method is for limiting development of HFPEF; while in other embodiments, the method is for treating HFPEF.

[009] In certain embodiments, the pharmaceutical composition does not include any omega-3 fatty acid therapeutic other than DHA or a pharmaceutically acceptable salt, ester, amide, epoxide, or prodrug thereof. In certain other embodiments, the pharmaceutical composition does not include any fatty acid therapeutic other than DHA or a pharmaceutically acceptable

salt, ester, amide, epoxide, or prodrug thereof. In yet other embodiments, the pharmaceutical composition comprises DHA or a pharmaceutically acceptable salt, ester, amide, epoxide, or prodrug thereof in an amount of at least 80%, at least 85%, at least 90%, at least 95% or at least 99.9% by weight of total fatty acids. In yet other embodiments, the pharmaceutical composition does not include any fatty acid therapeutic or any ω-3 fatty acid therapeutic, for the indication of the instant invention, other than DHA or a pharmaceutically acceptable salt, ester, amide, epoxide, or prodrug thereof.

[0010] In certain particular embodiments, the pharmaceutical composition comprises purified or synthesized DHA or a pharmaceutically acceptable salt, ester, amide, epoxide, or prodrug thereof. In certain other embodiments, DHA, or a pharmaceutically acceptable salt, ester, amide, epoxide, or prodrug thereof, is administered to the patient at a concentration of from about 5 mg/kg of body weight/day to about 50 mg /kg of body weight/day. In certain other particular embodiments, the dose is about 600 mg/day to about 1000 mg/day of DHA, or in certain other particular embodiments, about 800 mg/day. In other preferred embodiments, the pharmaceutical composition comprises DHA epoxides, including without limitation EpDPEs [epoxy docosapentaenoic acid]. In yet other preferred embodiments, the pharmaceutical composition comprises 19(20)EpDPE. In certain other embodiments, the patient to be administered the pharmaceutical composition is normotensive or hypotensive.

[0011] In another aspect, the invention provides methods of treating or limiting development of fibrosis comprising administering to a patient having or at risk of developing fibrosis an amount effective of a pharmaceutical composition comprising docosahexaenoic acid (DHA) or pharmaceutically acceptable salts, esters, amides, epoxides, and prodrugs thereof, to treat or limit development of fibrosis. In certain embodiments, the fibrosis is liver fibrosis, kidney fibrosis, cardiac fibrosis or lung fibrosis. In certain preferred embodiments, the fibrosis is cardiac fibrosis. In certain particular embodiments, the cardiac fibrosis is fibrosis in the ventricle. In certain other preferred embodiments, the method is for limiting development of fibrosis; while in other embodiments, the method is for treating fibrosis. In certain particular embodiments, the method is for limiting development of cardiac fibrosis; while in other particular embodiments, the method is for treating cardiac fibrosis. In certain particular embodiments, the cardiac fibrosis is ventricular fibrosis.

[0012] In certain additional embodiments of this aspect, the pharmaceutical composition does not include any omega-3 fatty acid therapeutic other than DHA or a pharmaceutically

acceptable salt, ester, amide, epoxide, or prodrug thereof. In certain other embodiments of this aspect, the pharmaceutical composition does not include any fatty acid therapeutic other than DHA or a pharmaceutically acceptable salt, ester, amide, epoxide, or prodrug thereof. In certain other embodiments, the pharmaceutical composition comprises DHA or a pharmaceutically acceptable salt, ester, amide, epoxide, or prodrug thereof in an amount of at least 80%, at least 85%, at least 90%, at least 95% or at least 99.9% by weight of total fatty acids. In certain particular embodiments, the pharmaceutical composition comprises purified or synthesized DHA or a pharmaceutically acceptable salt, ester, amide, epoxide, or prodrug thereof. In certain other embodiments, DHA, or a pharmaceutically acceptable salt, ester, amide, epoxide, or prodrug thereof, is administered to the patient at a concentration of from about 5 mg /kg of body weight/day to about 50 mg /kg of body weight/day. In certain other particular embodiments, the dose is about 600 mg/day to about 1000 mg/day of DHA, and in certain other particular embodiments, about 800 mg/day. In yet other embodiments, the pharmaceutical composition comprises DHA epoxide, including without limitation EpDPEs [epoxy docosapentaenoic acid]. In yet other preferred embodiments, the pharmaceutical composition comprises 19(20)EpDPE. In certain other embodiments, the patient to which the pharmaceutical composition is administered is normotensive or hypotensive.

[0013] Any and all embodiments disclosed herein are applicable to any and all aspects of the invention.

[0014] Specific embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] **Figure 1** shows results demonstrating that fish oil prevented pressure overload-induced cardiac dysfunction. **Fig. 1A** shows a bar graph measuring the omega-3 content (EPA+DHA % of total fatty acids) of red blood cells or left ventricle (LV) determined by gas chromatography four weeks following transverse aortic constriction (TAC) surgery. **Fig. 1B** shows the measurements of fractional shortening (FS) determined by echocardiography before and 1-, 2- and 4 weeks after TAC. **Fig. 1C** and **1D** show contractility (± dP/dt) and end-diastolic pressure determined from hemodynamic measurements four weeks after TAC. Data are presented as mean ± SEM with n=6 in control/sham and fish oil/sham groups and

n=8-9 in control/TAC and fish oil/TAC groups. In A, C and D, data were analyzed by two-way ANOVA with Tukey's post-hoc test.

[0016] **Figure 2** shows results demonstrating that fish oil prevented pressure overload-induced cardiac fibrosis in mice. **Fig. 2A** showed microphotographs of ventricular cross sections stained with picrosirius red four weeks following TAC. Upper penal: Entire LV section. Scale bar=500 μm. Lower panel: High magnification of indicated collagen area. Scale bar=100 μm. Quantitative measurement of fibrosis in the left ventricle is shown in **Fig. 2B** using Image J and presented as the fibrosis area/total area. **Fig. 2E** shows the heart weight to body weight ratio (HW/BW) calculated four weeks after TAC. In B and E, n=6 in Ctrl diet/Sham and Fish oil/Sham groups, and n=9 in Ctrl diet/TAC and Fish oil/TAC groups. Fig. C, D, F and G show the levels of mRNA expression in left ventricle of collagen I (**Fig. 2C**), collagen III (**Fig. 2D**), atrial natriuretic peptide (ANP) (**Fig. 2F**), and brain natriuretic peptide (BNP) (**Fig. 2G**) measured by real-time PCR 3- or 7-days after TAC (n=5 each group). Data were analyzed by two-way ANOVA with Tukey's post-hoc test and are presented as mean ± SEM.

[0017] **Figure 3** shows results demonstrating that fish oil blocked pressure overload-induced non-myocyte proliferation and myofibroblast transformation. **Fig. 3A** presents microphotographs of immunofluorescence images of ventricular sections stained with an antibody to Ki67 (top panel, arrows), as an indicator for fibroblast proliferation, phalloidin (top panel, labeling cardiac myocytes), and DAPI (second panel, labeling nuclei) four weeks following TAC. The merged images are shown in the third panel with arrows marking interstitial co-labeling of Ki67 and DAPI (third panel). Scale bar=20 μm. **Fig. 3B** shows microphotographs of immunohistochemistry images of ventricular sections stained with an antibody to α-SMA four weeks after TAC to measure myofibroblast transformation. Scale bar=200 μm. **Fig. 3C** shows the number of Ki67 positive cells from 15-20 fields per heart. **Fig. 3D** shows the percent area of α-SMA positive staining in the whole sections. Data were analyzed by two-way ANOVA with Tukey's post-hoc test and are presented as mean ± SEM with n=4 per group.

[0018] **Figure 4** shows results demonstrating that fish oil did not block pressure overload-induced TGF-β1 production or phosphorylation of Smad2. **Fig. 4A** is a bar graph showing ventricular levels of the active form of TGF-β1 measured by ELISA three days following transverse aortic constriction surgery (n=5-6 mice per group). **Fig. 4B** illustrates the levels of

phospho- and total Smad2 measured by western blot analysis three days or four weeks after TAC. **Fig. 4C** shows densitometric quantification of Smad2 phosphorylation/total Smad2 three days or four weeks after TAC (n=6-8 mice per group). Data were analyzed by two-way ANOVA with Tukey's post-hoc test and are presented as mean ± SEM.

[0019] **Figure 5** presents results demonstrating that EPA and DHA inhibited the TGF-β1-stimulated fibrotic response in isolated adult mouse cardiac fibroblasts. **Fig. 5A** shows the ω-3 PUFA content (EPA + DHA) in cultured cardiac fibroblasts treated for 48 hr with EPA (10 μM), DHA (10 μM), arachidonic acid (AA, 10 μM), or the oleic acid (OA, 10 μM), and treated for a final 24 hr with TGF-β1 (1 ng/ml) (n=3). **Fig. 5B** shows the levels of proliferation of cardiac fibroblasts treated as in A (n=3-4) measured by counting live cells using the trypan blue exclusion assay. **Fig. 5C** shows the levels of collagen synthesis determined by the [^{3}H]-proline incorporation assay in cardiac fibroblasts treated as in A (n=9-42). **Fig. 5D** shows the levels of myofibroblast transformation in cardiac fibroblasts treated as in A, measured by α-SMA immunostaining (green, asterisks) with counterstains for fibroblast specific protein 1 (FSP1, red, arrows) and nuclei (DAPI, blue). **Fig. 5E** shows the quantification of α-SMA positive cells as a percentage of total FSP1 positive cells (4 slides for each group). Data were analyzed by one-way ANOVA with Dunnett's post-hoc test in A-C and Tukey's post-hoc test in E. Data are presented as mean ± SEM.

[0020] **Figure 6** presents the results demonstrating that EPA and DHA inhibited the TGF-β1-stimulated fibrotic response through the cGMP/PKG pathway. **Fig. 6A** shows the levels of cGMP measured by ELISA in cultured cardiac fibroblasts treated for 48 hr with EPA or DHA (10-50 μM), using AA (10-20 μM), and OA (10-20 μM) (n=3-7) as control. **Fig. 6B** shows the results of collagen synthesis determined by [^{3}H]-proline incorporation in cardiac fibroblasts treated for 48 hr with EPA (10 μM), DHA (10 μM), 8-bromo-cGMP (1 mM), and/or the guanylyl cyclase inhibitor DT-3 (1 μM) and for the final 24 hr with TGF-β1 (1 ng/ml) (n=4-20). **Fig. 6C** shows the levels of Smad2 and Smad3 phosphorylation (Smad2 Ser465/467; Smad3 Ser423/425) and total Smad2/3 detected by western blot analysis in cultured cardiac fibroblasts treated for 24 hr with EPA or DHA (10 μM) and for an additional 30 min or 24 hr with fatty acids and TGF-β1 (1 ng/ml). **Fig. 6D** and **6E** show the quantification of Smad2 (D) or Smad3 (E) phosphorylation relative to total Smad2/3 (n=3). Data are presented as mean ± SEM. Means were compared by paired Student *t* test in B, one-way ANOVA with Dunnet's post-hoc test in A and Tukey's post-hoc test in D and E.

[0021] **Figure 7** shows the results demonstrating that EPA and DHA blocked nuclear translocation of phosphorylated Smad2 and Smad3 through the cGMP/PKG pathway. **Fig. 7A** shows cellular localization of phospho-Smad detected by staining for phospho-Smad2 (green, first row), Smad4 (red, second row) or phospho-Smad3 (green, fourth row) with a nuclear counterstain (DAPI, merged images in the third and fifth rows) in cultured cardiac fibroblasts treated for 24 hr with EPA (10 μM) (images not shown), DHA (10 μM), 8-bromo-cGMP (1 mM), and/or the guanylyl cyclase inhibitor DT-3 (1 μM) and for an additional 30 min with fatty acids and TGF-β1 (1 ng/ml). **Fig. 7B** and **Fig. 7C** show the percentage of nuclei positive staining for phosphorylated Smad2 and Smad3 (counting 50-200 cells per field, n=5-33 in B and n=4-31 in C). Data were analyzed by one-way ANOVA with Tukey's post-hoc test and are presented as mean ± SEM.

[0022] **Figure 8** shows results demonstrating that EPA and DHA increased NOx production, phosphor-eNOS expression and eNOS expression in cardiac fibroblasts. **Fig. 8A** shows the levels of nitrite/nitrate (NOx) determined using a Total Nitrate/Nitrite Fluorometric method (Cayman Chemical, Ann Arbor, MI) in cultured cardiac fibroblasts treated for 24 hr with EPA (10 μM), DHA (10 μM), 8-bromo-cGMP (0.1 mM), or 8-bromo-cGMP (1 mM) (n=4-14). **Fig. 8B** shows the levels of phospho-eNOS (endothelial nitric oxide synthase) and eNOS detected by western blot analysis in cultured cardiac fibroblasts treated for 24 hr with EPA or DHA (10 μM). **Fig. 8C** and **Fig. 8D** show the quantification of phospho-eNOS or eNOS protein levels relative to GAPDH (n=7-9). Data were analyzed by one-way ANOVA with Tukey's post-hoc test and are presented as mean ± SEM.

[0023] **Figure 9** shows the results demonstrating that EPA and DHA reduced the TGF-β1-induced Smad-responsive promoter activity. **Fig. 9A** shows PAI-1 mRNA levels in left ventricle analyzed by real-time PCR three days and seven days after TAC. Data were analyzed by two-way ANOVA with Tukey's post-hoc test and are presented as mean ± SEM (n=5 per group). **Fig. 9B** shows luciferase activity detected in cardiac fibroblasts transfected with VSV-g pseudotyped lentivirus particles expressing the firefly luciferase gene under the control of a CMV promoter and tandem repeats of the Smad transcriptional response element (TRE). Cells were treated with TGF-β1 (1 ng/ml) with or without 24 hr pretreatment of DHA or EPA. Data were analyzed by one-way ANOVA with Tukey's post-hoc test and are presented as mean ± SEM (n=5-6 per group).

[0024] **Figure 10** shows results demonstrating that EPA and DHA did not block TGF-β1-induced activation of ERK1/2. **Fig. 10A** shows the levels of phosphorylated ERK1/2 (Thr202/Tyr204) and total ERK1/2 detected by western blot analysis in cultured cardiac fibroblasts treated for 24 hr with EPA or DHA (10 μM) and for an additional 1 hr with fatty acids and TGF-β1 (1 ng/ml). **Fig. 10B** shows the quantification of phosphorylated ERK1/2 relative to total ERK1/2. Data were analyzed by one-way ANOVA with Tukey's post-hoc test and are presented as mean ± SEM (n=5 per group).

[0025] **Figure 11** presents results demonstrating that only DHA inhibited the TGF-β1-stimulated fibrotic response in isolated adult mouse cardiac fibroblasts. Cells were treated with no exogenous FAs (No FA), oleic acid (OA, 10 μM), arachidonic acid (AA, 10 μM), EPA (10 μM), or DHA (10 μM) and treated for a final 24 hr with TGF-β1 (1 ng/ml). Collagen production was measured by [^{3}H]-proline incorporation. To establish the potential for mediation by $CYP_{epoxygenase}$, cells were incubated with PPOH, a partial inhibitor of the epoxygenase ("$CYP_{epoxygenase}$ inhibitor").

[0026] **Figure 12** shows the chemical structures of selected compounds. **Fig. 12A** shows the structure of docosahexaenoic acid, **Fig. 12B** shows the structures of exemplary epoxide compounds, and **Fig. 12C** shows the structures of exemplary prodrugs of DHA or DHA epoxide.

DETAILED DESCRIPTION OF THE INVENTION

[0027] All publications, patents and patent applications cited herein are hereby expressly incorporated by reference for all purposes.

[0028] Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press) and *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA).

[0029] As used herein, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. For example, reference to "a pharmaceutically acceptable salt" of DHA means one or more pharmaceutically acceptable salts thereof.

