Delta-6-desaturase Links Polyunsaturated Fatty Acid Metabolism With Phospholipid Remodeling and Disease Progression in Heart Failure

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Background—Remodeling of myocardial phospholipids has been reported in various forms of heart failure for decades, but the mechanism and pathophysiological relevance of this phenomenon have remained unclear. We examined the hypothesis that δ-6 desaturase (D6D), the rate-limiting enzyme in long-chain polyunsaturated fatty acid biosynthesis, mediates the signature pattern of fatty acid redistribution observed in myocardial phospholipids after chronic pressure overload and explored plausible links between this process and disease pathogenesis.

Methods and Results—Compositional analysis of phospholipids from hearts explanted from patients with dilated cardiomyopathy revealed elevated polyunsaturated fatty acid product/precursor ratios reflective of D6D hyperactivity, manifesting primarily as lower levels of linoleic acid with reciprocally higher levels of arachidonic and docosahexaenoic acids. This pattern of remodeling was attenuated in failing hearts chronically unloaded with a left ventricular assist device. Chronic inhibition of D6D in vivo reversed similar patterns of myocardial polyunsaturated fatty acid redistribution in rat models of pressure overload and hypertensive heart disease and significantly attenuated cardiac hypertrophy, fibrosis, and contractile dysfunction in both models. D6D inhibition also attenuated myocardial elevations in pathogenic eicosanoid species, lipid peroxidation, and extracellular receptor kinase 1/2 activation; normalized cardiolipin composition in mitochondria; reduced circulating levels of inflammatory cytokines; and elicited model-specific effects on cardiac mitochondrial respiratory efficiency, nuclear factor κB activation, and caspase activities.

Conclusions—These studies demonstrate a pivotal role of essential fatty acid metabolism in myocardial phospholipid remodeling induced by hemodynamic stress and reveal novel links between this phenomenon and the propagation of multiple pathogenic systems involved in maladaptive cardiac remodeling and contractile dysfunction. (Circ Heart Fail. 2014;7:172-183.)

Key Words: fatty acid synthases ■ heart failure ■ hypertension ■ inflammation ■ metabolism

Heart failure (HF) is a complex, multifactorial syndrome characterized by progressive cardiac remodeling and contractile dysfunction that leads to an impaired matching of blood supply to tissue demands. Antecedent hypertension is present in the majority of cases,1 supporting a paradigm by which chronic hemodynamic overload of the myocardium results in initial compensatory adaptations that ultimately become maladaptive, leading to pathological hypertrophy, fibrosis, and mechanical failure. Several well-established pathogenic systems have been implicated in the transition from adaptive cardiac hypertrophy to maladaptive remodeling and failure, including chronic inflammation,2 oxidative stress,3 impaired myocardial energetics,4 and apoptosis.5 However, the molecular triggers and precise contribution of these processes remain areas of intense investigation and debate.

Clinical Perspective on p 183

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Alterations in the fatty acid composition of myocardial phospholipids have been reported in various forms of cardiac pathology for >25 years, including human cardiomyopathies and animal models of pressure-overload hypertrophy, hypertensive heart disease, postinfarct remodeling, diabetic cardiomyopathy, and senescence. Interestingly, despite distinct pathogeneses, genetic backgrounds, and biochemical techniques, the pattern of phospholipid remodeling has consistently manifested as a proportional loss of the essential polyunsaturated fatty acid (PUFA) linoleic acid (18:2n6; LA) and α-linolenic acid (18:3n3), respectively (Figure 1). Serum PUFA ratios reflective of systemic D6D hyperactivity have been reported in patients with hypertension and are highly predictive of overall cardiovascular mortality in humans. However, no experimental studies have investigated the role of this enzyme in myocardial phospholipid remodeling or the pathogenesis of heart disease. Herein, we present the first comprehensive analysis of phospholipid composition in the failing human heart with and without mechanical unloading support and investigated the effects of chronic pharmacological D6D inhibition in rodent models of pressure-overload hypertrophy and hypertensive heart disease. These studies demonstrate a central role of D6D in myocardial phospholipid remodeling resulting from hemodynamic stress and reveal novel links between this process and disease progression at the molecular, organ, and systemic levels.

Methods

Human Heart Tissue

Left ventricular (LV) tissue was obtained by an institutional review board-approved protocol maintained by the University of Colorado Denver Cardiac Tissue Bank. Hearts donated for research purposes were obtained under written consent from family members of organ donors or by direct written consent from patients undergoing cardiac transplantation. Patient characteristics are presented in Table I in the Data Supplement.

Animal Models

Lean male spontaneously hypertensive HF (Mcco/Hsd) rats were obtained from a colony maintained at the University of Colorado. Spontaneously hypertensive HF rats that were selected for these studies were based on their well-characterized development of progressive hypertensive cardiomyopathy that shares many of the hallmark biochemical and pathophysiological features of dilated cardiomyopathy (DCM) in humans. Two cohorts of animals were studied: (1) at 21 to 22 months of age when rats exhibit pathological cardiac hypertrophy progressing toward dilated HF and (2) after thoracic aortic banding at 3 months of age to induce hemodynamic stress and pathological hypertrophy in the absence of age-related pathology (transverse aortic constriction [TAC]). Cohorts of HF and TAC (2 weeks after surgery) animals were each divided and matched on echocardiography parameters before being semirandomly assigned to receive the D6D inhibitor (SC-26196 [SC]) or no drug for 4 weeks. The effect of D6D inhibition was also examined in 3-month-old spontaneously hypertensive HF rats exposed to a sham TAC surgery (Sham) that were followed along with TAC groups for 4 weeks as an experimental control. All animals were provided Purina 5001 chow and water ad libitum for the duration of the study. At the conclusion of the study, animals were euthanized with a lethal dose of sodium pentobarbital (150 mg/kg IP) followed by midline thoracotomy and removal of the heart. All procedures were approved by the Animal Care and Use Committee at Colorado State University and/or University of Colorado Boulder in strict compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication No. 85-23, revised 1996).