[0030] "Heart failure" as used herein means inability of the heart to supply sufficient blood flow to meet the needs of the body. "Preserved ejection fraction" means that the patient does

not have a significant reduction in ventricular ejection fraction compared to that of a control (*e.g.*, a healthy individual) or compared to an average value from a healthy population. In certain embodiments, ejection fraction in patients classified as HFPEF is $\geq 45\%$.

[0031] "Treating" a patient having a disease or disorder means accomplishing one or more of the following: (a) reducing the severity of the disease; (b) arresting the development of the disease or disorder; (c) inhibiting worsening of the disease or disorder; (d) limiting or preventing recurrence of the disease or disorder in patients that have previously had the disease or disorder; (e) causing regression of the disease or disorder; (f) improving or eliminating the symptoms of the disease or disorder; and (f) improving survival. In certain preferred embodiments, the disease or disorder is HFPEF. In certain particular embodiments, the inventive methods cause regression of HFPEF and improve patient survival by reducing cardiac fibrosis and reducing diastolic pressure.

[0032] "Limiting development" means any reduction in development of a disease or disorder, including but not limited to (a) decreasing the rate of development of a disease or disorder; (b) delaying the onset of development of the disease or disorder; (c) reducing the risk of developing a disease or disorder; and (d) preventing development of the disease or disorder. In certain preferred embodiments, the disease or disorder is HFPEF and the inventive methods described herein reduce the risk of developing HFPEF. In certain particular embodiments, the inventive methods reduce the risk of developing HFPEF by preventing developing cardiac fibrosis and maintaining the efficiency of blood filling into the heart ventricle during diastole.

[0033] A patient at risk of developing HFPEF is any patient with one or more symptoms of HFPEF (including but not limited to shortness of breath, leg swelling, and exercise intolerance), or one or more other risk factors, including but not limited to, a genetic predisposition to HFPEF, a family member with HFPEF; and one or more disorders including, but not limited to, myocardial infarction, cardiomyopathy, ventricular diastolic dysfunction, ventricular systolic dysfunction, ventricular systolic stiffening, vascular stiffening and dysfunction, left atrial dysfunction, pulmonary hypertension, autonomic dysfunction (e.g., chronotropic incompetence; sympathetic hyperactivation), skeletal muscle dysfunction (e.g., impaired vasodilation; sympathetic hyperactivation and ergoreflex stimulation), anemia, and symptoms thereof.

[0034] In certain embodiments of any of the aspects of the invention, a "subject" or "patient" refers to a mammal in need of the intervention of the inventive method. In certain particular embodiments, the mammal is a human. In certain other embodiments, the patient is normotensive or hypotensive. In certain further embodiments, the patient's heart muscle shows normal contractile capability that leads to normal ejection fraction of the heart.

[0035] As used herein, the term "amount effective," "effective amount" or a "therapeutically effective amount" refers to an amount of a therapeutic compound sufficient to achieve the stated desired result, for example, treating or limiting development of HFPEF, tissue fibrosis or cardiac fibrosis. The amount of the compound which constitutes an "effective amount" or "therapeutically effective amount" may vary depending on the severity of the disease, the condition or age of the patient to be treated, or the route of administration, but can be determined routinely by one of ordinary skill in the art. In certain embodiments, DHA is administered to the patient at a concentration of from about 5 mg/kg of body weight/day to about 50 mg/kg of body weight/day.

[0036] In certain embodiments, the pharmaceutical composition comprises DHA epoxide including without limitation epoxydocosapentaenoic acids (EpDPEs) at the 19(20) position or the 4(5), 7(8), 10(11), 13(14), or 16(17) position and all enantiomers thereof. The structures of exemplary epoxide compounds are shown in Figure 12 B.

[0037] A prodrug is an inactive or less active form of the active ingredient that is metabolized to the active form in the body. Prodrugs of DHA suitable for use in the instant invention include without limitation esters of DHA or esters of DHA epoxide. Exemplary prodrugs are shown in Figure 12 C, wherein R is without limitation ethyl, methyl, H, sugar or other carbohydrates, glycerol or as glycerol-lipid per phospholipids, triglycerides, or cholesterol.

[0038] Omega-3 fatty acids are essential polyunsaturated fatty acids vital for normal metabolism and cannot be synthesized *de novo* by mammals. Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) include without limitation α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). These three polyunsaturated fatty acids have 3, 5, or 6 double bonds starting from the third carbon atom from the methyl group in a carbon chain of 18, 20, or 22 carbon atoms, respectively. Fish oils and plant oils are common sources of ω-3 PUFAs. Although fish oils have been considered beneficial to cardiovascular

health in general, to the best knowledge of the inventors, the effect of purified or synthetic DHA on heart failure and fibrosis has not been reported.

[0039] In certain advantageous embodiments, the pharmaceutical composition comprises purified, isolated or synthetic DHA. The use of purified or synthetic DHA avoids the possibility of environmental or other sources of contamination. Purified or synthetic DHA is commercially available from, for example, Sigma (St. Louis, MO) or can be obtained based on the disclosure of, for example, Journal of the American Oil Chemists Society, 1997, 74:1435-40. In certain particular embodiments, the DHA for use in the claimed invention is purified or synthesized DHA that is at least 90%, at least 95%, at least 98%, or at least 99% pure. In certain particular embodiments, the pharmaceutical composition comprising purified or synthesized DHA without any other ω-3 PUFAs. In certain other embodiments, the composition comprising purified or synthesized DHA without any other fatty acids.

[0040] The terms purified DHA and isolated DHA are used interchangeably throughout the application. The term refers to DHA obtained from a source, including but not limited to its natural source such as fish oils or plant oils, by one or more chemical and/or physical means. The purified or isolated DHA is separated from other components and impurities from its source or from the compounds or impurities introduced during the isolation process. A pharmaceutically acceptable salt, ester, amide or prodrug of DHA suitable for use in the instant invention can be naturally existing in the source and purified from the source or can be chemically synthesized or prepared.

[0041] The term "fatty acid therapeutic" or "omega-3 fatty acid therapeutic" refers to a fatty acid (or omega-3 fatty acid) that is present in a pharmaceutical composition and exerts a therapeutic effect and is not merely present as an inert excipient or diluent or a minor contaminant without any therapeutic effect. In certain particular embodiments, the method comprising the step of administering to a patient in need thereof a pharmaceutical composition that does not include any fatty acid (or omega-3 fatty acid) therapeutic for the recited indication of the instant invention other than DHA or a pharmaceutically acceptable salt, ester, amide or prodrug thereof. In certain embodiments, minor contaminating fatty acids may be present in an amount not exceeding 0.01%, 0.05%, 0.1%, 0.15%, 0.5%, 1%, 2%, 3% or 5% of total fatty acids, wherein the contaminating fatty acids do not exert any therapeutic effect. In certain other embodiments, the pharmaceutical composition comprises DHA or a pharmaceutically acceptable salt, ester, amide, epoxide, or prodrug thereof in an

amount of at least 80%, at least 85%, at least 90%, at least 95% or at least 99.9% by weight of total fatty acids. In certain preferred embodiments, the method comprising the step of administering to a patient in need thereof a pharmaceutical composition that does not include any fatty acid (or omega-3 fatty acid) therapeutic other than DHA or a pharmaceutically acceptable salt, ester, amide or prodrug thereof. In certain other preferred embodiments, the pharmaceutical composition does not include any fatty acid (or omega-3 fatty acid) other than DHA or a pharmaceutically acceptable salt, ester, amide or prodrug thereof.

[0042] Previous studies purportedly reported that diet supplemented with ω-3 PUFAs inhibited left ventricle perivascular fibrosis or reduced cardiac fibrosis in a hypertensive animal model. See Fischer *et al.* (Hypertension, 2008, 51:540-546) and Medeiros et al. (Prostaglandins Other Lipid Mediat. 2005, 78:231-248). These studies showed significant reduction of systemic arterial blood pressure as a result of diet containing ω-3 PUFAs. However, the studies were conducted in animals that had already developed hypertension. Thus, a conclusion cannot be drawn that any purported anti-fibrotic effect reported was independent of blood pressure. In addition, the animals were fed with a mixture of fatty acids and no conclusion can be drawn that any anti-fibrotic effect or anti-hypertensive effect is attributed to DHA.

[0043] The inventors of the instant application unexpectedly discovered that, *inter alia*, the effect of DHA on heart failure is independent of blood pressure. The inventors unexpectedly discovered that ω-3 PUFAs prevented pressure overload-induced cardiac dysfunction and cardiac fibrosis in animals that were normotensive. In particular, it was surprisingly discovered by the inventors of the instant application that DHA reduced TGF-β1-induced Smad responsive promoter activity and that DHA greatly inhibited TGF-β1-induced collagen synthesis in cardiac fibroblasts.

[0044] Thus, in certain particular embodiments, the invention provides methods of treating HFPEF comprising administering to a patient having or at risk of developing HFPEF an amount effective of a pharmaceutical composition comprising DHA wherein the pharmaceutical composition differentially or selectively reduced diastolic pressure or maintained normal diastolic pressure in the patient. In certain other particular embodiments, the invention provides methods of limiting the development of HFPEF comprising administering to a patient at risk of developing HFPEF an amount effective of a

pharmaceutical composition comprising DHA wherein the pharmaceutical composition differentially or selectively maintained normal diastolic pressure in the patient.

[0045] Further in accordance with the instant invention, methods are provided for treating or limiting development of HFPEF, comprising the step of treating or limiting development of diastolic dysfunction by administering to a patient an amount effective of a pharmaceutical composition comprising DHA or pharmaceutically acceptable salts, esters, amides, epoxides, and prodrugs thereof, to treat or limit development of HFPEF. In a related aspect, the invention provides methods of treating or limiting development of diastolic dysfunction comprising administering to a patient administering to a patient having or at risk of developing diastolic dysfunction an amount effective of a pharmaceutical composition comprising DHA or pharmaceutically acceptable salts, esters, amides, epoxides, and prodrugs thereof, to treat or limit development of diastolic dysfunction.

[0046] The term normotensive as used herein refers to normal systolic and/or diastolic blood pressures. In certain particular embodiments, a normotensive patient has an end point systolic pressure of no more than 139 mmHg and no less than 90 mmHg. In certain other particular embodiments, the normotensive patient has an end point diastolic pressure of no more than 89 mmHg and no less than 60 mmHg.

[0047] The term hypotensive as used herein refers to systolic and/or diastolic blood pressures that are lower than the normal values. In certain particular embodiments, a hypotensive patient has a systolic pressure lower than 90 mmHg. In certain other particular embodiments, the hypotensive patient has a diastolic pressure lower than 60 mmHg.

[0048] The term hypertensive as used herein refers to systolic and/or diastolic blood pressures that are higher than the normal values. In certain particular embodiments, a hypertensive patient has a systolic pressure of at least 140 mmHg. In certain other particular embodiments, the hypertensive patient has a diastolic pressure of at least 90 mmHg.

[0049] The instant inventors also surprisingly discovered that DHA inhibits transforming growth factor-β1 (TGF-β1)-induced cardiac fibroblast proliferation and myofibroblast transformation Thus, in other aspects, the instant invention provides methods for treating or limiting development of fibrosis comprising the step of administering to a patient having or at risk of developing fibrosis an amount effective of a pharmaceutical composition comprising docosahexaenoic acid (DHA) or pharmaceutically acceptable salts, esters, amides, epoxides, and prodrugs thereof, to treat or limit development of fibrosis. Applicable tissue fibrosis

includes without limitation fibrosis in the lung, kidney, liver, and heart. In certain particular embodiments, the invention provides methods for inhibiting cardiac fibrosis. In related embodiments, the invention provides methods of treating or limiting development of fibrosis comprising the step of inhibiting cardiac fibroblast proliferation, myofibroblast transformation, inhibiting collagen expression or activating the cGMP/PKG pathway, by administering to a patient having or at risk of developing fibrosis an amount effective of a pharmaceutical composition comprising DHA or pharmaceutically acceptable salts, esters, amides, epoxides, and prodrugs thereof, to treat or limit development of fibrosis.

[0050] In a related aspect, the invention provides methods of treating or limiting development of diastolic dysfunction. In certain embodiments, the methods of treating or limiting development of diastolic dysfunction comprises the step of inhibiting cardiac fibrosis by administering to a patient having or at risk of developing fibrosis an amount effective of a pharmaceutical composition comprising docosahexaenoic acid (DHA) or pharmaceutically acceptable salts, esters, amides, epoxides, and prodrugs thereof, to treat or limit development of diastolic hypertension.

[0051] In a further aspect, the invention provides uses of docosahexaenoic acid (DHA) or pharmaceutically acceptable salts, esters, amides, epoxides, and prodrugs thereof for the preparation for a medicament for treating or limiting development of HFPEF. In yet another aspect, the invention provides uses of DHA or pharmaceutically acceptable salts, esters, amides, epoxides, and prodrugs thereof for the preparation for a medicament for treating or limiting development of fibrosis. In certain embodiments, the fibrosis is liver fibrosis, kidney fibrosis, cardiac fibrosis or lung fibrosis. In certain preferred embodiments, the fibrosis is cardiac fibrosis. In certain other particular embodiments, the fibrosis is fibrosis in the ventricle.

[0052] The pharmaceutical compositions used in the inventive methods can be specially formulated for oral administration in solid or liquid form or for intravenous injection. Optimal pharmaceutical compositions can be determined by one skilled in the art depending upon, for example, the intended route of administration, delivery format and desired dosage. *See, for example*, REMINGTON'S PHARMACEUTICAL SCIENCES, *Id.*

[0053] The DHA or pharmaceutical acceptable salts, esters, amides, epoxides and prodrugs thereof can be incorporated in a conventional systemic dosage form, such as a tablet, capsule, soft gelatin capsule, elixir or injectable formulation. The dosage forms may also include the

necessary physiologically acceptable carrier material, excipient, lubricant, buffer, surfactant, antibacterial, bulking agent (such as mannitol), antioxidants (ascorbic acid or sodium bisulfite) or the like. Oral dosage forms are preferred, although parenteral forms can be used as well.

[0054] Suitable surfactants include without limitation Tween 20, Tween 80, a polyethylene glycol or a polyoxyethylene polyoxypropylene glycol, such as Pluronic F-68. The salt or buffering agent may be any salt or buffering agent, such as for example sodium chloride, or sodium/potassium phosphate, respectively. Preferably, the buffering agent maintains the pH of DHA or acceptable salts, esters, amides, epoxides and prodrugs thereof. The salt and/or buffering agent is also useful to maintain the osmolality at a level suitable for administration to a human.

[0055] The drug is administered preferably orally, in particular in the form of soft gelatin capsules. Other types of formulation for oral administration are also suitable for the purposes of the invention; for example hard capsules or tablets, in which the ω-3 fatty acid is adsorbed on solid supports or micro-particles.

[0056] The dosage forms of capsules can be prepared with coatings and shells such as enteric coatings and other coatings well-known in the pharmaceutical formulating art. They may optionally contain opacifying agents and may also be of a composition such that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

[0057] Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups and elixirs. In addition to the active compounds, the liquid dosage forms may contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethyl formamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan and mixtures thereof.

[0058] Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, tragacanth and mixtures thereof.

[0059] The pharmaceutical composition of the invention may be administered to a patient by sustained release, as is known in the art. Sustained release administration is a method of drug delivery to achieve a certain level of the drug over a particular period of time.

[0060] Pharmaceutical compositions of this invention for parenteral injection comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol and the like), injectable organic esters (such as ethyl oleate) and suitable mixtures thereof. Proper fluidity can be maintained, for example, by the use of coating materials such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants.