Inhibition of D6D In Vivo

Rats were administered the potent, orally active D6D inhibitor SC (a gift from Dr Mark Obukowicz, Pfizer Corporation) at a dose previously reported to inhibit D6D enzyme activity selectively with no effect on other desaturase enzymes in rodents in vivo (100 mg/kg per day mixed in chow for 4 weeks), based on daily food consumption records taken >3 to 4 weeks before treatment.

Echocardiography and Blood Pressure

Transthoracic echocardiography was performed under light isoflurane anesthesia before and after the 4-week experimental period using a 12-MHz pediatric transducer connected to a Hewlett Packard Sonos 5500 Ultrasound as previously described. Tail cuff blood pressure measurements were obtained in the Sham and HF groups using the Kent Coda 6 system (Kent Scientific, Torrington, CT) in lightly isoflurane-anesthetized rats.

Lipid Analyses

See the Expanded Methods in the Data Supplement for a detailed description of lipid analyses. Briefly, phospholipids were extracted by thin layer chromatography (total) or liquid chromatography (individual species) for compositional analysis by gas chromatography (fatty acid composition) or electrospray ionization mass spectrometry.
Mitochondrial Isolation and Respiratory Function
Mitochondria were freshly isolated from ∼300 mg of LV tissue by differential centrifugation in the absence of proteases and assayed for respiratory function using a Clark-type electrode system (Strathkelvin) with pyruvate+malate as substrates as previously described.19

Biochemical Analyses
Immunoblotting was performed by standard methods using commercially available antibodies. Serum glucose and free fatty acids were determined by colorimetric assays (Biovision). Caspase activities were determined in 30 mg of LV homogenates by luminescence assay (Promega). Myocardial hydroxyproline was quantitated as a marker of collagen (fibrosis) in 30 to 40 mg of septal tissue by the colorimetric assay of Switzer and Summer.21 Serum cytokines were determined in 80 μL of sample by ELISA cytokine array (Raybiotech). Quantitative real-time polymerase chain reaction was performed using 2X SYBR Green quantitative polymerase chain reaction Master Mix and validated gene-specific primers (listed in Table II in the Data Supplement) with resulting data normalized to 18S rRNA and analyzed according to the comparative (ΔΔCt) Ct method.

Statistical Analyses
All data are presented as group mean±SE. Human heart data were compared by 1-way ANOVA with Tukey HSD tests post hoc when appropriate. Data from the animal studies were analyzed by separate 2 (condition) X 2 (drug) ANOVAs for determination of main and interaction effects of SC treatment and TAC or HF versus Sham cohorts, followed by Dunnett test for comparison of group means versus Sham control. Mean differences between SC-treated and untreated groups were compared by independent sample t tests for each condition. Pre- to post-treatment differences in echocardiography parameters were examined by paired t tests for each condition cohort. Pearson correlations were calculated to determine the associations between phospholipid fatty acid composition and lipid peroxidation adducts. All reported P values are 2 sided. Statistical significance was established at P<0.05, using SPSS 21 version software (IBM) for all analyses.

Results
Phospholipid Indices of D6D Activity Are Elevated in the Failing Human Heart and Are Reversed by Mechanical Unloading
In the absence of dietary supplementation, desaturation and elongation of LA and α-linolenic acid provide the majority of long-chain PUFAs present in mammalian tissues. The most abundant of these are AA and DHA, which along with LA account for >90% of PUFAs in membrane phospholipids, their primary site of incorporation. D6D catalyzes rate-limiting steps in this pathway (Figure 1); therefore, changes in specific tissue phospholipid PUFAs reflect chronic changes in D6D activity in vivo.16 Reduced levels of cardiolipin molecular species normally enriched with LA acyl chains have been reported in hearts explanted from patients with dilated and ischemic cardiomyopathies.6,7 However, whether these changes occur in other phospholipid classes or are reflective of a global redistribution of PUFAs in the total myocardial phospholipid pool was not addressed. Therefore, we performed a detailed compositional analysis of myocardial phospholipids from patients with DCM (n=8) with or without mechanical unloading with a LV assist device for ≥4 months before explant (n=4; Figure 2).

Patient characteristics are presented in Table I in the Data Supplement. Hearts from patients with DCM exhibited markedly lower proportions of total phospholipid LA, with correspondingly higher levels of AA, DHA, and PUFAs, product/precursor ratios reflective of D6D hyperactivity, when compared with donor hearts from age-matched individuals with no cardiac pathology (nonfailing; n=8). Comparatively minor differences were seen in saturated and monoensaturated fatty acid species (Figure I in the Data Supplement).

Figure 2. Phospholipid polyunsaturated fatty acid (PUFA) desaturation in human heart failure. Gas chromatographic analysis of total phospholipid fatty acids extracted from human left ventricular tissue revealed a loss of phospholipid linoleic acid (LA) paralleled by elevations in arachidonic acid (AA), docosahexaenoic acid (DHA), and PUFAs in hearts explanted from patients with dilated cardiomyopathy (DCM; n=8) when compared with nonfailing donor hearts (NF; n=8), which was partially reversed in patients implanted with a left ventricular assist device (LVAD) for 4 to 10 months (n=4). *P<0.05 vs NF. †P<0.05 vs DCM. PL indicates phospholipid.
Chronic LV assist device support was associated with significantly higher phospholipid LA levels and reduced AA, DHA, and D6D product/precursor ratios when compared with DCM. Compositional analysis of the 3 major phospholipid species present in the mammalian heart, phosphatidylcholine, phosphatidylethanolamine, and cardiolipin revealed similar patterns of fatty acid redistribution observed in total phospholipids, although the magnitude of changes and relative abundance of individual fatty acids varied considerably between classes (Figure I in the Data Supplement).

D6D Inhibition Reverses Phospholipid PUFA Remodeling in TAC and HF Groups
As seen in patients with DCM, hearts from both TAC and HF animals exhibited markedly lower phospholipid LA content, with corresponding elevations in AA, DHA, and D6D product/precursor ratios in total myocardial phospholipids when compared with Sham controls (Figure 3A and 3B). Chronic administration of the D6D inhibitor ameliorated these effects of TAC and HF, bringing total phospholipid LA, AA, DHA, and D6D activity indices to near Sham control levels. Despite intrinsic differences in baseline fatty acid composition, similar patterns of PUFA redistribution were seen within individual phospholipid classes in both models that were reversed by D6D inhibition (Figure II in the Data Supplement). Interestingly, only minor effects of D6D inhibition were seen on phospholipid PUFA composition in the Sham rats, suggesting that basal levels of PUFAs in membranes are highly defended, perhaps aided by an incomplete inhibition of D6D enzymatic activity in vivo (for a 24-hour period) and/or trace amounts of AA and DHA present in rodent chow (Purina 5001).

D6D Inhibition Normalizes the Cardiolipin Molecular Species Profile in Mitochondria
Alterations in the highly regulated fatty acid composition of cardiolipin may have particular relevance in cardiac pathologies. Therefore, cardiolipin molecular species were also examined in cardiac mitochondria isolated from animals in this study. As previously reported, a marked loss of the predominant tetra-linoleoyl species (L₄CL) was seen in TAC and HF mitochondria, which corresponded to elevations in species containing AA and/or DHA (HUFA [CL]), without any...
appreciable effect on total cardiolipin content (Figure 3C; Figure IV in the Data Supplement). D6D inhibition completely normalized the cardiolipin molecular species profile in TAC and HF, restoring L,CL and reducing highly unsaturated fatty acid (cardiolipin) species to Sham control levels in both groups. These data provide novel evidence for a central role of LA desaturation and/or endogenous highly unsaturated fatty acid production in modulating cardiolipin composition in the hypertrophied and failing heart.

Myocardial Free AA and Eicosanoid Contents
Once liberated from phospholipids by phospholipase enzymes, AA can serve as a substrate for multiple oxygenase enzymes and nonenzymatic oxidation pathways capable of generating a host of bioactive eicosanoid species with complex effects on inflammatory, cardiovascular, and transcriptional regulation.23,24 Both TAC and HF groups elicited significant increases in free AA and several eicosanoid species in the heart, all of which were reduced to near control levels with D6D inhibition (Figure 3D). Particularly significant changes were seen in 12- and 15-hydroxyeicosatetraenoic acid, thromboxane A2 (TXA2), and isoprostanes, which are formed via 12-/15-lipoygenase, cyclooxygenase-2, and the nonenzymatic peroxidation of AA by reactive oxygen species, respectively.

Serum and Hepatic Phospholipids, Circulating Cytokines, and Animal Characteristics
Analysis of total serum and liver phospholipids revealed similar patterns of PUFA distribution seen in cardiac phospholipids in TAC and HF groups (Figure II in the Data Supplement), suggesting that myocardial changes may be reflective of elevated systemic (eg, hepatic) D6D activity. No significant effects of D6D inhibition were observed on body weight, serum glucose, free fatty acids, or blood pressure in any of the groups (Table). However, significant elevations in serum interferon-γ and serum monocyte chemotactic protein-1 in TAC and HF groups were attenuated with SC treatment, consistent with an anti-inflammatory effect of D6D inhibition.18

D6D Inhibition Attenuates Maladaptive Cardiac Remodeling and Contractile Dysfunction
Echocardiography performed before and after the 4-week experimental period revealed significant progression of LV dilatation and contractile dysfunction in the untreated TAC and HF animals that were significantly attenuated or reversed by D6D inhibition (Figure 4A and 4B). These improvements corresponded to lower final heart weights in the treated TAC and HF rats when compared with untreated animals and a trend for lower wet lung weights suggestive of reduced pulmonary congestion (Figure 4C; Table III in the Data Supplement). Notably, a mild degree of cardiac hypertrophy and reduced fractional shortening is evident in the Sham spontaneously hypertensive HF rats when compared with other rat strains at this age2,17 that was unaffected by D6D inhibition, indicating that this treatment attenuates the progression of more marked pathological remodeling and dysfunction seen after chronic hemodynamic stress. Myocardial fibrosis, assessed by tissue hydroxyproline content, was also reduced by D6D inhibition in TAC and HF rats (Figure 5B), which paralleled histological evidence of reduced interstitial and perivascular collagen deposition (Figure 5B; Figure VII in the Data Supplement). TAC and HF significantly increased mRNA expression of atrial natriuretic peptide and myocardial extracellular-regulated activated kinase 1/2 phosphorylation when compared with Sham controls, which was largely prevented by D6D inhibition treatment in both models (Figure 5C and 5D), TAC cohorts, and to a lesser extent HF, were both associated with myocardial activation of nuclear factor κB (NFκB), indicated by degradation of its endogenous inhibitory regulator I kBα, which was significantly attenuated in TAC rats but not in HF rats (Figure 5E).

Cardiac Mitochondrial Respiration and Caspase Activities
Reduced capacity and efficiency of oxidative phosphorylation in cardiac mitochondria may impair myocardial bioenergetics, thereby contributing to the development and progression of HF.4 However, TAC rats had no effect on mitochondrial state

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Data are means±SEM. BP indicates blood pressure; FFA, free fatty acids; HF, heart failure; IFNγ, interferon γ; IL, interleukin; MCP-1, macrophage chemotactic protein-1; ND, not determined; SC, SC-26196; TAC, transverse aortic constriction; and TIMP-1, TIMP metalloproteinase inhibitor 1.

*P<0.05 vs Sham.
†P<0.05 vs untreated.
Phospholipid Remodeling in Heart Failure

Conversely, HF cohorts were associated with depressed state 3 respiration, respiratory control ratio, and oxidative phosphorylation efficiency (ADP/O ratio) when compared with TAC rats and Sham controls. D6D inhibition decreased state 4 respiration and restored respiratory control ratio and ADP/O to near Sham control levels in HF mitochondria but had no effect on state 3 respiration. Immunoblotting of mitochondrial proteins for respiratory complex subunits revealed a deficiency in complexes 1 and 2 in HF consistent with reduced respiratory capacity in this group (Figure 6B), which was similarly unaffected by D6D inhibition (n=10; B). SC treatment resulted in significantly lower heart weights in TAC and HF animals and decreased pulmonary congestion in HF rats (C). *P<0.05 vs baseline (A), Pre-Tx (B), or Sham (C). †P<0.05 vs untreated cohort.