[0061] These compositions may also contain adjuvants such as preservative, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms can be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid and the like. It may also be desirable to include isotonic agents such as sugars, sodium chloride and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

[0062] The Examples disclosed herein are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

EXAMPLES

Experimental Procedure

1. Animals and Statistic Analysis

[0063] Male C57BL/6 mice (5 weeks, Jackson Laboratory) were fed a control or a fish oil supplemented diet for eight weeks (provided by Dyets, Bethlehem, PA). Both diets contained 4% by weight test oil (control diet: 40 g corn oil per kg; fish oil supplemented diet: 12 g menhaden oil plus 28 g corn oil per kg), but were otherwise identical. The fish oil diet

provided 1% energy as EPA+DHA. After eight weeks on the assigned diet, mice were randomly allocated into 4 groups using a 2 x 2 factorial design and subjected to transverse aortic constriction (TAC) surgery (groups: control/sham, control/TAC, fish oil/sham, and fish oil/TAC; n=22-25/group). The TAC mice developed diastolic dysfunction and systolic dysfunction and significant interstitial fibrosis which are common in HFPEF.

[0064] Transverse aortic constriction surgery was performed without intubation under anesthesia with isoflurane, as previously described (O'Connell *et al.*, J Clin Invest. 2003, 111:1783-1791, O'Connell *et al.*, J Clin Invest. 2006,116:1005-1015). Following the surgery, mice were continually fed the assigned diets and sacrificed after three, seven, or 28 days.

[0065] Unless specified otherwise, results are reported as means ± SEM. Mean values were compared by one-way or two-way ANOVA followed by Tukey's post-hoc test or Dunnett's post-hoc test as appropriate. $P<0.05$ was considered significant. For analysis of fractional shortening (Figure 1B), longitudinal analysis with restricted maximum likelihood estimation method and an unstructured correlation matrix for the repeated measurements was used (SAS version 9.2, Cary, NC).

2. Measurement of Cardiac Function

[0066] Echocardiography and hemodynamics were performed as described previously (Tang *et al.*, Circulation, 2005, 112:3122-3130). Briefly, echocardiographic measurements were performed under anesthesia (3% isoflurane induction, 1% maintenance) using a Visual Sonics Vevo 660 High-Resolution Imaging System (Visual Sonics, Toronto, Canada) with a 30-MHz linear array transducer (model RMV-707). Left ventricular dimensions and heart rate were measured from 2-D short-axis M-mode tracings at the level of the papillary muscle. Left ventricular mass and functional parameters were calculated using the above primary measurements. Left ventricular hemodynamics were measured under anesthesia (3% isoflurane induction, 1% maintenance) using a Millar catheter inserted into the left ventricle via the carotid artery. After stabilization, heart rate (HR), left ventricular end-systolic pressure and end-diastolic pressure were measured from the left ventricular pressure waveform, and ±dP/dt and tau were calculated.

3. Isolation and Culture of Cardiac Fibroblasts

[0067] Adult mouse cardiac fibroblasts were isolated using a previously described procedure (O'Connell *et al.*, J Clin Invest. 2006, 116:1005-1015) with the following modifications.

Briefly, following perfusion and digestion of the heart with collagenase II (Worthington Biochemical Corp, Lakewood, NJ), dissociated cells (myocytes and non-myocytes) were sedimented by gravity, and the supernatant, rich in cardiac fibroblasts, was collected and centrifuged for 5 min at 1000 rpm. Fibroblasts were resuspended in Dulbecco's Modification of Eagle's Medium (DMEM, Fisher Scientific, Pittsburgh, PA) with 15% fetal bovine serum (FBS, Fisher Scientific) and plated on laminin-coated 60 mm dishes (Becton Dickinson, Franklin Lakes, NJ). After one hour, the culture medium was changed, which removed weakly adherent cells, including any myocytes and endothelial cells. After 24 hours, the culture medium was changed again, and the fibroblast cultures were grown to 85% confluence, at which point the medium was replaced with DMEM containing 0.1% FBS. After 24 hours in reduced serum medium, fibroblast cultures were treated with fatty-acids or other agonist/antagonists as indicated.

4. Fatty Acids Preparation

[0068] Free fatty acids (DHA, EPA, arachidonic acid and oleic acid, Sigma, St. Louis, MO) for *in vitro* studies were dissolved in 100% ethanol to make a stock solution (100 mg/ml). Aliquots (5 µl/tube) of the stock solution were stored in PCR tubes under a blanket of nitrogen and sealed with parafilm and stored at -80 ºC. Before use, the aliquots were diluted with 0.2% BSA/PBS and then further diluted in culture medium.

5. Real-time Quantitative PCR

[0069] Gene expression was measured by the Genomic-Microarray/qPCR Core at The Burnham Institute for Medical Research (La Jolla, CA). Briefly, RNA from the left ventricle (three and seven days post-surgery) was extracted using the RNeasy Fibrous Tissue Mini Kit (QIAGEN, Valencia, CA). Oligo (dT) primed cDNA synthesis was performed using Superscript III (Invitrogen, Carlsbad, CA). Expression of collagen I, collagen III, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), PAI-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts were measured using the TaqMan Gene Expression Assay kit using validated primers (Applied Biosystems, Foster City, CA).

6. Immunohistochemistry and Immunocytochemistry

[0070] The heart tissues were excised, cannulated through the aorta and perfused with buffer (1% adenosine, 2% BDM, and 0.04% heparin in PBS). Hearts were fixed in formalin overnight, embedded in paraffin and sectioned (5 µm) by the Histology Core at The Burnham

Institute for Medical Research (La Jolla, CA). To measure fibrosis, tissue sections were stained with picrosirius red. High resolution images were obtained through Aperio Scanscope software (Burnham Institute, La Jolla, CA) and cardiac fibrosis was quantified using Image J (NIH website). Alpha-SMA staining was performed on formalin-fixed, paraffin-embedded left ventricular sections (5 μm).

[0071] After deparaffinization, rehydration, heat-induced epitope retrieval and blocking of endogenous peroxidase activity, tissue sections were incubated with antibodies to α-SMA (1:100, Dako, Carpinteria, CA) and an HRP-labeled secondary antibody. Dako EnVision+ System-HRP (DAB) was used to visualize positive α-SMA staining. The sections were counterstained with hematoxylin to visualize nuclei. Images were captured using an inverted microscope (Olympus IX71). The positive areas of α-SMA staining were quantified by Image-Pro plus (Media Cybernetics, Bethesda, MD).

[0072] Ki67 staining for proliferation was performed on formalin-fixed, paraffin-embedded left ventricular sections (5 μm). After deparaffinization, re-hydration and heat-induced epitope retrieval, sections were incubated with antibodies to Ki67 (1:200, Abcam, Cambridge, MA) and a goat anti-rabbit AlexaFluor 488 secondary antibody (1:400, Invitrogen). Images were captured using confocal microscopy (FV1000, Olympus). Quantitative data were obtained by measuring co-localization of 4',6-diamidino-2-phenylindole (DAPI, nuclear staining) with Ki67 in the interstitial area of the left ventricle.

[0073] Staining for fibroblast-specific protein-1 (FSP-1), α-SMA or Smad was performed on cultured cardiac fibroblasts fixed with 4% paraformaldehyde and permeabilized with ice-cold methanol. After blocking with 5% normal serum from the same species as the secondary antibody, fibroblasts were incubated with primary antibody [phospho-Smad3 (Ser423/425) (1:400, Cell Signaling Technology, Danvers, MA); phospho-Smad2 (ser465/467) (1:500, Millipore, Billerica, MA); Smad4 (1:100, Santa Cruz Biotechnology, Inc, Santa Cruz, CA); FSP1 (1:100, Abcam); and α-SMA (1:200, Dako)] and fluorochrome-conjugated secondary antibodies. Fibroblasts were counterstained with DAPI to visualize nuclei. Images were captured using confocal microscopy (FV1000, Olympus).

7. Western Blot Analysis

[0074] Samples from the left ventricle and cultured adult cardiac fibroblasts were homogenized and incubated in Tissue Extraction Reagent I (Invitrogen, Carlsbad, CA) and Cell Extraction Buffer (Invitrogen) with a protease inhibitors cocktail (Sigma, Saint Louise,

MO) and 1mM PMSF (phenylmethylsulfonyl Fluoride) on ice for 15 minutes. The lysates were centrifuged at 13,000 g for 10 minutes at 4°C. The supernatant was collected, aliquoted, and stored at -80°C until time of use. Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Samples were mixed with Laemmli buffer containing 5% β-mercaptoethanol and were evenly loaded onto SDS-PAGE gels. Proteins were transferred to PVDF membranes. Membranes were incubated overnight at 4°C with primary antibodies specific for phospho-Smad2, phospho-Smad3, Smad2/3, phospho-eNOS, total eNOS, phospho-ERK1/2, total ERK1/2 (all from Cell Signaling Technology, Inc., Danvers, MA) in 5% BSA. A horseradish peroxidase conjugated secondary antibody was incubated for 1 h at room temperature in 5% milk and processed for chemiluminescent detection using an ECL Advanced Western Blotting Kit (GE Healthcare, Piscataway, NJ). Protein abundance on western blots was quantified by densitometry with the Quantity One program from Bio-Rad (Hercules, CA).

Example 1 Fish Oil Prevented Pressure Overload-Induced Cardiac Dysfunction

[0075] Before inducing pressure overload, mice were fed a diet supplemented with ω-3 PUFAs or control diet for eight weeks. Omega-3 content in the red blood cells and left ventricle was measured as previously described (Duda *et al.*, Cardiovasc Res. 2009, 81:319-327). As shown in Figure 1A and Tables 1-2, ω-3 PUFAs-supplemented diet increased the ω-3 content (EPA+DHA % of total fatty acids) in both red blood cells (3.4 fold) and left ventricle (2.4 fold) relative to mice on the control diet. Aortic constriction decreased the ω-3 content in red blood cells and heart in mice on the control diet ($p<0.01$), whereas aortic constriction had no effect on the fish oil groups (Figure 1A and Tables 1-2).

[0076] Aortic constriction induced contractile dysfunction in mice fed with the control diet by four weeks following surgery. For analysis of fractional shortening (FS), a repeated measures model was used. As shown in Figures 1B-D, aortic constriction induced contractile dysfunction in mice fed with the control diet as evidenced by a 38% decrease in fractional shortening (FS), a 29% decrease in dP/dt max, a 27% decrease in dP/dt min, and a 6.5-fold increase in end-diastolic pressure (all at least $p<0.05$ relative to sham). In contrast, TAC-induced contractile dysfunction was mitigated or prevented in mice fed the fish oil diet (Figures 1B-D). Fractional shortening in Ctrl diet/Sham animals was not changed (0.7 ± 0.8%, $P>0.2$) compared to baseline, nor was FS in Fish oil/Sham animals (1.5 ± 1.1%, $P=0.2$)

(Figure 1B). TAC decreased FS by -4.4 ± 1.0% per week (P=0.0003), and fish oil prevented the effect of TAC by 3.1 ± 1.5% per week (p=0.043), almost three-quarters of the effect of TAC surgery (Figures 1C and D). There was no significant difference in pressure gradients induced by aortic constriction between the control diet and fish oil diet groups (Table 3). In short, dietary supplementation with fish oil protected against pressure overload-induced contractile dysfunction. Tables 1-3 are shown below.

Table 1: Fatty Acid Profiles from Red Blood Cells[a]

Fatty acid[b]	Structure	Control diet		Fish oil diet	
		Sham (n=6)	TAC (n=9)	Sham (n=6)	TAC (n=9)
Myristic acid	C14:0	0.26 ± 0.03	0.25 ± 0.01	0.31 ± 0.01 [A]	0.29 ± 0.01 [C]
Palmitic acid	C16:0	29.3 ± 0.20	29.7 ± 0.27	31.6 ± 0.44 [A]	32.4 ± 0.28 [C]
Palmitoleic acid	C16:1n7	0.83 ± 0.08	0.84 ± 0.06	0.79 ± 0.04	0.79 ± 0.03
trans Palmitoleic acid	C16:1n7t	0.05 ± 0.00	0.06 ± 0.00	0.07 ± 0.01 [A]	0.08 ± 0.00 [C]
Stearic acid	C18:0	12.8 ± 0.43	13.3 ± 0.24	12.4 ± 0.10	11.5 ± 0.13 [B,C]
Oleic acid	C18:1n9	14.1 ± 0.40	15.3 ± 0.34 [A]	13.3 ± 0.10	13.7 ± 0.14 [C]
trans Oleic acid	C18:1t	0.19 ± 0.02	0.17± 0.01	0.18 ± 0.02	0.15 ± 0.01
Linoleic acid (n-6)	C18:2n6	11.1 ± 0.53	10.5 ± 0.34	10.2 ± 0.12	10.3 ± 0.16
trans Linoleic acid (n-6)	C18:2n6t	0.24 ± 0.02	0.28 ± 0.02	0.32 ± 0.04	0.17 ± 0.02 [B,C]
α-Linolenic acid (n-3)	C18:3n3	0.05 ± 0.01	0.05 ± 0.00	0.04 ± 0.01 [A]	0.03 ± 0.00 [C]
γ-Linolenic acid (n-6)	C18:3n6	0.08 ± 0.01	0.09 ± 0.00	0.10 ± 0.01	0.13 ± 0.01 [B,C]
Eicosenoic acid	C20:1n9	0.48 ± 0.01	0.52 ± 0.01	0.45 ± 0.02	0.38 ± 0.01 [B,C]
Eicosadienoic acid (n-6)	C20:2n6	0.30 ± 0.02	0.28 ± 0.01	0.20 ± 0.01 [A]	0.18 ± 0.00 [C]
Eicosatrienoic acid (n-6)	C20:3n6	1.26 ± 0.08	1.37 ± 0.04	1.13 ± 0.05	1.06 ± 0.02 [C]
Arachidonic acid (AA, n-6)	C20:4n6	19.3 ± 0.63	18.4 ± 0.34	10.6 ± 0.29 [A]	10.4 ± 0.14 [C]
Eicosapentaenoic acid (EPA, n-3)	C20:5n3	0.05 ± 0.01	0.08 ± 0.04	3.59 ± 0.14 [A]	3.64 ± 0.11 [C]
Docosatetraenoic aicd (n-6)	C22:4n6	2.17 ± 0.10	2.26 ± 0.06	0.51 ± 0.00 [A]	0.50 ± 0.01 [C]
Docosapentaenoic acid (n-3)	C22:5n3	0.42 ± 0.06	0.26 ± 0.02 [A]	2.16 ± 0.02 [A]	2.14 ± 0.03 [C]
Docosapentaenoic acid (n-6)	C22:5n6	2.17 ± 0.24	2.61 ± 0.06 [A]	0.24 ± 0.01 [A]	0.28 ± 0.00 [C]
Docosahexaenoic acid (DHA, n-3)	C22:6n3	4.41 ± 0.22	3.45 ± 0.11 [A]	11.5 ± 0.09 [A]	11.6 ± 0.11 [C]
Lignoceric acid	C24:0	0.17 ± 0.01	0.16 ± 0.01	0.18 ± 0.01	0.22 ± 0.01 [B,C]
Nervonic acid	C24:1n9	0.15 ± 0.01	0.12 ± 0.01	0.15 ± 0.02	0.17 ± 0.01 [C]
Composite Indices					
Omega-3 Content (DHA+EPA)		4.45 ± 0.23	3.54 ± 0.13 [A]	15.1 ± 0.11 [A]	15.2 ± 0.17 [C]
Total Saturated		42.6 ± 0.42	43.4 ± 0.40	44.5 ± 0.40 [A]	44.3 ± 0.29
Total Monounsaturated		15.8 ± 0.43	17.0 ± 0.40 [A]	15.0 ± 0.14	15.3 ± 0.14 [C]
n-3 Polyunsaturated		4.93 ± 0.29	3.84 ± 0.15 [A]	17.3 ± 0.11 [A]	17.4 ± 0.19 [C]
n-6 Polyunsaturated		36.7 ± 0.64	35.7 ± 0.36	23.2 ± 0.31 [A]	23.0 ± 0.26 [C]
n-3 : n-6		0.135 ± 0.008	0.108 ± 0.005	0.745 ± 0.011 [A]	0.756 ± 0.011 [C]
EPA : AA		0.003 ± 0.001	0.004 ± 0.002	0.342 ± 0.021 [A]	0.350 ± 0.011 [C]
EPA : DHA		0.010 ± 0.002	0.022 ± 0.009	0.313 ± 0.014 [A]	0.314 ± 0.009 [C]

[A] $P<0.05$ verus Control diet/sham; [B] $P<0.05$ verus Fish oil diet/sham; [C] $P<0.05$ verus Control diet/TAC. All measurements are means ± SEM, and significance was determined using the two-way ANOVA with post tukey test.