D6D Inhibition Reduces Cardiac Lipoxidative Stress

Aldehyde products of PUFA peroxidation, such as malondialdehyde and 4-hydroxynonenal, are common markers of oxidative stress that correlate closely with the incidence and severity of HF in humans.25 The peroxidizability of PUFAs increases exponentially with their double-bond content26; therefore, the calculated cardiac membrane peroxidizability index increased significantly in TAC and HF groups and was normalized to Sham levels by D6D inhibition (Figure 7A). Consistent with this observation, malondialdehyde- and hydroxynonenal-protein adducts were elevated in TAC and HF hearts and were significantly reduced by D6D inhibition (Figure 7B). Myocardial malondialdehyde levels correlated positively with the relative proportion of DHA in myocardial phospholipids across the experimental groups, whereas a strong negative correlation was seen between hydroxynonenal and phospholipid LA (Figure 7C). D6D inhibition also tended to decrease myocardial superoxide dismutase and glutathione peroxidase enzyme contents (Figure 7D), collectively suggesting a decrease in myocardial oxidative stress.

Cardiac and Hepatic D6D Expression

Although our lipid analyses support a central role of D6D in myocardial phospholipid remodeling associated with pressure overload, the mechanism driving D6D activity in response to hemodynamic stress is less clear. Expression of D6D is low in the heart, and while detectable, we found no evidence of upregulation at the protein or mRNA levels or any significant changes in expression of downstream elongation/desaturation.
enzymes in TAC, HF, or human DCM (Figure V in the Data Supplement). Interestingly, LV assist device treatment significantly reduced myocardial D6D mRNA expression when compared with DCM and nonfailing controls, despite no detectable changes in enzyme protein content, suggesting potential regulatory role of hemodynamic unloading on at least enzyme expression in the human heart. Hepatic D6D activity might have influenced myocardial membrane composition by altering the distribution of PUFA supplied to the heart from the circulation (Figure II in the Data Supplement); however, liver D6D expression was also unaffected by TAC or HF groups (Figure V in the Data Supplement). Little is known about post-translational regulation of D6D activity, but putative mechanisms are currently being investigated in our laboratory.

**Discussion**

The present study demonstrates a pivotal role of essential fatty acid metabolism through D6D in generating the signature pattern of phospholipid PUFA redistribution associated with cardiac pathology in multiple experimental models, and show that it is closely linked to hemodynamic stress in the failing human heart. The remarkable phenotypic effects of D6D inhibition in TAC and HF animals highlight the pathophysiological importance of this process and provide novel insight into the mechanisms responsible for maladaptive remodeling and contractile dysfunction in the pressure-overloaded myocardium. Although previous studies have associated serum markers of D6D activity with coronary artery disease risk and inflammation, the present study is the first to demonstrate a role for this enzyme in regulating myocardial membrane composition and responses to pathological stress.

The proportional loss of phospholipid LA is the most marked and consistent manifestation of membrane remodeling associated with cardiac overload across species, tissues, and phospholipid classes observed herein and in previous studies. The reversibility of this effect by D6D inhibition in TAC and HF animals highlight the pathophysiological importance of this process and provide novel insight into the mechanisms responsible for maladaptive remodeling and contractile dysfunction in the pressure-overloaded myocardium. Although previous studies have associated serum markers of D6D activity with coronary artery disease risk and inflammation, the present study is the first to demonstrate a role for this enzyme in regulating myocardial membrane composition and responses to pathological stress.

**Figure 5.** Myocardial fibrosis, hypertrophic, and inflammatory signaling. Masson trichrome staining of left ventricular tissue from transverse aortic constriction (TAC) and heart failure (HF) animals revealed interstitial and perivascular fibrosis that was markedly reduced with δ-6 desaturase (D6D) inhibition (A), which paralleled a significant attenuation of elevated myocardial hydroxyproline (collagen) content in TAC and HF animals (n=6 per group; B). SC-26196 (SC) treatment prevented significant elevations in atrial natriuretic peptide (ANP) expression (C) and extracellular receptor kinase (ERK) phosphorylation (D) in TAC and HF and attenuated loss of the endogenous nuclear factor κB inhibitor and inhibitory regulator IκBα (IκBα; E) in TAC (n=4–6 per group). *P<0.05 vs Sham. †P<0.05 vs untreated cohort. CM indicates Coomassie.
D6D-dependent increases in myocardial levels of free AA and its eicosanoid derivatives observed in TAC and HF animals may have contributed to maladaptive remodeling in these cohorts. Several lines of evidence implicate myocardial AA-derived eicosanoids in the development of cardiac fibrosis and failure. Among these, the pathogenic roles of TXA2 and 12-/15-hydroxyeicosatetraenoic acid have been established through the development of transgenic mice with cardiomyocyte-specific overexpression of cyclooxygenase-2 and 12/15-lipoxygenase, respectively; both of which develop marked cardiac fibrosis, hypertrophy, and contractile dysfunction. Myocardial AA promotes extracellular receptor kinase signaling and cardiac hypertrophy via activation of specific intracellular G-protein–coupled receptors by TXA2 and reactive oxygen species–derived isoprostanes in cardiomyocytes. Overproduction of 12-hydroxyeicosatetraenoic acid also increases extracellular receptor kinase activity, hypertrophy, and fibronectin content in cardiac fibroblasts, suggesting a potential role in myocardial fibrosis. Therefore, D6D inhibition might have attenuated maladaptive remodeling by reducing extracellular receptor kinase signaling by multiple AA-derived eicosanoid species. Eicosanoids also serve as potent initiators and propagators of inflammatory signaling, many of which converge on the NFκB pathway. Cardiomyocyte NFκB signaling has been implicated in the pathogenesis of cardiac hypertrophy and failure and has been associated with cyclooxygenase-2 induction in the failing human heart. Therefore, NFκB may mediate temporal and model-specific effects that are differentially modulated by altered myocardial eicosanoid levels and membrane PUFA composition.