[a] Reported as percent of total fatty acid.

[b] Most common name given. Omega-9, omega-6 and omega-3 FAs are indicated as n9, n6, and n3, respectively

Table 2 Fatty Acid Profiles from LV Tissues[a]

Fatty acid[b]	Structure	Control diet		Fish oil diet	
		Sham (n=6)	TAC (n=9)	Sham (n=6)	TAC (n=9)
Myristic acid	C14:0	0.23 ± 0.02	0.23 ± 0.01	0.31 ± 0.02 [A]	0.30 ± 0.03 [C]
Palmitic acid	C16:0	13.5 ± 0.21	13.7 ± 0.10	15.0 ± 0.26 [A]	14.9 ± 0.20 [C]
Palmitoleic acid	C16:1n7	0.50 ± 0.07	0.56 ± 0.04	0.65 ± 0.07	0.66 ± 0.06
trans Palmitoleic acid	C16:1n7t	0.03 ± 0.00	0.03 ± 0.00	0.037 ± 0.002 [A]	0.044 ± 0.001 [B,C]
Stearic acid	C18:0	16.6 ± 0.39	17.0 ± 0.15	16.0 ± 0.27	16.2 ± 0.25 [C]
Oleic acid	C18:1n9	12.2 ± 0.76	12.9 ± 0.47	9.99 ± 0.46 [A]	10.6 ± 0.35 [C]
trans Oleic acid	C18:1t	0.17 ± 0.01	0.16± 0.00	0.15 ± 0.00 [A]	0.14 ± 0.00 [C]
Linoleic acid (n-6)	C18:2n6	17.4 ± 0. 51	16.6 ± 0.33	11.7 ± 0.14 [A]	12.4 ± 0.45 [C]
trans Linoleic acid (n-6)	C18:2n6t	0.07 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.07 ± 0.00
α-Linolenic acid (n-3)	C18:3n3	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00
γ-Linolenic acid (n-6)	C18:3n6	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.02 ± 0.01 [B,C]
Eicosenoic acid	C20:1n9	0.58 ± 0.04	0.57 ± 0.02	0.45 ± 0.02 [A]	0.46 ± 0.03 [C]
Eicosadienoic acid (n-6)	C20:2n6	0.33 ± 0.01	0.30 ± 0.01 [A]	0.24 ± 0.01 [A]	0.25 ± 0.01 [C]
Eicosatrienoic acid (n-6)	C20:3n6	0.75 ± 0.08	0.80 ± 0.02	0.56 ± 0.01 [A]	0.54 ± 0.01 [C]
Arachidonic acid (AA, n-6)	C20:4n6	10.2 ± 0.15	10.6 ± 0.15	3.85 ± 0.08 [A]	3.83 ± 0.16 [C]
Eicosapentaenoic acid (EPA, n-3)	C20:5n3	0.01 ± 0.00	0.01 ± 0.00	0.49 ± 0.02 [A]	0.47 ± 0.03 [C]
Docosatetraenoic aicd (n-6)	C22:4n6	1.18 ± 0.09	1.21 ± 0.03	0.13 ± 0.01 [A]	0.13 ± 0.00 [C]
Docosapentaenoic acid (n-3)	C22:5n3	0.60 ± 0.04	0.55 ± 0.07	2.38 ± 0.04 [A]	2.37 ± 0.06 [C]
Docosapentaenoic acid (n-6)	C22:5n6	9.84 ± 1.05	12.3 ± 0.25 [A]	0.42 ± 0.01 [A]	0.41 ± 0.01 [C]
Docosahexaenoic acid (DHA, n-3)	C22:6n3	15.5 ± 0.94	12.3 ± 0.59 [A]	37.5 ± 0.61 [A]	36.0 ± 0.59 [C]
Lignoceric acid	C24:0	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.06 ± 0.00 [B,C]
Nervonic acid	C24:1n9	0.06 ± 0.00	0.07 ± 0.00	0.07 ± 0.01	0.11 ± 0.01 [B,C]
Composite Indices					
Omega-3 Content (DHA+EPA)		15.5 ± 0.94	12.3 ± 0.59 [A]	37.9 ± 0.60 [A]	36.5 ± 0.59 [C]
Total Saturated		30.4 ± 0.41	31.0 ± 0.20	31.3 ± 0.16 [A]	31.4 ± 0.17
Total Moncunsaturated		1.33 ± 0.08	1.39 ± 0.04	1.36 ± 0.07	1.42 ± 0.07
n-3 Polyunsaturated		16.2 ± 0.96	12.9 ± 0.66 [A]	40.3 ± 0.58 [A]	38.9 ± 0.64 [C]
n-6 Polyunsaturated		39.8 ± 0.67	41.9 ± 0.36 [A]	17.0 ± 0.16 [A]	17.6 ± 0.48 [C]
n-3 : n-6		0.408 ± 0.030	0.309 ± 0.018	2.371 ± 0.040 [A]	2.227 ± 0.092 [C]
EPA : AA		0.001 ± 0.000	0.001 ± 0.000	0.124 ± 0.006 [A]	0.125 ± 0.010 [C]
EPA : DHA		0.000 ± 0.000	0.001 ± 0.000	0.013 ± 0.001 [A]	0.013 ± 0.001 [C]

[A] $P<0.05$ verus Control diet/sham; [B] $P<0.05$ verus Fish oil diet/sham; [C] $P<0.05$ verus Control diet/TAC. All measurements are means ± SEM, and significance was determined using the two-way ANOVA with post tukey test.

[a] Reported as percent of total fatty acid.

[b] Most common name given. Omega-9, omega-6 and omega-3 FAs are indicated as n9, n6, and n3, respectively

Table 3 Echocardiographic and Hemodynamic Assessment of Cardiac Structure and Function After 4 Weeks of TAC

	Control diet		Fish oil diet	
	Sham	**TAC**	**Sham**	**TAC**
	n=6	**n=9**	**n=6**	**n=9**
BW (g)	26 ± 0.5	27 ± 0.5	26 ± 0.5	26 ± 0.4
HW (mg)	133 ± 7	197 ± 12[A]	118 ± 2	160 ± 10[B,C]
HW/BW (mg/g)	5.1 ± 0.2	7.3 ± 0.4[A]	4.5 ± 0.1	6.0 ± 0.3[B,C]
Echocardiography	**n=6**	**n=9**	**n=6**	**n=9**
HR	610 ± 19	608 ± 22	640 ± 9	641 ± 10
IVSth (mm)	0.87 ± 0.03	1.21 ± 0.04[A]	0.88 ± 0.02	1.11 ± 0.05[B]
LVPWth (mm)	0.86 ± 0.01	1.27 ± 0.06[A]	0.86 ± 0.01	1.05 ± 0.03[B,C]
LVEDD (mm)	3.37 ± 0.08	3.79 ± 0.19[A]	3.31± 0.06	3.66 ± 0.09
LVESD (mm)	1.87 ± 0.11	2.76 ± 0.28[A]	1.62 ± 0.12	2.01 ± 0.11[C]
FS(%)	45 ± 2	28 ± 4[A]	51 ± 3	45 ± 3[C]
Hemodynamics	**n=6**	**n=8**	**n=6**	**n=8**
ESP (mmHg)	110 ± 2	186 ± 7[A]	118 ± 6	206 ± 9[B]
EDP (mmHg)	1.8 ± 1.6	13.4 ± 3.5[A]	2.0 ± 0.7	2.5 ± 0.9[C]
dP/dT max (mmHg/sec)	11979 ± 753	8485 ± 538[A]	11450 ± 897	12144 ± 631[C]
dP/dT min (mmHg/sec)	-11380 ± 1101	-8340 ± 549[A]	-11038 ± 764	-11088 ± 654[C]
Tau_w	3.9 ± 0.9	8.5 ±1.6[A]	7.0 ± 1.9	4.2 ± 0.9[C]
PG(mmHg)		67.2 ± 8.8		76.4 ± 12.0

[A]$P<0.05$ verus Control diet/sham; [B]$P<0.05$ verus Fish oil diet/sham; [C]$P<0.05$ verus Control diet/TAC. All measurements are means ± SEM, and significance was determined using the two-way ANOVA with post tukey test. BW, body weight; HW, heart weight; IVSth, intraventricular septal thickness; LVPWth, left ventricular posterior wall thickness; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; FS, fractional shortening; HR, heart rate; ESP, end-systolic pressure; EDP, end-diastolic pressure; PG, pressure gradient.

Example 2 Fish Oil Prevented Pressure Overload-Induced Cardiac Fibrosis in Mouse Left Ventricle

[0077] To determine if dietary supplementation with fish oil prevented pressure overload-induced cardiac fibrosis, left ventricle tissue cross sections were stained with picrosirius red to detect collagen distribution four weeks after surgery. In mice fed the control diet, aortic constriction induced significant fibrosis. The collagen content was increased 4.6-fold over sham (Figures 2A-B). This was accompanied by a significant increase in collagen I and III expression at both three and seven days following surgery (collagen I: 5.3-fold at three days, 4.2-fold at seven days; collagen III: 7.2-fold at three days, 8.0-fold at seven days) (Figures 2C-D). However, in mice fed the fish oil diet, fibrosis was increased only 2.0-fold over sham and there was no induction of collagen expression. Fish oil diet resulted in 63% less fibrosis compared to the mice fed the control diet (Figure 2B). Thus, fish oil feeding prevented fibrosis induced by pressure overload.

[0078] Aortic constriction induced a hypertrophic response as evidenced by a 42% increase in heart weight-to-body weight (HW/BW) ratio in mice fed the control diet and a 31% increase in HW/BW ratio in mice fed the fish oil diet (Figure 2E). See also Duda *et al.*, Cardiovasc Res. 2007, 76:303-310. However, after adjusting for the effect of fish oil, aortic constriction increased the HW/BW ratio by 1.9±0.3 in both control and fish oil groups (Figure 2E). Conversely, in mice fed the control diet, aortic constriction significantly induced expression of the hypertrophic marker genes atrial and brain natriuretic peptide (ANP and BNP, respectively), which was not observed in mice fed the fish oil diet (Figures 2F-G, the slight increase in ANP and BNP expression after constriction as compared to sham treatment is not statistically significant).

Example 3 Fish Oil Blocked Pressure Overload-Induced Non-Myocyte Proliferation and Myofibroblast Transformation

[0079] To determine how fish oil feeding affected cardiac fibroblast proliferation and transformation after aortic constriction, ventricular sections were stained for interstitial expression of Ki67 (excluding myocytes, which were counter-stained with phalloidin) as a marker of non-myocyte proliferation and stained for α-SMA as a marker of fibroblast

transformation (excluding the vascular staining) (Figures 3A-B). In mice fed the control diet, aortic constriction induced a significant increase in the number of interstitial Ki67 positive cells and a significant increase in α-SMA positive area, whereas dietary supplementation with fish oil prevented non-myocyte proliferation and fibroblast transformation (Figures 3C-D). These findings indicate that fish oil prevented fibroblast proliferation and transformation, which is consistent with the effect of fish oil on fibrosis and contractile dysfunction in this model.

Example 4 Fish Oil Did Not Block Pressure Overload-Induced TGF-β1 Production or Phosphorylation of Smad2

[0080] To determine if dietary supplementation with fish oil interfered with TGF-β1 signaling following aortic constriction, the levels of the active form of TGF-β1 were measured three days after TAC and the Smad2 phosphorylation levels were measured three days or four weeks after TAC. TGF-β1 levels were measured by ELISA (R&D Systems, Minneapolis, MN) as directed by the product insert. Absorbance was measured at 450 nm on a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA) and data collected using SoftMax (Molecular Devices) software for data analysis. Cyclic GMP levels were quantified using the acetylation protocol for a competitive Cyclic GMP EIA kit (Cayman Chemical, Ann Arbor, MI). All procedures were performed as directed in the product insert with modifications as indicated. Briefly, samples were diluted 1:1 with EIA buffer included in the kit. Absorbance was measured at 405 nm on a SpectraMax Plus 384 microplate reader (Molecular Devices) and data collected using SoftMax Pro 5.3 (Molecular Devices).

[0081] As shown in Figures 4A-C, aortic constriction increased TGF-β1 levels and Smad2 phosphorylation to a similar degree in both control and fish oil groups (Figures 4A-C). The Smad3 phosphorylation levels were not detected in the heart tissue at three days or four weeks post-TAC (data not shown). Therefore, dietary supplementation with fish oil did not interrupt TGF-β1 production and Smad2 phosphorylation induced by aortic constriction.

Example 5 EPA and DHA Inhibited TGF-β1-stimulated Fibrotic Response in Isolated Adult Mouse Cardiac Fibroblasts

[0082] To explain the anti-fibrotic effects of dietary fish oil observed *in vivo*, EPA- and DHA-mediated inhibition of pro-fibrotic TGF-β1 signaling was investigated in isolated adult mouse cardiac fibroblasts. EPA (10 μM) and DHA (10 μM) significantly increased the ω-3 content (EPA + DHA) in cardiac fibroblasts by 2.3-fold and 1.65-fold, respectively. Neither arachidonic acid (ω-6 PUFA) nor oleic acid (ω-9 PUFA) altered the ω-3 content (Figure 5A).

[0083] The effect of TGF-β1 on fibroblast proliferation and collagen synthesis was tested. Cardiac fibroblasts (3 x 10^5 cells/dish) were cultured and treated with TGF-β1 as above. After 48 hours, fibroblasts were harvested by trypsinization and the cell number was determined by trypan blue exclusion. Collagen synthesis was measured by incorporation of [^{3}H]-proline. Cardiac fibroblasts were cultured in 24-well plates (2.5 x 10^4 cells/well), and after 24 hours of culture in reduced serum medium, fibroblasts were treated with TGF-β1 (1ng/ml) to induce collagen synthesis. After 8 hours, [^{3}H]-proline (1 μCi/ml, Perkin Elmer, Waltham, MA) was added. After 40 hours, fibroblasts were rinsed three times with PBS and fixed with ice cold 10% TCA for 30 min. Cell precipitates were washed with phosphate-buffered saline and solubilized in 0.2 N NaOH at room temperature for one hour. Incorporation of [^{3}H]-proline was determined by liquid scintillation spectrometry (Beckman LS 6500, Fullerton, CA).

[0084] As shown in Figures 5B-E, TGF-β1 (1 ng/ml) significantly increased proliferation of cardiac fibroblasts by 142% (P<0.01, Figure 5B), collagen synthesis by 190% (P<0.01, Figure 5C), and myofibroblast transformation by 66% (P<0.01, Figures 5D-E). In the absence of TGF-β1, EPA and DHA at 10 μM had no effect on these parameters. However, both EPA and DHA (10 μM) blocked TGF-β1-induced cardiac fibroblast proliferation, prevented collagen synthesis, and significantly decreased myofibroblast transformation (all P<0.01 vs. TGF-β1-treated cardiac fibroblasts, Figures 5B-E). Other fatty acids, arachidonic acid (10 μM) and oleic acid (10 μM), did not significantly affect TGF-β1-induced proliferation and collagen synthesis in cardiac fibroblasts (Figures 5B-C). These results demonstrate that the anti-fibrotic effects of ω-3 PUFAs were mediated by suppression of TGF-β1-induced fibrosis in cardiac fibroblasts.

Example 6 EPA and DHA Inhibited TGF-β1-stimulated Fibrotic Response Through the cGMP/PKG Pathway

[0085] The cGMP/PKG signaling pathway plays a counter-regulatory role against TGF-β1-induced cardiac fibrosis. To determine whether ω-3 PUFAs would activate cGMP/PKG signaling and block pro-fibrotic TGF-β1 signaling, cultured cardiac fibroblasts were treated with either EPA or DHA (10-50 μM). As shown in Figure 6A, both EPA and DHA induced dose-dependent increases in cGMP production (all $P<0.01$), but neither of the control fatty acids, arachidonic acid and oleic acid (10 or 20 μM), was able to induce cGMP production (Figure 6A). Furthermore, EPA (10 μM) and DHA (10 μM) as well as 8-bromo-cGMP (1 mM), which activates PKG (protein kinas G), blocked the TGF-β1-induced collagen synthesis (Figure 6B). Conversely, a peptide-based inhibitor of PKG, DT-3 (1 μM) significantly diminished the anti-fibrotic effect of EPA, DHA and cGMP (Figure 6B). In summary, these results indicate that ω-3 PUFAs inhibited pro-fibrotic TGF-β1 signaling likely by at least inducing cGMP/PKG signaling.

[0086] Smad2 and Smad3 phosphorylation and subsequent translocation to the nucleus are required for TGF-β1 signaling. Since EPA and DHA demonstrated an inhibitory effect on TGF-β1-induced fibrosis (Figure 5B-E), the effects of EPA and DHA on the TGF-β1-induced phosphorylation of Smad2 and Smad3 were tested. In cultured cardiac fibroblasts, TGF-β1 treatment for 30 minutes induced robust phosphorylation of Smad2 (2.7 fold, $P<0.01$) and Smad3 (2.2 fold, $P<0.01$) (Figures 6C-E). Longer exposure (24 hours) to TGF-β1 induced phosphorylation of Smad2 but not Smad3 (Figure 6C-E). However, EPA and DHA (10 μM) failed to block TGF-β1-induced phosphorylation of Smad2 and Smad3 (Figure 6C-E) at either 30 minutes or 24 hours, which was consistent with the finding that fish oil did not inhibit TAC-induced phosphorylation of Smad2 *in vivo* (Figures 4B-C). Thus, the inhibitory effect of EPA and DHA on TGF-β1-induced cardiac fibrosis was not likely due to inhibition of Smad phosphorylation.

Example 7 EPA and DHA Blocked Nuclear Translocation of Phosphorylated Smad2 and Smad3 Through the cGMP/PKG Pathway

[0087] Since TGF-β1-induced phosphorylation of Smads was not blocked by DHA and EPA, the ability of EPA and DHA to block TGF-β1-induced nuclear translocation of phospho-Smad2 and -Smad3 was analyzed. In cultured cardiac fibroblasts, TGF-β1 increased the nuclear localization of phospho-Smad2 (66%, $P<0.01$) and phospho-Smad3 (83%, $P<0.01$)

(Figure 7). TGF-β1-induced nuclear translocation of both Smads was significantly blocked by EPA and DHA (phospho-Smad2: reduced to 15% with DHA; to 13% with EPA, and phospho-Smad3: reduced to 14% with DHA; to 13% with EPA) (Figure 7). Furthermore, 8-bromo-cGMP (1 mM) blocked TGF-β1-induced translocation of both phospho-Smad2 and -Smad3, whereas DT-3 reversed the effects of EPA and DHA on nuclear translocation of Smads in cardiac fibroblasts treated with TGF-β1 (Figure 7). Therefore, EPA and DHA inhibition of TGF-β1-induced fibrosis was likely mediated by blockage of phospho-Smad nuclear translocation.

Example 8 EPA and DHA Increased NOx Production, Phospho-eNOS and eNOS Expression in Cardiac Fibroblasts

[0088] Omega-3 PUFAs increase nitric oxide (NO) synthesis in humans. Nitric oxide increases cGMP production through activation of soluble guanylyl cyclase (sGC). See Ritchie *et al.*, Pharmacol Ther. 2009, 124:279-300, Tsai *et al.*, Pharmacol Ther. 2009,122:216-238, Harris et al., Am J Clin Nutr. 1997, 65:459-464). Thus, whether EPA and DHA increase NO production in cardiac fibroblasts was tested. Because NO is rapidly metabolized, total intracellular levels of nitrite (NO_2^-) and nitrate (NO_3^-), a stable NO metabolites, were measured as an index of total NO production. Twenty-four hours of treatment with EPA and DHA (10 μM) significantly increased nitrite and nitrate levels (NOx) in cardiac fibroblasts (70% in EPA and 65% in DHA). Whether NO production was induced with an increase in intracellular levels of cGMP was also analyzed. As expected, the cGMP analog, 8-Br-cGMP, (0.1 and 1 mM) did not induce NO production in cardiac fibroblasts (Figure 8A). This demonstrates that the increase of NO was not a result of increased intracellular cGMP.

[0089] To determine if EPA and DHA enhance expression of phospho-eNOS (endothelial nitric oxide synthase that generates NO in blood vessels) and eNOS, phospho-eNOS and eNOS protein levels in cardiac fibroblasts were analyzed. Treatment with 10 μM DHA or EPA significantly increased phospho-eNOS and eNOS protein levels in cardiac fibroblasts (Figures 8B-D).

[0090] To examine other possible mechanisms involved in cGMP production and degradation, the effects of EPA and DHA on particulate guanylyl cyclase activity, cGMP-specific phosphodiesterase activity, and mRNA levels of ANP and BNP were analyzed in

cardiac fibroblasts. In the cell, NO-sensitive soluble guanylyl cyclase and natriuretic peptide-sensitive particulate guanylyl cyclase produce cGMP, whereas cGMP-specific phosphodiesterases hydrolyze cGMP. As shown above, DHA increases NO synthesis and phospho-eNOS and eNOS expression levels (see also Stebbins *et al.*, J Cardiovasc Pharmacol Ther. 2008, 13:261-268). The results presented above also show that EPA and DHA did not increase particulate guanylyl cyclase activity or ANP/BNP production. Additionally, EPA and DHA did not inhibit cGMP-specific phosphodiesterase activity. EPA (10 μM) and DHA (10 μM) did not increase particulate guanylyl cyclase activity and did not inhibit cGMP-specific phosphodiesterase activity (data not shown). In addition, EPA (10 μM) and DHA (10 μM) significantly decreased ANP mRNA and did not change the BNP mRNA in cardiac fibroblasts (data not shown).

[0091] In conclusion, the results in Figure 8 show that EPA and DHA increased phospho-eNOS and eNOS expression, thereby promoting NO production in cardiac fibroblasts.

Example 9 Omega-3 PUFAs Reduced Smad-responsive Promoter Activity *in vivo* and *in vitro*

[0092] The effect of omega-3 PUFAs on Smad-responsive promoter activity was examined by measuring the mRNA levels of a Smad-responsive gene PAI-1 (plasminogen activator inhibitor type 1), and by measuring the luciferase activity in a Smad-responsive luciferase assay. As shown in Figure 9A, mice fed with the control diet exhibited increased PAI-1 mRNA levels after TAC, whereas the effect of TAC on Smad-responsive PAI-1 mRNA expression was not detected in mice fed with fish oil.

[0093] TGF-β1-induced Smad-responsive promoter activity was also determined by measuring Smad-induced luciferase activity. Passage one cardiac fibroblasts were seeded into a 96-well tissue culture plate (5×10^3 cells/well) and were transduced with commercially available lentivirus particles (SABiosciences, Frederick, MD) expressing the firefly luciferase gene under the control of a minimal (m) CMV promoter and tandem repeats of the SMAD transcriptional response element (AGCCAGACA). After 24 hours of culture in reduced serum medium, fibroblasts were pretreated with vehicle or fatty acids for 24 hours, followed by TGF-β1 (1 ng/ml) treatment for 48 hours. Fibroblasts were rinsed with PBS and lysed prior to measurement of luciferase activity using the Luciferase Assay System provided by Promega (Madison, WI) and a luminometer (Zylux, Huntsville, AL).

[0094] As shown in Figure 9B, TGF-β1 increased the luciferase activity in the transfected cardiac fibroblasts, and 24 hr pretreatment of DHA or EPA prevented TGF-β1-induced luciferase activity. In conclusion, ω-3 PUFAs inhibited TGF-β1-induced Smad-responsive transcriptional activity.

Example 10 EPA and DHA Did Not Block TGF-β1-induced Activation of ERK1/2

[0095] TGF-β1-induced phosphorylation of ERK1/2 is also involved in the TGF-β1-induced fibrotic response in fibroblasts (Liu et al., Mol Pharmacol. 2006, 70:1992-2003). Thus, the effect of omega-3 PUFAs on the TGF-β1-induced phosphorylation of ERK1/2 was tested in cardiac fibroblasts after one hour treatment of TGF-β1 with or without pretreatment of EPA and DHA. TGF-β1 (1 ng/ml) induced significant phosphorylation of ERK1/2 in cardiac fibroblasts whereas EPA and DHA did not block this effect (Figure 10A-B).

Example 11 DHA, But Not EPA, AA or OA Reduced Collagen Synthesis

[0096] TGF-β1-induced collagen synthesis was measured in the absence of exogenous FAs (no FA), in the presence of oleic acid (OA, 10 μM), arachidonic acid (AA, 10 μM), EPA (10 μM), or DHA (10 μM) and treated for a final 24 hr with TGF-β1 (1 ng/ml) and incubation with [^{3}H]-proline. To establish the potential for mediation by $CYP_{epoxygenase}$, adult mouse cardiac fibroblasts were incubated with PPOH, a partial inhibitor of the epoxygenase. As shown in Figure 11, only DHA reduced collagen synthesis by 87% (90, 82; geometric SE) in mouse cardiac fibroblasts. Further, incubation with PPOH resulted in a 133% (73, 215; geometric SE) increase in collagen synthesis, indicating that the effect of DHA on inhibiting collagen production was mediated by $CYP_{epoxygenase.}$

[0097] It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

WHAT IS CLAIMED IS:

1. A method for treating or limiting development of heart failure with preserved ejection fraction (HFPEF), comprising administering to a patient having or at risk of developing HFPEF an amount effective of a pharmaceutical composition comprising docosahexaenoic acid (DHA) or pharmaceutically acceptable salts, esters, amides, epoxides, and prodrugs thereof, to treat or limit development of HFPEF.

2. The method of claim 1, wherein the pharmaceutical composition does not include any omega-3 fatty acid therapeutic other than DHA or a pharmaceutically acceptable salt, ester, amide, epoxide, or prodrug thereof.

3. The method of claim 1 or 2, wherein the pharmaceutical composition does not include any fatty acid therapeutic other than DHA or a pharmaceutically acceptable salt, ester, amide, epoxide, or prodrug thereof.

4. The method of any one of claims 1-3, wherein the pharmaceutical composition comprises purified or synthesized DHA.

5. The method of any one of claims 1-4, wherein DHA is administered to the patient at a concentration of from about 5 mg/kg of body weight/day to about 50 mg/kg of body weight/day.

6. The method of any one of claims 1-5, wherein the pharmaceutical composition comprises DHA epoxide.

7. The method of any one of claims 1-6, wherein the pharmaceutical composition comprises 19(20) DHA epoxide.

8. The method of any one of claims 1-7 wherein the patient is normotensive or hypotensive.

9. The method of any one of claims 1-8, wherein the method is for limiting development of HFPEF.

10. The method of any one of claims 1-8, wherein the method is for treating HFPEF.

11. A method of treating or limiting development of fibrosis comprising administering to a patient having or at risk of developing fibrosis an amount effective of a pharmaceutical composition comprising docosahexaenoic acid (DHA) or pharmaceutically acceptable salts, esters, amides, epoxides, and prodrugs thereof, to treat or limit development of fibrosis.

12. The method of claim 11, wherein the pharmaceutical composition does not include any omega-3 fatty acid therapeutic other than DHA or a pharmaceutically acceptable salt, ester, amide, epoxide, or prodrug thereof.

13. The method of claim 11 or 12, wherein the pharmaceutical composition does not include any fatty acid therapeutic other than DHA or a pharmaceutically acceptable salt, ester, amide, epoxide, or prodrug thereof.

14. The method of any one of claims 11-13, wherein the pharmaceutical composition comprises purified or synthesized DHA.

15. The method of any one of claims 11-14, wherein DHA is administered to the patient at a concentration of from about 5 mg/kg of body weight/day to about 50 mg/kg of body weight/day.

16. The method of any one of claims 11-15, wherein the pharmaceutical composition comprises DHA epoxide.

17. The method of any one of claims 11-16, wherein the pharmaceutical composition comprises 19(20) DHA epoxide.

18. The method of any one of claims 11-17, wherein the patient is normotensive or hypotensive.

19. The method of any one of claims 11-18, wherein the fibrosis is liver fibrosis, kidney fibrosis, cardiac fibrosis or lung fibrosis.

20. The method of any one of claims 11-19, wherein the method is for limiting development of fibrosis.

21. The method of any one of claims 11-20, wherein the fibrosis is cardiac fibrosis.

22. The method of claim 21, wherein the cardiac fibrosis is fibrosis in the ventricle.

23. The method of any one of claims 11-19, wherein the method is for treating fibrosis.

24. The method of claim 23, wherein the fibrosis is cardiac fibrosis.

25. The method of claim 24, wherein the cardiac fibrosis is fibrosis in the ventricle.

Figure 1

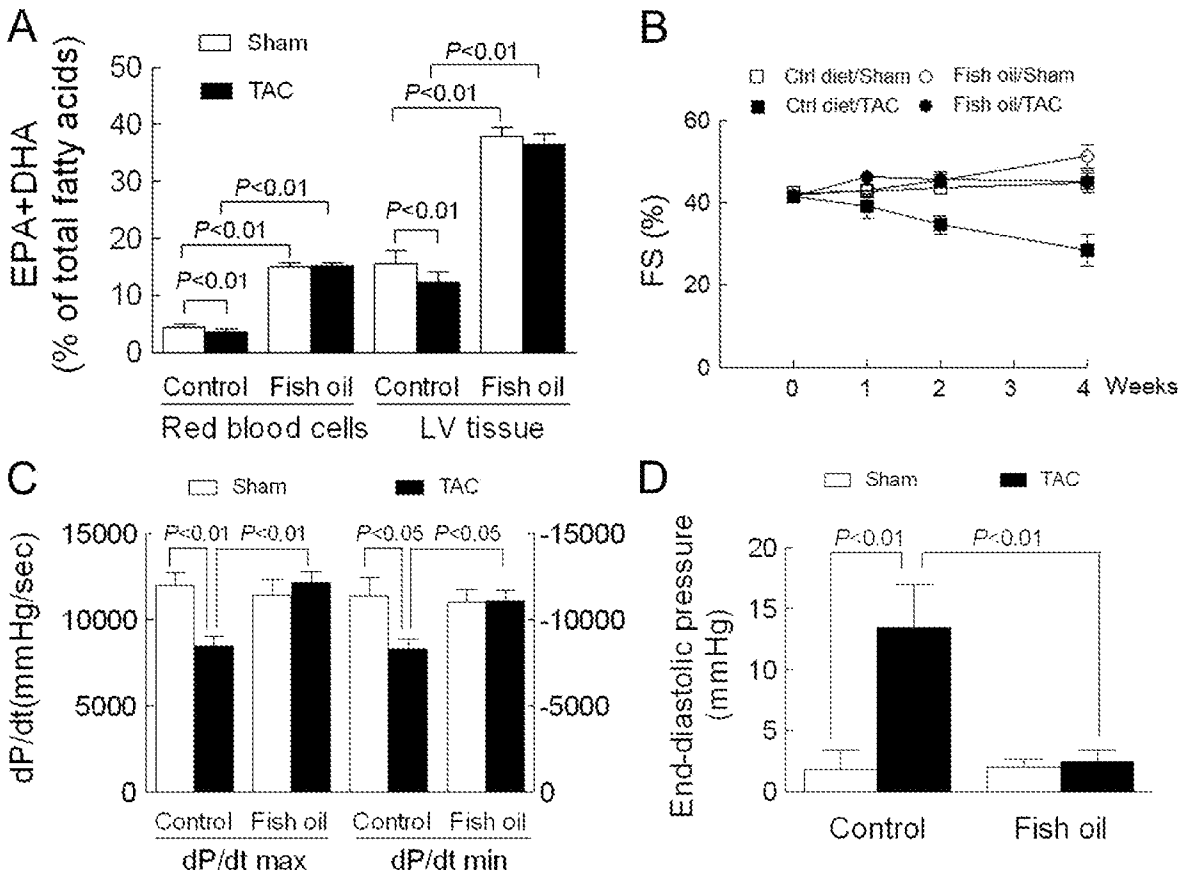

A
EPA+DHA (% of total fatty acids)
Sham
TAC
P<0.01
Control
Fish oil
Red blood cells
LV tissue
B
Ctrl diet/Sham
Fish oil/Sham
Ctrl diet/TAC
Fish oil/TAC
FS (%)
Weeks
C
dP/dt(mmHg/sec)
P<0.05
dP/dt max
dP/dt min
D
End-diastolic pressure (mmHg)