In addition to its suppression of free AA and eicosanoid levels, D6D inhibition reversed several significant changes in the fatty acid composition of individual phospholipid classes that could have altered functional properties of membranes in which they reside. Although a comprehensive evaluation of

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**Figure 6.** Mitochondrial respiration and caspase activities. A, State 3 (phosphorylating) and state 4 (uncoupled) respiration, respiratory control ratio (RCR; state3/4) and phosphorylation efficiency (ADP phosphorylated per O2 consumed [ADP/O]) in cardiac mitochondria isolated from each of the experimental groups (n=4–8 per group). B, Protein expression of subunits from each of the 5 respiratory complexes (Cl–V) obtained by immunoblotting 10 μg of mitochondrial protein (n=4 per group). C, Caspase activities in myocardial homogenates assayed by luminescence assay (n=4–6 per group). *P<0.05 vs Sham. †P<0.05 vs untreated. HF indicates heart failure; and TAC, transverse aortic constriction.
these is beyond the scope of this investigation, the reversal of PUFA remodeling in cardiolipin is particularly relevant and warrants further discussion.

Cardiolipin is a dimeric tetra-acyl phospholipid found exclusively in mitochondria, where it provides critical structural and functional support to proteins involved in oxidative phosphorylation and regulates apoptotic signaling by binding cytochrome c to the inner mitochondrial membrane. The majority of cardiolipin molecular species in the healthy mammalian heart contain 4 LA acyl chains (L4CL). Several studies report that this LA enrichment is lost in states of cardiac pathology, which has been suggested as a potential contributor to mitochondrial dysfunction and disease progression. It is well established that the compositional uniformity of cardiolipin results from a series of deacylation/reacylation reactions after de novo biosynthesis in mitochondria. At least 3 enzymes have been identified that are capable of catalyzing these reactions; however, none has exhibited the anticipated specificity for LA acyl chains that predominate in the mammalian heart. It was recently suggested that the LA enrichment may arise from an enzymatic equilibrium distribution of fatty acids exchanged between multiple phospholipid species. Our data are consistent with this hypothesis, demonstrating that changes in cardiolipin composition coincide with changes in phosphatidylcholine, phosphatidylethanolamine, and total phospholipid fractions reflective of a global redistribution of membrane PUFAs. Notwithstanding changes in specific acyltransferase enzymes implicated in aberrant cardiolipin remodeling, our findings indicate that the loss of L4CL in the pressure overloaded and failing heart results from increased flux of PUFAs through D6D and downstream elongation/desaturation enzymes in the heart and/or liver. This ultimately reduces the bioavailability of phospholipid LA for cardiolipin remodeling in the heart, favoring an exchange of LA for long-chain PUFA products of this pathway (ie, AA and DHA).
Reduced capacity and efficiency of oxidative phosphorylation in cardiac mitochondria may contribute to the development and progression of HF, and aberrant cardiolipin remodeling has been postulated as a mechanism for these defects. However, despite a significant loss of ΔυCL, TAC had no effect on mitochondrial respiratory function in the presence or in the absence of D6D inhibition. Impaired oxidative phosphorylation capacity was seen in HF mitochondria, but this was unaffected by D6D inhibition and may have resulted from the loss of complexes 1 and 2 that deliver reducing equivalents to respiratory chain. Reductions in oxidative phosphorylation efficiency may also contribute to cardiac dysfunction in HF. Therefore, increases in respiratory control ratio and ADP/O with D6D inhibition, although relatively modest, could have contributed to improvements in cardiac function observed in HF groups. Taken together, these findings indicate that the LA enrichment of cardiolipin, at least within the range observed in these studies, does not significantly influence the oxidative phosphorylation capacity of cardiac mitochondria but may support efficient respiratory coupling. The observed dissociation of mitochondrial respiratory parameters from changes in cardiolipin composition and cardiac function in TAC and HF groups further argues against their direct relationship in these models.

Apopotic loss of cardiomyocytes during maladaptive cardiac remodeling may hasten the progression of pathological hypertrophy to decompensated failure. Myocardial activities of caspase-9 and caspase-3/7 were elevated in both TAC and HF groups but were only attenuated by D6D inhibition in TAC groups. This model-specific effect argues against an independent effect of PUFA metabolism or phospholipid (ie, cardiolipin) remodeling on myocardial apoptotic signaling in vivo but parallels the pattern of myocardial NFκB activation seen in these animals. This is consistent with evidence linking NFκB activity and apoptotic signaling in the pressure-overloaded failing heart. However, the extent to which caspase activities reflect cumulative apoptotic myocyte loss that contributed to the observed changes in cardiac structure and function is unclear. Nevertheless, the absence of changes in caspase activities in HF, despite marked improvements in cardiac structure and function with D6D inhibition, suggests that mechanisms other than apoptotic signaling mediate the pathogenic effects of phospholipid remodeling in this model.

Membrane PUFAs are a primary target of reactive oxygen species, which react with hydrogens in methylene groups adjacent to their double bonds, triggering an autocatalytic series of oxidation events leading to chain breaks and reactive aldehyde formation. Serum levels of lipid peroxidation products, such as malondialdehyde, are positively associated with New York Heart Association clinical class in patients with HF, suggesting that this process may be prognostic importance. The susceptibility of PUFAs to peroxidation increases exponentially with double-bond content; therefore, modification of membrane PUFA composition could influence the extent of aldehyde formation in states of oxidative stress. Consistent with this hypothesis, D6D inhibition significantly attenuated increases in myocardial malondialdehyde- and hydroxynonenal-protein adducts in TAC and HF groups, which correlated closely with changes in phospholipid DHA and LA levels, respectively. Interestingly, although D6D inhibition completely abolished increases in myocardial malondialdehyde in both models, it led to significant, but less robust reductions in hydroxynonenal. Malondialdehyde and hydroxynonenal are typically used interchangeably as markers of oxidative stress in tissues; however, they differ with regard to their origin and cellular toxicities. Malondialdehyde is formed primarily from the autocatalytic peroxidation of DHA and during TXA2 synthesis, and is thought to be toxic primarily by forming mutagenic DNA-adducts. Hydroxynonenal is derived from the peroxidation of n6 PUFAs, primarily AA, and exerts its pathological effects by cross-linking proteins and promoting inflammatory and fibrogenic signaling. Relatively minor changes in phospholipid AA and the large increases in LA might explain the less pronounced decreases in hydroxynonenal with D6D inhibition, whereas dramatic changes in DHA and TXA2, parallel similar changes in malondialdehyde. Therefore, modulation of membrane PUFA content and composition seem to strongly influence the extent and pattern of lipid aldehyde species that accumulate in the heart during states of oxidative stress.

In summary, our studies demonstrate a central role of essential fatty acid metabolism through D6D in generating the signature pattern of PUFA redistribution in myocardial phospholipids widely reported in cardiac pathologies, and suggests important pathophysiological consequences of this phenomenon in HF. Although precisely defining how cardiac and hepatic lipid metabolism interact to influence myocardial membrane composition and disease progression in hypertensive heart disease will require further investigation, a primary pathogenic effect of this process may be an increase in myocardial free AA, favoring production of multiple eicosanoid species implicated in hypertrophic and fibrotic remodeling. An exchange of membrane LA for highly unsaturated AA and DHA may also promote lipid peroxidation and alter critical membrane-dependent processes, such as the efficiency of oxidative phosphorylation and perhaps others not evaluated herein. The observed model-specific effects of D6D inhibition on mitochondrial respiration, NFκB, and apoptotic signaling, despite eliciting consistent benefits on cardiac structure and function, argue against primary roles of these systems in mediating the pathogenic effects of phospholipid remodeling in HF. Similar patterns of PUFA redistribution observed in myocardial and serum phospholipids highlight the potential prognostic value of serum D6D activity ratios associated with hypertension and cardiovascular morality in the epidemiological literature. In conclusion, targeting the D6D pathway may represent an integrative new approach to the study and treatment of HF and perhaps other pathologies associated with this signature pattern of phospholipid PUFA remodeling.

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Disclosures

None.

References

CLINICAL PERSPECTIVE

The present study implicates enhanced metabolism of polyunsaturated fatty acids through δ-6 desaturase (D6D) in the signature pattern of myocardial phospholipid remodeling associated with cardiac overload and failure. Serum indices of D6D hyperactivity have been linked to cardiovascular mortality and hypertension in epidemiological studies, but this is the first experimental study to investigate the role of this enzyme in cardiovascular disease. Our findings highlight the importance of endogenous polyunsaturated fatty acid metabolism and myocardial membrane remodeling in the maladaptive responses to pressure overload and provide the basis for integrative new approaches to the study and treatment of heart failure. In general, consistent patterns of cardiac membrane remodeling in humans and rodent models support future translational studies aimed at defining the mechanisms that regulate D6D activity in response to cardiac overload and dissecting out adaptive versus maladaptive pathways that emanate from this important metabolic enzyme. Targeting D6D may also help to resolve longstanding controversy about the effects of dietary polyunsaturated fatty acids on cardiac health and disease, particularly in light of accumulating evidence for common single-nucleotide polymorphisms in the D6D gene (Fads2) that influence the metabolism of polyunsaturated fatty acids, low-grade inflammation and cardiometabolic risk in the context of the modern Western diet.
Delta-6-desaturase Links Polyunsaturated Fatty Acid Metabolism With Phospholipid Remodeling and Disease Progression in Heart Failure

Catherine H. Le, Christopher M. Mulligan, Melissa A. Routh, Gerrit J. Bouma, Melinda A. Frye, Kimberly M. Jeckel, Genevieve C. Sparagna, Joshua M. Lynch, Russell L. Moore, Sylvia A. McCune, Michael Bristow, Simona Zarini, Robert C. Murphy and Adam J. Chicco

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/content/7/2/382.full.pdf

Data Supplement (unedited) at:
http://circheartfailure.ahajournals.org/content/suppl/2013/11/27/CIRCHEARTFAILURE.113.000744.DC1
Correction

In the article “Delta-6-desaturase Links Polyunsaturated Fatty Acid Metabolism With Phospholipid Remodeling and Disease Progression in Heart Failure” by Le et al, which was published in the January 2014 issue (Circ Heart Fail 2014;7:172–183), a correction was needed.

On page 172, in the Conclusions section of the Abstract, the word “although” was erroneously included. The sentence has been revised as follows: “These studies demonstrate a pivotal role of essential fatty acid metabolism in myocardial phospholipid remodeling induced by hemodynamic stress and reveal novel links between this phenomenon and the propagation of multiple pathogenic systems involved in maladaptive cardiac remodeling and contractile dysfunction.”

The compositor apologizes for the error.

These corrections have been made to the current online version of the article, which is available at http://circheartfailure.ahajournals.org/content/7/1/172.full.
Supplemental Material for

Delta-6-desaturase links PUFA metabolism with phospholipid remodeling and disease progression in heart failure

Catherine H. Le, BS1,2*, Christopher M. Mulligan, MS1,3*, Melissa A. Routh, MS1,2, Gerrit J. Bouma, PhD4, Melinda A. Frye, PhD4, Kimberly M. Jeckel, PhD4, Genevieve C. Sparagna, PhD6, Joshua M. Lynch, MS6, Russell L. Moore, PhD5, Sylvia A. McCune, PhD5, Michael Bristow, MD, PhD7, Simona Zarini, PhD8, Robert C. Murphy, PhD8, Adam J. Chicco, PhD1-5†

Supplemental Methods

Human Heart Tissue
Left ventricular tissue from previously frozen human hearts was obtained by an Institutional Review Board-approved protocol maintained by the University of Colorado Denver Cardiac Tissue Bank. Hearts donated for research purposes were obtained under written consent from family members of organ donors or by direct written consent from end-stage DCM patients undergoing cardiac transplantation. Patient characteristics are presented in Table S1.