Figure 2

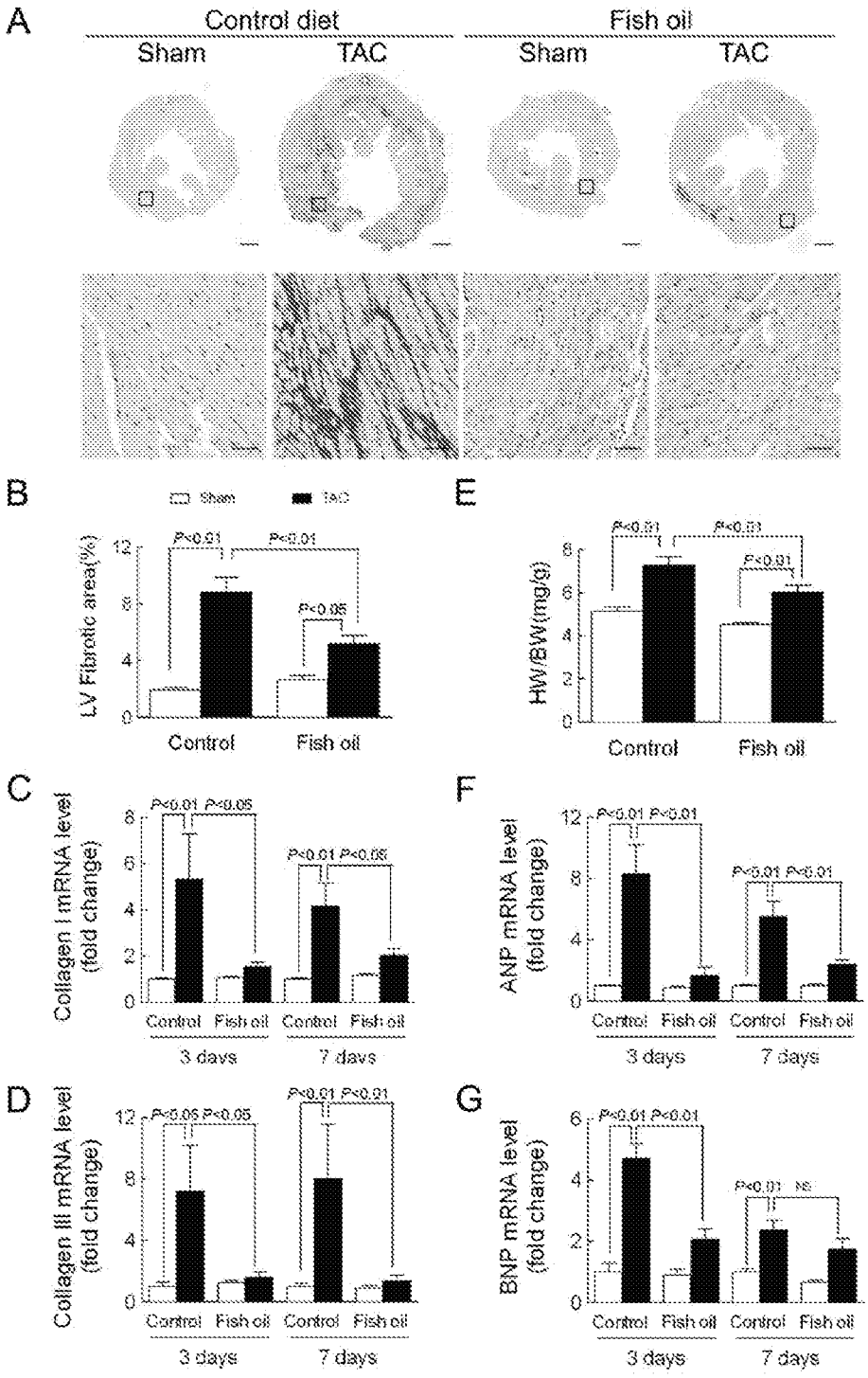

A
Control diet
Fish oil
Sham
TAC
Sham
TAC
B
Sham
TAC
LV Fibrotic area(%)
P<0.01
P<0.01
P<0.05
Control
Fish oil
E
HW/BW(mg/g)
P<0.01
P<0.01
P<0.01
Control
Fish oil
C
Collagen I mRNA level (fold change)
P<0.01 P<0.05
P<0.01 P<0.05
Control Fish oil Control Fish oil
3 days
7 days
F
ANP mRNA level (fold change)
P<0.01 P<0.01
P<0.01 P<0.01
Control Fish oil Control Fish oil
3 days
7 days
D
Collagen III mRNA level (fold change)
P<0.05 P<0.05
P<0.01 P<0.01
Control Fish oil Control Fish oil
3 days
7 days
G
BNP mRNA level (fold change)
P<0.01 P<0.01
P<0.01 NS
Control Fish oil Control Fish oil
3 days
7 days

Figure 3

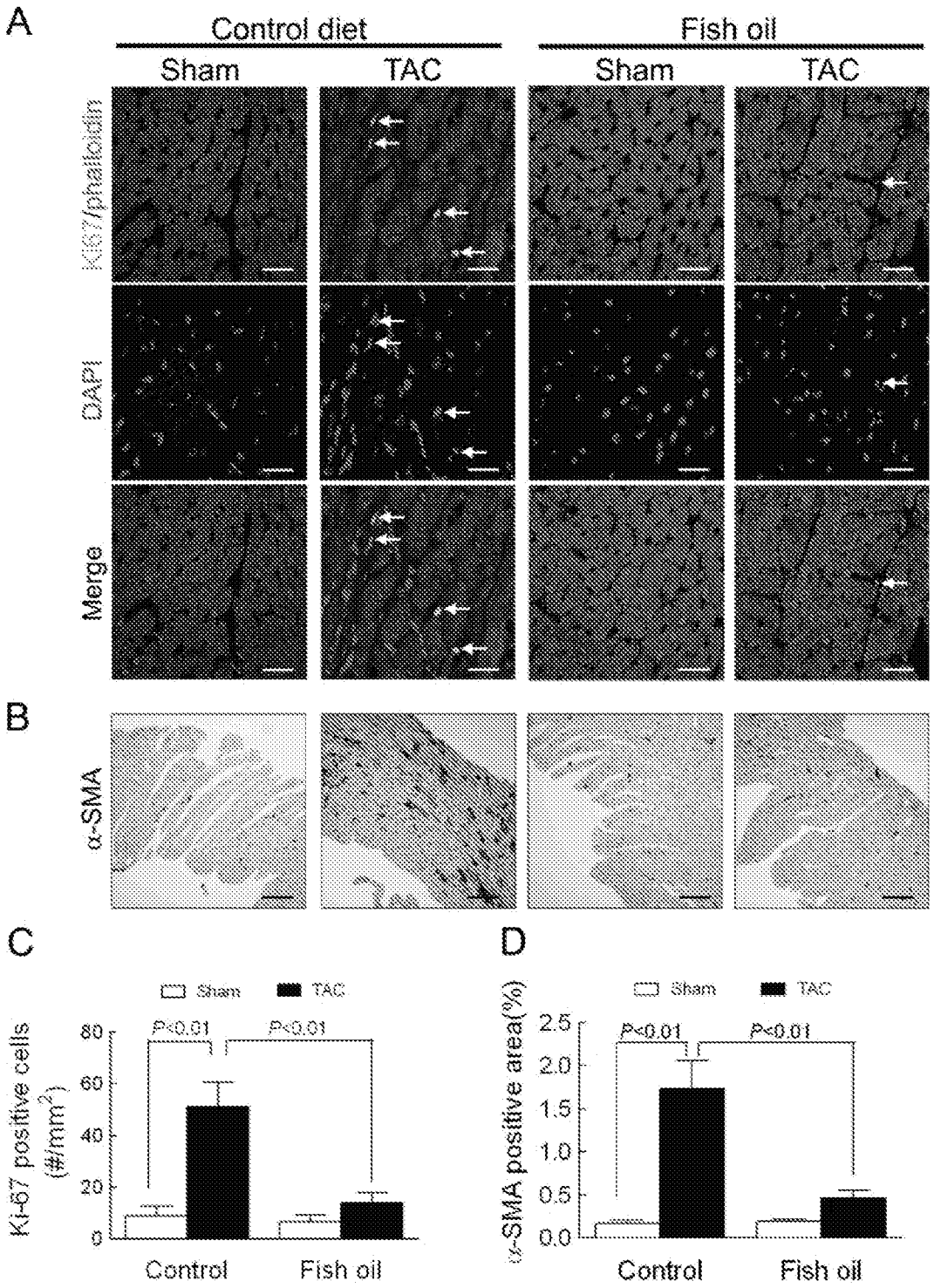

A
Control diet
Fish oil
Sham
TAC
Sham
TAC
Ki67/phalloidin
DAPI
Merge
B
α-SMA
C
Sham
TAC
P<0.01
P<0.01
Ki-67 positive cells (#/mm2)
80
60
40
20
0
Control
Fish oil
D
Sham
TAC
P<0.01
P<0.01
α-SMA positive area(%)
2.5
2.0
1.5
1.0
0.5
0.0
Control
Fish oil