Animal Models
Lean male spontaneously hypertensive heart failure (Mcc facet; SHHF) rats were obtained from a colony maintained at the University of Colorado by Dr. Sylvia McCune. SHHF rats were selected for these studies based on their well-characterized development of progressive hypertensive heart disease leading to pathologic hypertrophy and terminal heart failure by 22-24 months of age, sharing many of the hallmark biochemical and pathophysiological features of DCM in humans1, including phospholipid remodeling2. Two cohorts of animals were studied: 1) at 21-22 months of age when animals exhibit early signs of dilated heart failure (HF), and 2) following rapid induction of pathologic left ventricular hypertrophy by thoracic aortic banding at 3 months of age (TAC; see Supplemental Methods). HF rats were matched on echocardiographic parameters at 21 months of age prior to being semi-randomly assigned to treatment or control groups for the 4 week treatment period. TAC surgery was performed as previously described2 in 3 month old rats. Two weeks following confirmation of successful TAC (see Figure S3), two cohorts of animals were matched on aortic Doppler and M-mode echocardiography parameters to ensure uniform responses and baseline pathology before being randomly assigned to receive the D6D inhibitor or no drug for 4 weeks. The effect of the D6D inhibition was also examined in 3 month old SHHF rats exposed to a sham TAC surgery (Sham) that were followed along with TAC groups for 4 weeks as an experimental control. All animals were provided Purina 5001 chow and water ad libitum for the duration of the study. At the conclusion of the study, animals were sacrificed with a lethal dose of sodium pentobarbital (150 mg/kg i.p.) followed by midline thoracotomy and removal of the heart. All procedures were approved by the Animal Care and Use Committee at Colorado State University and/or University of Colorado Boulder in strict compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Inhibition of D6D in vivo
Rats were administered the potent, orally active D6D inhibitor SC-26196 (2,2-diphenyl-5-(4-[[1 E]-pyridin-3-yl-methylidene]amino)piperazin-1-yl)pentanenitrile a generous gift from Dr. Mark Obukowicz, Pfizer Corporation; at 100 mg/kg/d mixed in chow based on daily food consumption records taken over
3-4 weeks, A detailed description of the pharmacokinetics and selectivity of this compound for D6D over the other desaturase enzymes (D5D and stearoyl-CoA desaturase, D9D) has been reported elsewhere. The 100 mpk dose for 4 weeks was selected based on previous studies demonstrating full inhibition of D6D (LA to AA conversion) and expected changes in tissue PUFA levels in rodent models and pilot studies in our lab demonstrating less inhibition with 50 mpk and similar effects of 200 mpk in aged SHHF rats (data not shown).

**Echocardiography and Blood Pressure**

Transthoracic echocardiography was performed in isoflurane anesthetized rats prior to and following the 4 week experimental period using a 12 MHz pediatric transducer connected to a Hewlett Packard Sonos 5500 Ultrasound as previously described. Tail cuff blood pressure measurements were obtained in the Sham and HF groups using the Kent Coda 6 system (Kent Scientific, Torrington, CT) in lightly isoflurane-anesthetized rats.

**Lipid analyses**

To determine the global fatty acid profile of tissue phospholipids, lipids were extracted from ~40 mg of tissue or 100 µL serum using 4 ml of 2:1 Chloroform:Methanol and 1ml of water. The aqueous layer was removed and the lipid containing fraction was dried down using nitrogen and re-suspended in 0.5ml hexane. Thin layer chromatography was then used to separate out the phospholipid fraction using a 20cm x 20cm silica gel TLC plate in a 70:30:1 hexane:ethyl ether:acetic acid solution. The band associated with the phospholipid fraction was scraped and dissolved in 0.5ml hexane and 0.5ml 0.5N KOH. 2ml of 14% BF₃-methanol was then added and samples were heated at 100 °C for 30 minutes to obtain methyl esters for subsequent determination of fatty acid composition by gas chromatography. Separation of individual phospholipid classes was performed by normal phase liquid chromatography (Agilent Zorbax Rx-Sil column, 4.6 X 250mm, 5-micron) using a Hexane:Isopropanol:Potassium Acetate mobile phase gradient optimized for separation of PE, PC and CL by UV detection (206 nm). Fractions were collected based on elution time of known standards, evaporated under a nitrogen stream, and resuspended in hexane for GC analysis as described above.

GC analysis was performed using an Agilent Technologies DB-225 30m x 0.250mm x 0.25µm column (model 122-2232, J&W Scientific) on an Agilent 6890 Series Gas Chromatographer. The initial temperature of the oven was 120 °C with an initial ramp temperature of 10°C/min for 8 minutes, then 2.5°C/min for 4 minutes and held at 210°C for the remaining 6 minutes for a total run time of 20 min. The inlet split ratio was 15:1 with the column at constant flow and an initial flow, pressure, and velocity at 1.8ml/min, 23.59 psi, and 42 cm/sec, respectively.

Tissue PC and PE contents were determined by colorimetric phosphorus assay on LC fractions obtained from 20-30mg tissue as described above. Briefly, evaporated fractions were heated in perchloric acid overnight at 160 °C and cooled, followed by the sequential addition of 275 µl of H₂O, 41.7 µl of 20.2 mM Ammonium molybdate, and 41.7 µl of 0.568 M Ascorbic Acid, vortexing each for 20 seconds. The assay mix was then heated for 5 minutes at 100°C, cooled and read on a spectrophotometer at 800 nm for quantitation of total phosphorus based on a KH₂PO₄ standard curve.

Cardiolipin molecular species were determined in lipid extracts from 0.25 mg of mitochondrial protein isolated from rat left ventricle or 40 mg of LV protein (humans) by electrospray ionization mass spectrometry previously described in detail. “Total” cardiolipin represents the m/z sum of the 10 most prevalent CL species detected. Myocardial contents of free AA and eicosanoid species were quantified in lipid extracts obtained from 50 mg of LV tissue by LC/MS/MS methods developed in Dr. Murphy’s laboratory using deuterated standards as previously described.

**Mitochondrial isolation and respiratory function**

Mitochondria were freshly isolated from ~300 mg of left ventricular (LV) tissue by differential centrifugation in the absence of proteases as described previously. State 3 (ADP-stimulated) and state 4-
4 (ADP-limited) mitochondrial respiratory function was measured in isolated mitochondria (0.25 mg protein) using a Clark-type electrode system (Strathkelvin) at 30°C with pyruvate + malate as substrates as previously described.  