Figure 4

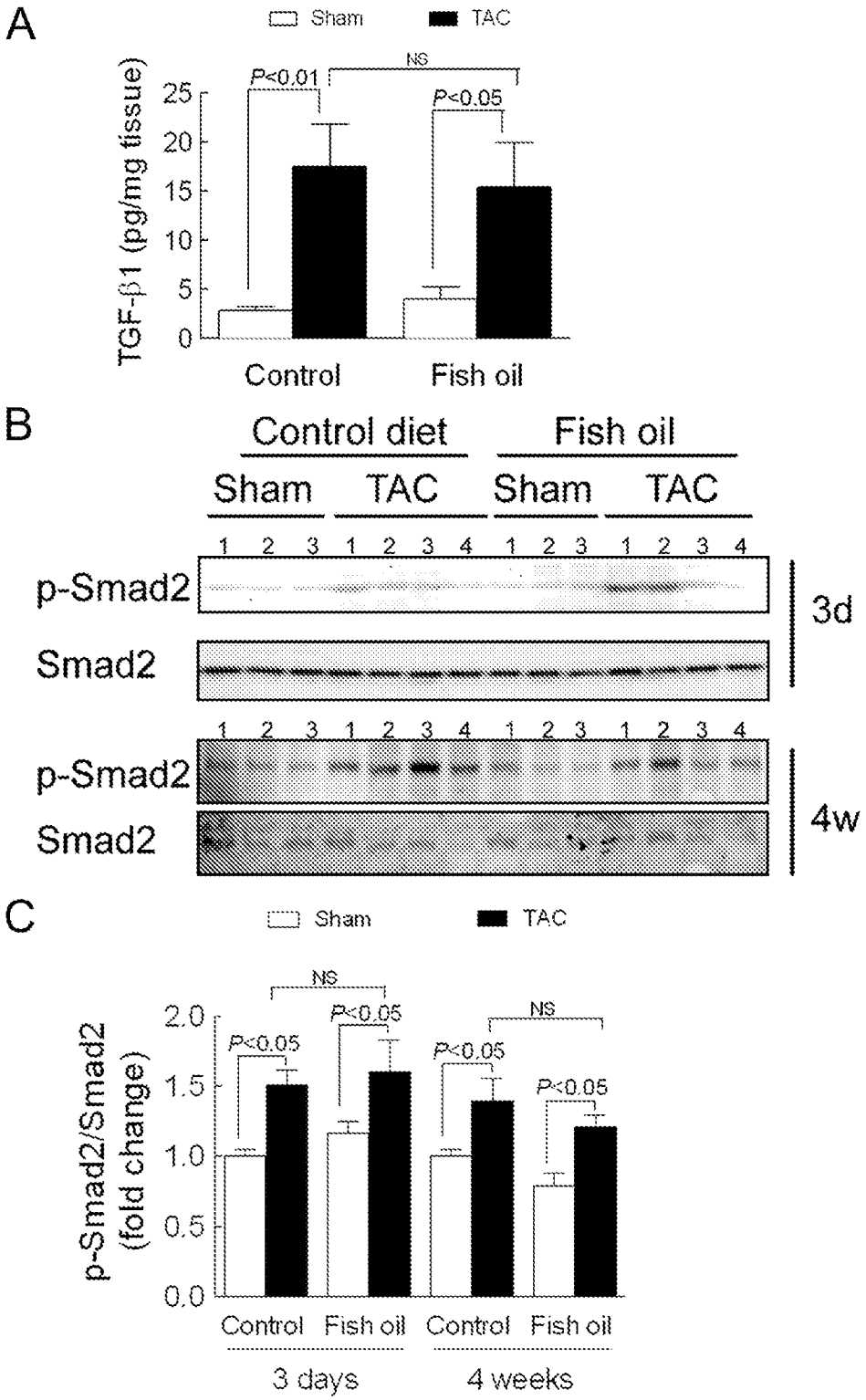


Figure 5

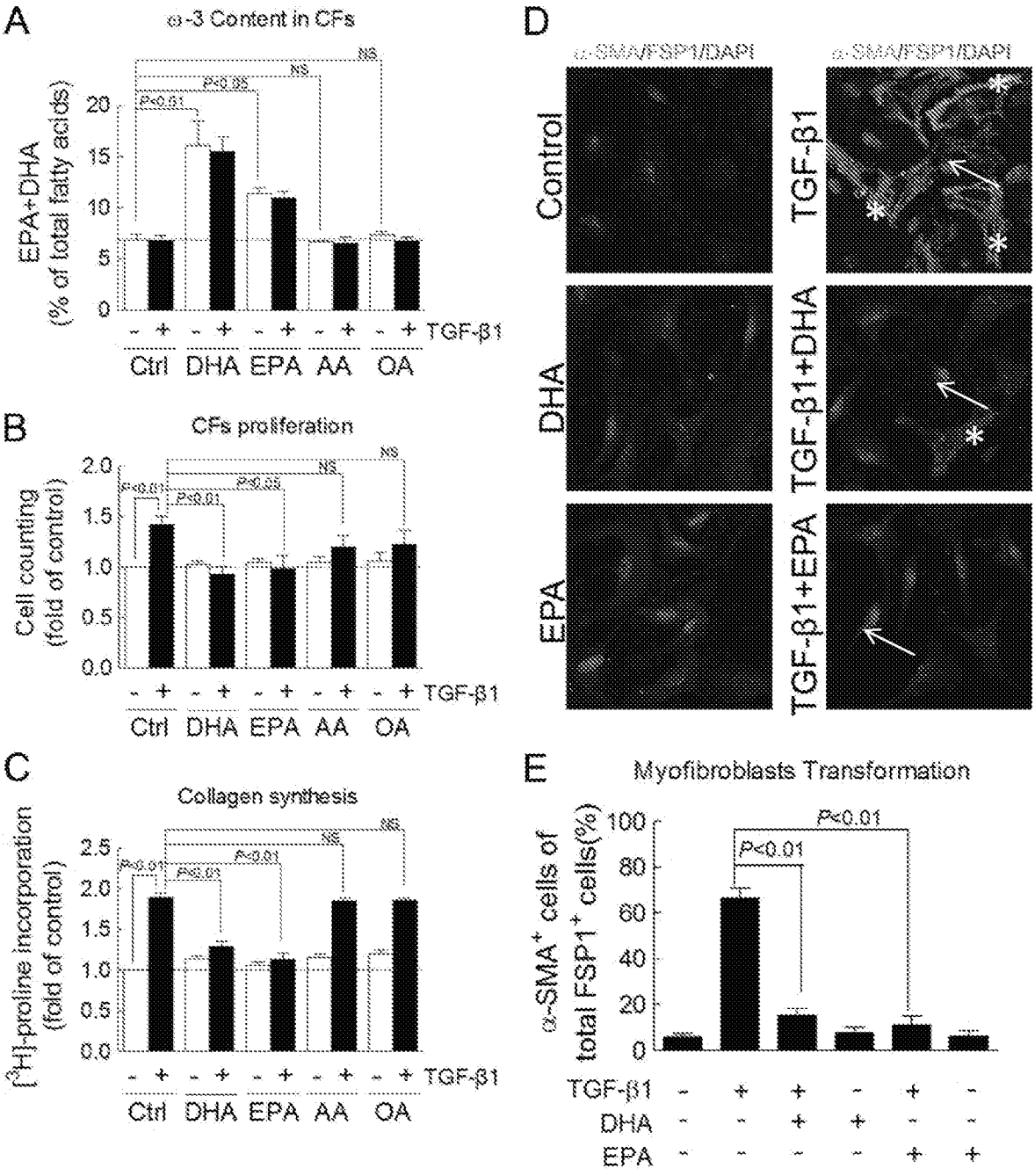

A
ω-3 Content in CFs
EPA+DHA (% of total fatty acids)
NS
P<0.05
P<0.01
TGF-β1
Ctrl DHA EPA AA OA
B
CFs proliferation
Cell counting (fold of control)
C
Collagen synthesis
[3H]-proline incorporation (fold of control)
D
α-SMA/FSP1/DAPI
Control
TGF-β1
DHA
TGF-β1+DHA
EPA
TGF-β1+EPA
E
Myofibroblasts Transformation
α-SMA+ cells of total FSP1+ cells(%)
TGF-β1
DHA
EPA

Figure 6

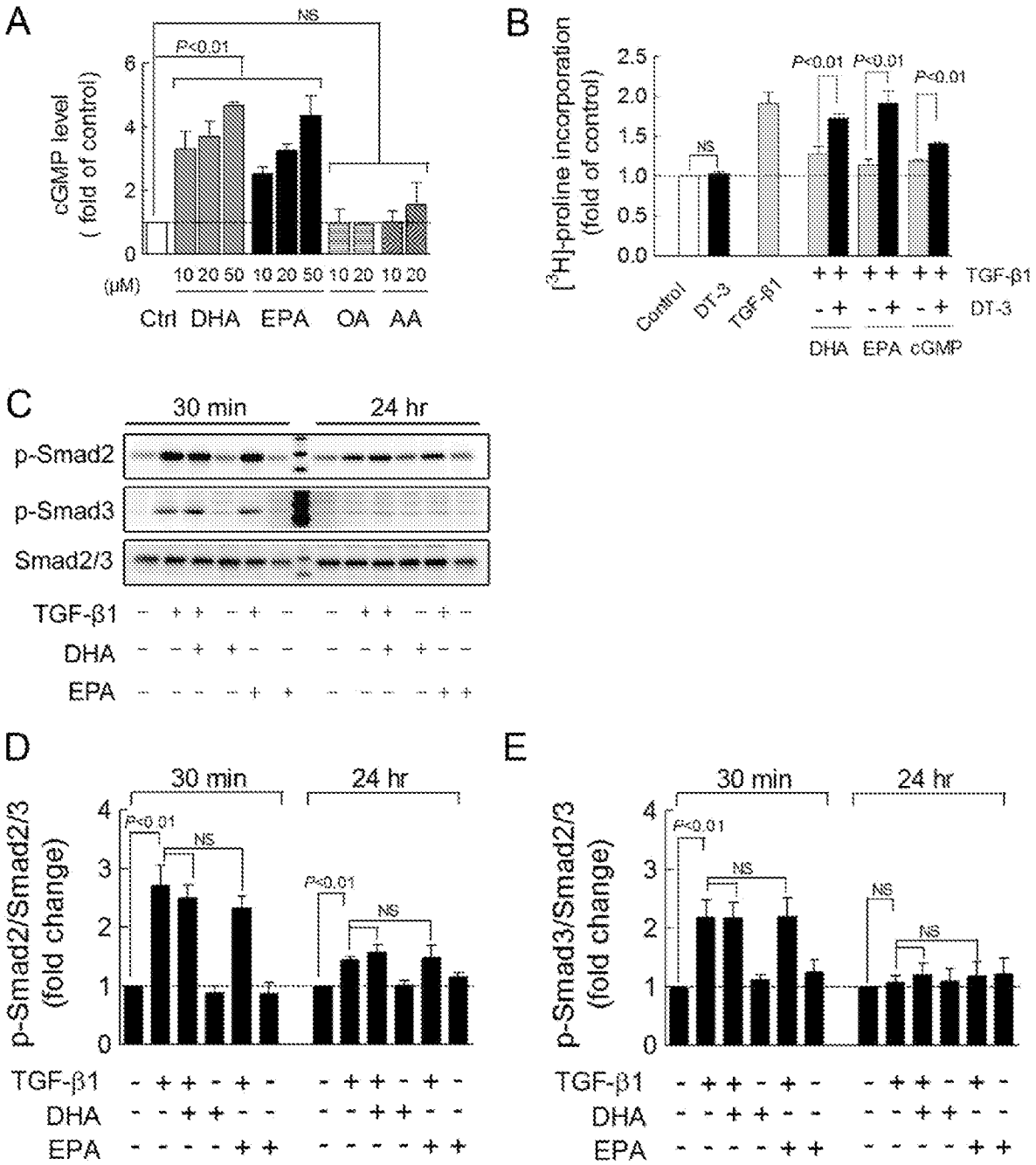

A
cGMP level (fold of control)
NS
P<0.01
(μM)
10 20 50 10 20 50 10 20 10 20
Ctrl DHA EPA OA AA
B
[3H]-proline incorporation (fold of control)
P<0.01 P<0.01 P<0.01
NS
Control DT-3 TGF-β1
TGF-β1
DT-3
DHA EPA cGMP
C
30 min 24 hr
p-Smad2
p-Smad3
Smad2/3
TGF-β1
DHA
EPA
D
p-Smad2/Smad2/3 (fold change)
30 min 24 hr
P<0.01 NS P<0.01 NS
TGF-β1
DHA
EPA
E
p-Smad3/Smad2/3 (fold change)
30 min 24 hr
P<0.01 NS NS NS
TGF-β1
DHA
EPA

Figure 7

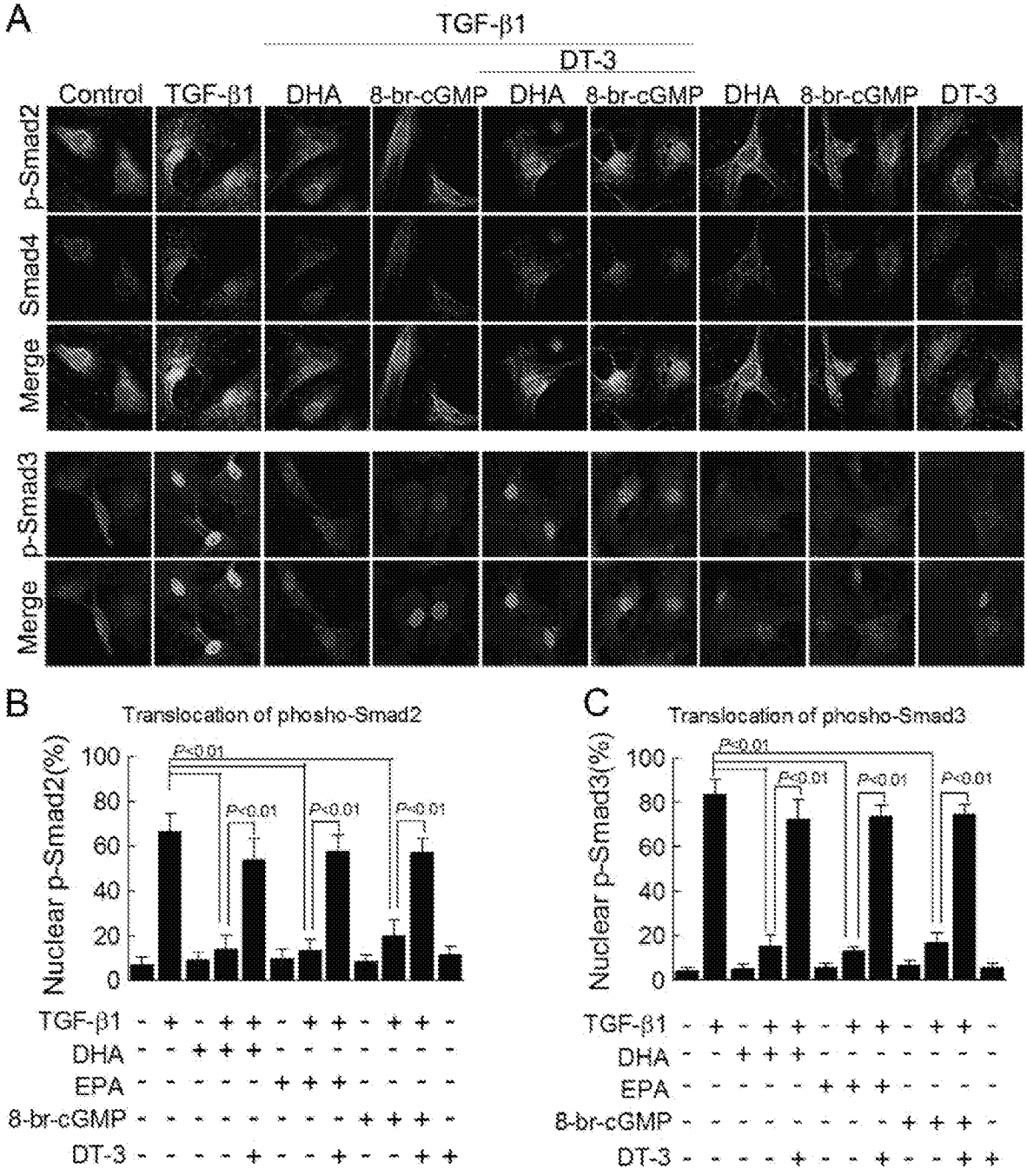

A
TGF-β1
DT-3
Control
TGF-β1
DHA
8-br-cGMP
DHA
8-br-cGMP
DHA
8-br-cGMP
DT-3
p-Smad2
Smad4
Merge
p-Smad3
Merge
B
Translocation of phosho-Smad2
Nuclear p-Smad2(%)
100
80
60
40
20
0
P<0.01
P<0.01
P<0.01
P<0.01
TGF-β1 - + - + + - + + - + + -
DHA - - + + + - - - - - - -
EPA - - - - - + + + - - - -
8-br-cGMP - - - - - - - - + + + -
DT-3 - - - - + - - + - - + +
C
Translocation of phosho-Smad3
Nuclear p-Smad3(%)
100
80
60
40
20
0
P<0.01
P<0.01
P<0.01
P<0.01
TGF-β1 - + - + + - + + - + + -
DHA - - + + + - - - - - - -
EPA - - - - - + + + - - - -
8-br-cGMP - - - - - - - - + + + -
DT-3 - - - - + - - + - - + +

Figure 8

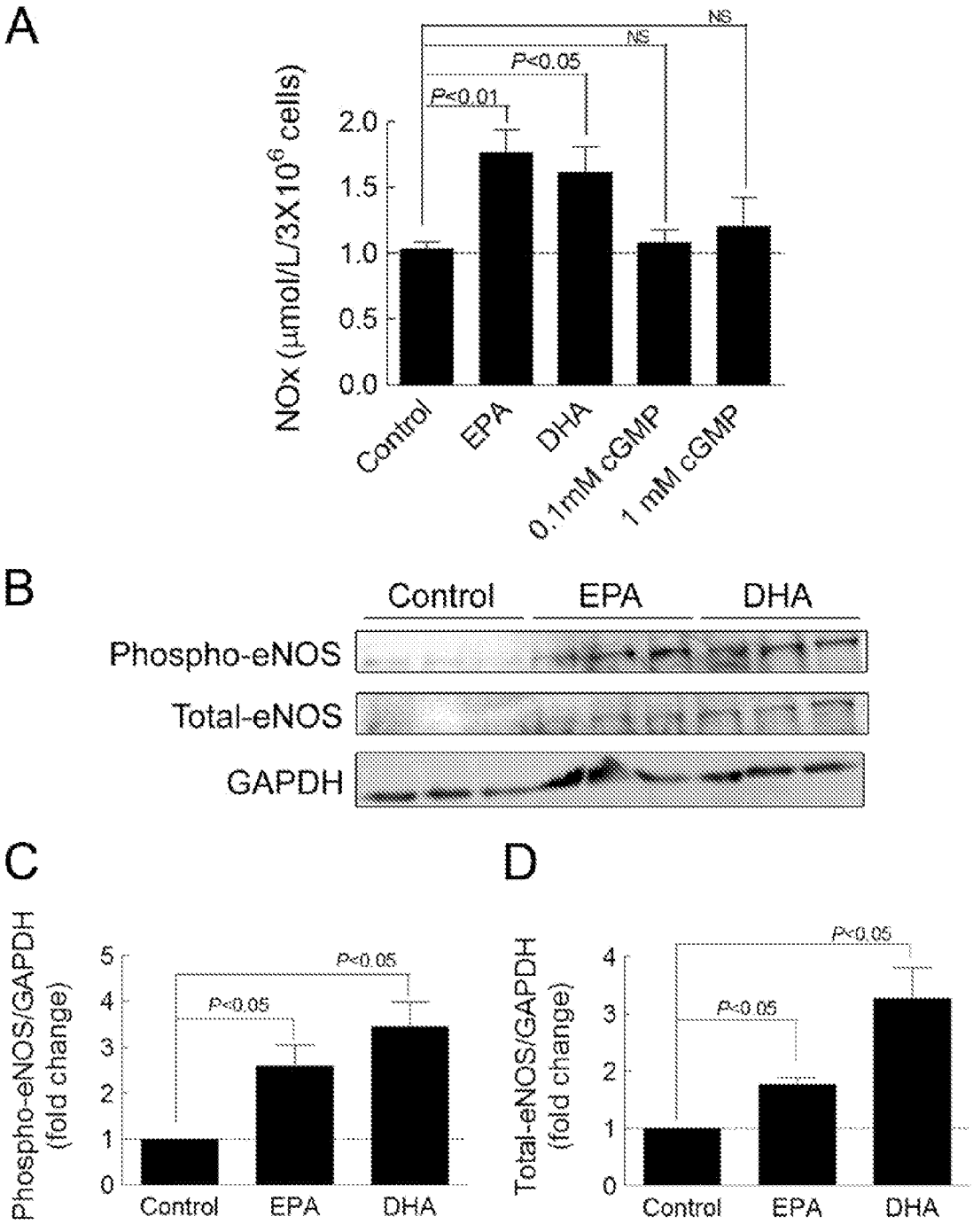
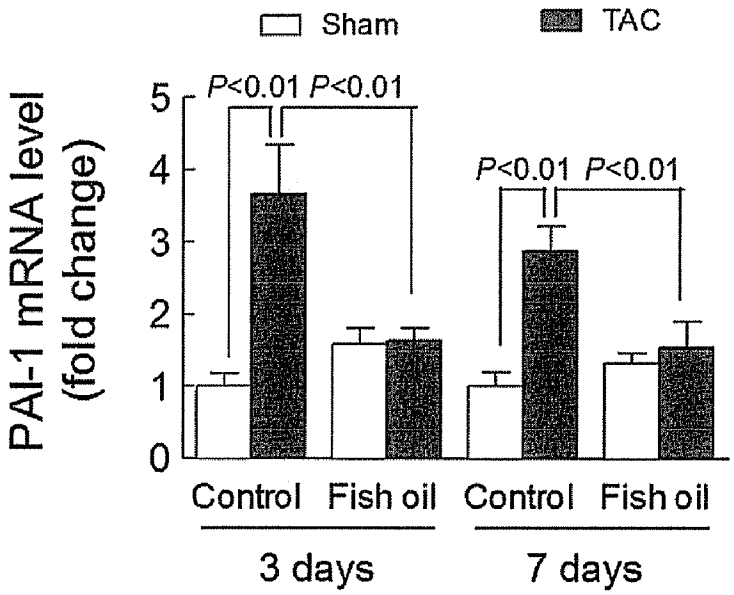

A
NS
NS
P<0.05
P<0.01
NOx (μmol/L/3X10^6 cells)
2.0
1.5
1.0
0.5
0.0
Control
EPA
DHA
0.1mM cGMP
1 mM cGMP
B
Control
EPA
DHA
Phospho-eNOS
Total-eNOS
GAPDH
C
Phospho-eNOS/GAPDH (fold change)
P<0.05
P<0.05
5
4
3
2
1
0
Control
EPA
DHA
D
Total-eNOS/GAPDH (fold change)
P<0.05
P<0.05
4
3
2
1
0
Control
EPA
DHA

Figure 9

A


Sham
TAC
PAI-1 mRNA level (fold change)
P<0.01 P<0.01
P<0.01 P<0.01
0
1
2
3
4
5
Control
Fish oil
Control
Fish oil
3 days
7 days

B

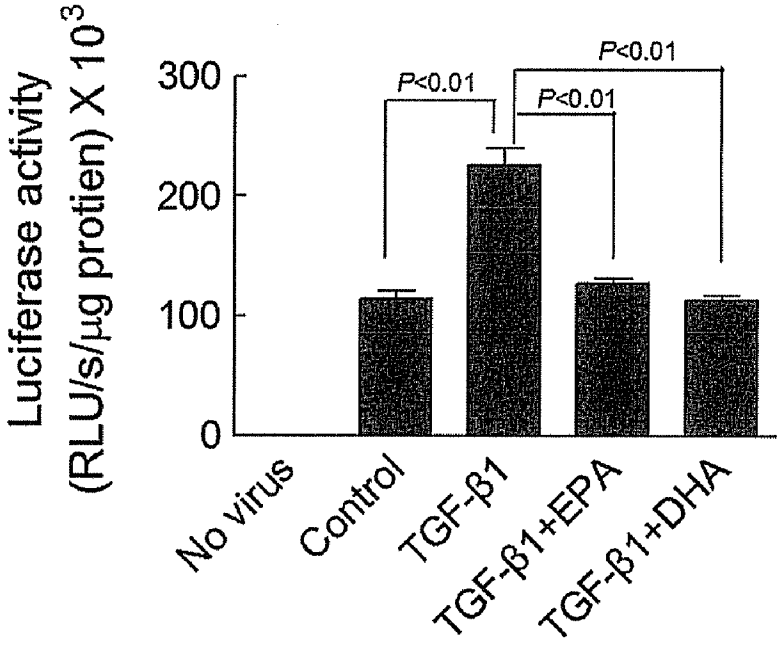
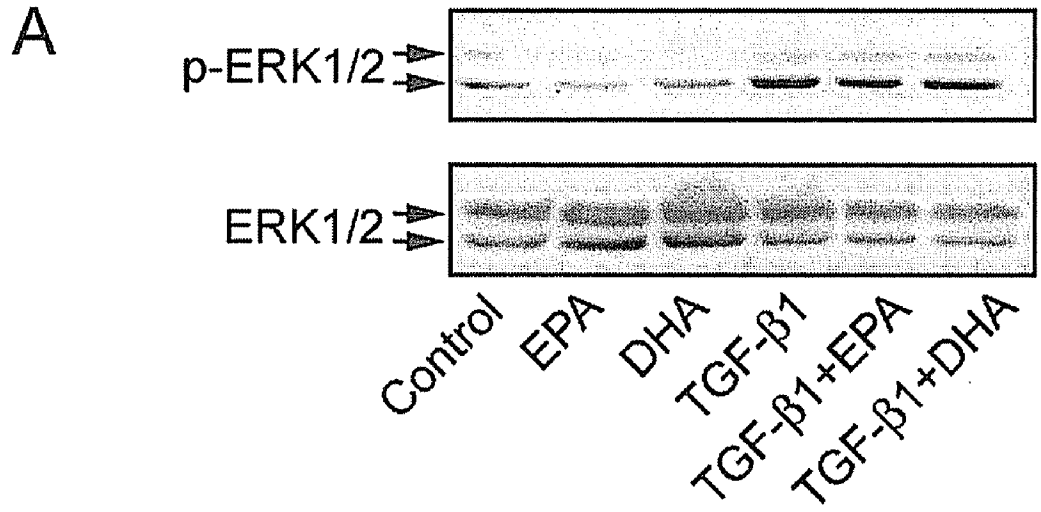

Luciferase activity (RLU/s/μg protien) X 10^3
P<0.01
P<0.01
P<0.01
0
100
200
300
No virus
Control
TGF-β1
TGF-β1+EPA
TGF-β1+DHA

Figure 10


A
p-ERK1/2
ERK1/2
Control
EPA
DHA
TGF-β1
TGF-β1+EPA
TGF-β1+DHA

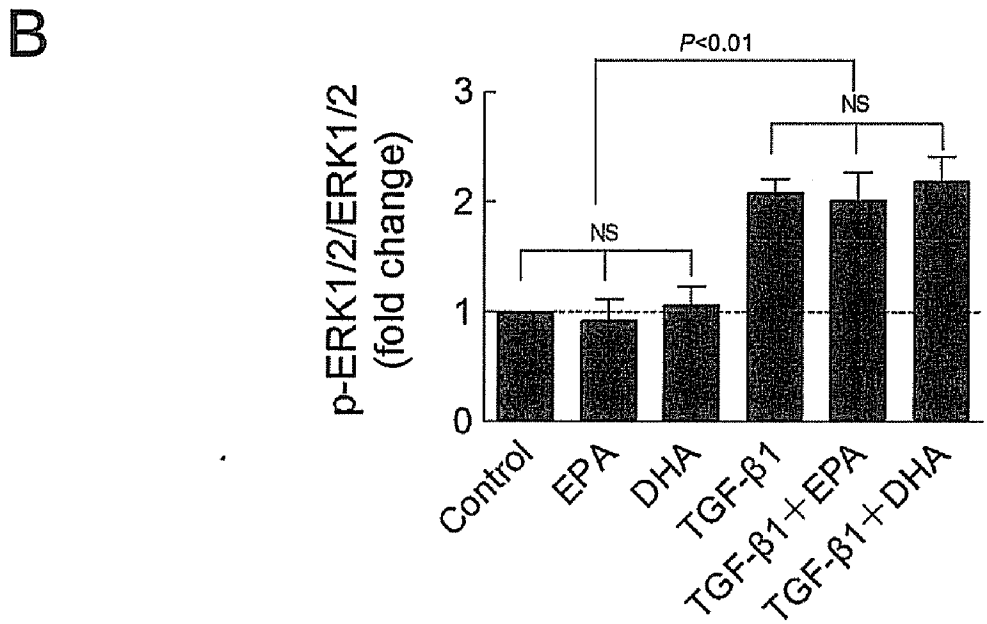
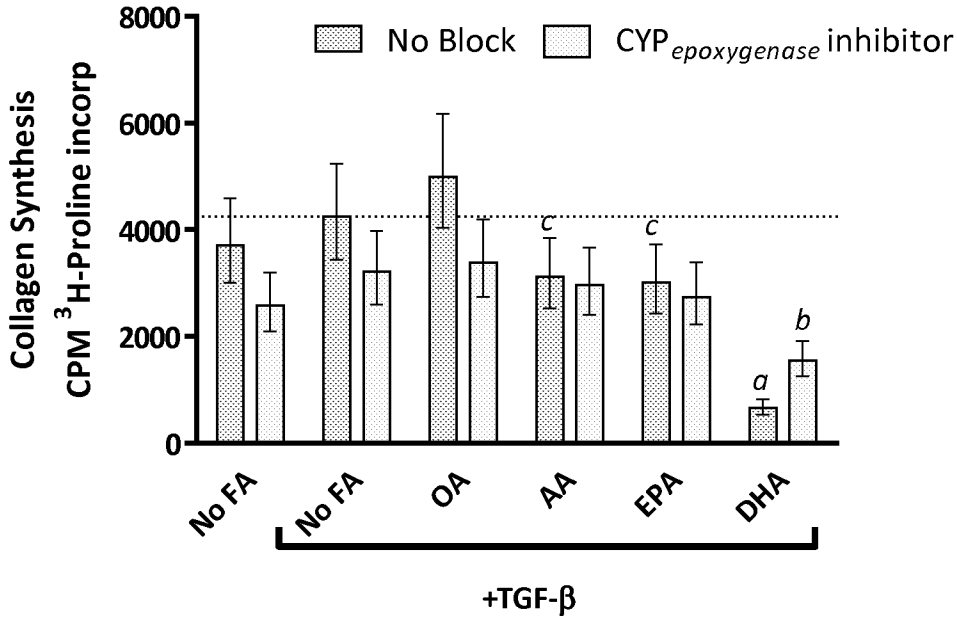

B
p-ERK1/2/ERK1/2 (fold change)
P<0.01
NS
NS
3
2
1
0
Control
EPA
DHA
TGF-β1
TGF-β1+EPA
TGF-β1+DHA

Figure 11



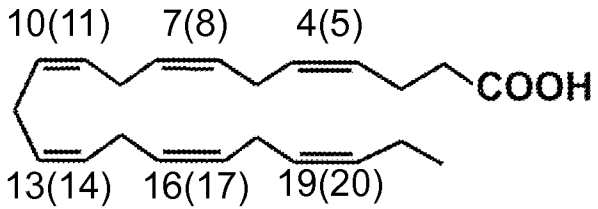
a: -87% (-90, -82) p <0.0001 vs. No FA + TGF-β

b: 133% (73, 215) p =0.009 vs. DHA + TGF-β

c: ns vs. No FA + TGF-β

Figure 12

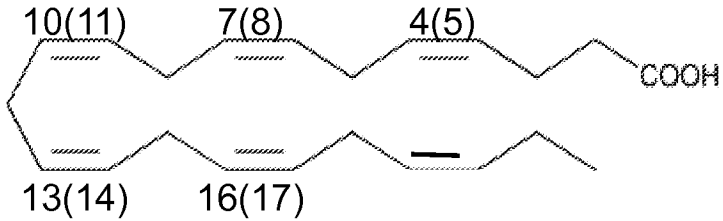
A.



Docosahexaenoic acid

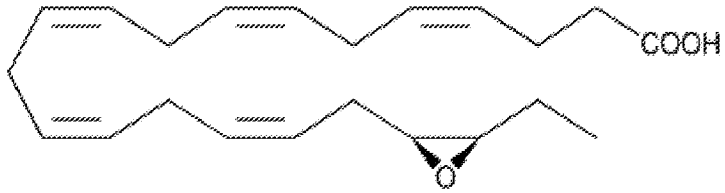
B.

Secondary



4(5); 7(8); 10(11); 13(14); 16(17) –EpDPE
(as all 4 enantiomers at each double bond)

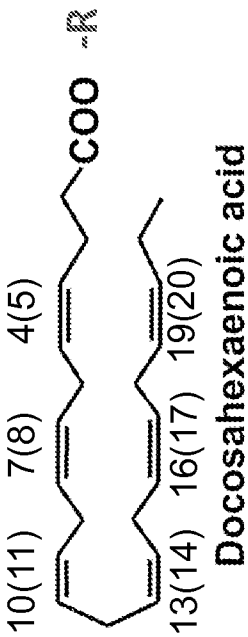
Primary



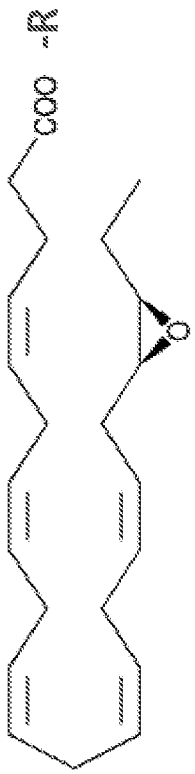
19(20)-epoxy Docosapentaenoic Acid;
19(20)EpDPE
(as all four enantiomers)

Figure 12 (Continued)

C.



Docosahexaenoic acid



19(20)-epoxy Docosapentaenoic Acid;
19(20)EpDPE
(as all four enantiomers)

PATENT COOPERATION TREATY

PCT

DECLARATION OF NON-ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT

(PCT Article 17(2)(a), Rules 13*ter*.1(c) and (d) and 39)

Applicant's or agent's file reference	**IMPORTANT DECLARATION**	Date of mailing (*day/month/year*)
11-1339-PCT		29 April 2013 (29.04.2013)
International application No.	International filing date (*day/month/year*)	(Earlist) Priority date *(day/month/year)*
PCT/US2013/023581	**29 January 2013 (29.01.2013)**	30 January 2012 (30.01.2012)

International Patent Classification (IPC) or both national classification and IPC

A61K 31/202(2006.01)i, A61K 31/20(2006.01)i, A61P 9/04(2006.01)i, A61P 9/00(2006.01)i

Applicant

O'CONNELL D. TIMOTHY et al

This International Searching Authority hereby declares, according to Article 17(2)(a), that **no international search report will be established** on the international application for the reasons indicated below.

1. ☒ The subject matter of the international application relates to:
   - a. ☐ scientific theories.
   - b. ☐ mathematical theories.
   - c. ☐ plant varieties.
   - d. ☐ animal varieties.
   - e. ☐ essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes.
   - f. ☐ schemes, rules or methods of doing business.
   - g. ☐ schemes, rules or methods of performing purely mental acts.
   - h. ☐ schemes, rules or methods of playing games.
   - i. ☒ methods for treatment of the human body by surgery or therapy.
   - j. ☐ methods for treatment of the animal body by surgery or therapy.
   - k. ☐ diagnostic methods practised on the human or animal body.
   - l. ☐ mere presentation of information.
   - m. ☐ computer programs for which this International Searching Authority is not equipped to search prior art.
2. ☒ The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out:

   ☐ the description ☒ the claims ☐ the drawings
3. ☐ A meaningful search could not be carried out without the sequence listing; the applicant did not, within the prescribed time limit:
   - ☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
   - ☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
   - ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b)
4. Further coments:

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