Biochemical analyses

Immunoblotting for D6D, (p)ERK, IκBα, MDA and HNE-adducts, and antioxidant enzymes were performed in 40-50 mg LV homogenates by standard methods using commercially available antibodies and chemiluminescent detection. Blot densities were normalized to total lane protein content (Coomassie or ponceau staining) to control for any differences in loading on each membrane, then expressed relative to at least 2 Sham samples on each gel to allow for comparisons between blotting experiments. The source, catalog number and blotting conditions used for each of the antibodies used are listed below.

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<th>Antibody</th>
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<th>Buffer</th>
<th>Temp.</th>
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<tr>
<td>CuZnSOD (Assay Designs, #SOD-101)</td>
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<td>MDA (Anti-Malondialdehyde)</td>
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<td>HNE (Anti-HNE-Michael Adducts Reduced)</td>
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<td>5%NFM</td>
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<tr>
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</tr>
<tr>
<td>ERK (Cell Signaling, #9102)</td>
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<tr>
<td>OXPHOS (Mitosciences, #MS604)</td>
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<td>IκBalpha (Abcam, #ab32518)</td>
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Serum glucose and free fatty acids were determined by colorimetric assays according to the manufacturer's instructions (Biovision). Caspase activities were determined in 30 mg of LV homogenates by luminescence assay (Promega). Myocardial hydroxyproline was quantitated as a marker of collagen (fibrosis) in 30-40 mg of septal tissue by the colorimetric assay of Switzer and Summer. Serum cytokines were determined in 80 μL of sample by ELISA cytokine array (Raybiotech).

Statistical analyses

All data are presented as group means ± standard error. Human heart data were compared by one way ANOVA with Tukey tests post hoc when appropriate. Rat data were analyzed by 3(condition) X 2(drug) ANOVA to determine main and interaction effects with Tukey tests post hoc for determination of significant group differences. Within-group differences in echocardiography data from pre- to post-treatment were compared by paired t-tests. Statistical significance was established at P < 0.05 for all analyses.
## Supplemental Tables

### Table S1. Patient Characteristics

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<th>Lab code</th>
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<th>BP (mmHg)</th>
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<tr>
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<td>56</td>
<td>LVAD 9 mo</td>
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Table S2. Primer sequences used for qRT-PCR

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<td>TGTCACAAGTTTGCTATTG</td>
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<td>ATCCCTTTCTACGGCATCT</td>
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<td>TAGGCTCTCTGGTAACTCT</td>
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<td>Rat</td>
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<td>GTTGAAAGGCTGACTGGT</td>
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<tr>
<td>fads1</td>
<td>Human</td>
<td>Reverse</td>
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<tr>
<td>Elovl5</td>
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<td>Forward</td>
<td>TACCACATGCCACTATGCT</td>
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Table S3. Animal Morphology

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<th>TAC+SC</th>
<th>HF</th>
<th>HF+SC</th>
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<tr>
<td>Body Weight, g</td>
<td>285 ± 8</td>
<td>289 ± 10</td>
<td>260 ± 8</td>
<td>259 ± 10</td>
<td>394 ± 10*</td>
<td>381 ± 11*</td>
</tr>
<tr>
<td>Heart, gww</td>
<td>1.18 ± 0.03</td>
<td>1.19 ± 0.06</td>
<td>1.44 ± 0.06*</td>
<td>1.25 ± 0.03†</td>
<td>1.77 ± 0.04*</td>
<td>1.60 ± 0.04†</td>
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<tr>
<td>Lungs, gww</td>
<td>1.45 ± 0.04</td>
<td>1.50 ± 0.09</td>
<td>2.21 ± 0.25*</td>
<td>1.99 ± 0.21*</td>
<td>2.42 ± 0.10*</td>
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<tr>
<td>Brain, gww</td>
<td>1.76 ± 0.03</td>
<td>1.74 ± 0.02</td>
<td>1.77 ± 0.01</td>
<td>1.79 ± 0.02</td>
<td>2.11 ± 0.02*</td>
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<tr>
<td>Heart/BW, g/kg</td>
<td>4.15 ± 0.10</td>
<td>4.12 ± 0.12</td>
<td>5.57 ± 0.25*</td>
<td>4.88 ± 0.20†</td>
<td>4.39 ± 0.09*</td>
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<td>Heart/Brain, g/g</td>
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<td>0.86 ± 0.04</td>
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<td>1.11 ± 0.11*</td>
<td>1.16 ± 0.05*</td>
<td>1.05 ± 0.04*</td>
</tr>
</tbody>
</table>

Data are means ± SEM. *P < 0.05 vs. Sham; †P < 0.05 vs. untreated.
Supplemental Figures

Figure S1. Fatty acid composition of phospholipid extracts from human left ventricle.

Data are means ± SEM of %total fatty acids from phospholipids extracted from 30-50 mg of left ventricular (LV) tissue by thin layer chromatography (total phospholipids) or HPLC (individual phospholipid classes) as described in Methods (n = 4-8/group).
Figure S2. Phospholipid fatty acid composition of rat heart, liver and serum

Data are means ± SEM of %total fatty acids from phospholipids extracted from 30-50 mg of left ventricular (LV) or liver tissue and 30 µL serum, by TLC (total phospholipids) or HPLC (individual phospholipid classes) as described in Methods (n = 4-8/group).
Figure S3. Aortic outflow Doppler on TAC mice.

Following successful TAC surgery, aortic outflow acceleration time (AT) decreases markedly, while total ejection time (ET) increases, resulting in a dramatic elevation in ET/AT indicative of severe aortic stenosis. Thus, the ET/AT ratio served as a basis for pre-treatment matching of TAC and TAC+SC groups 2 weeks following surgery to ensure similar extents of aortic constriction prior to beginning treatment. No change in AT or ET/AT from 12-16 weeks in TAC or TAC+SC indicates a nearly identical maintenance of aortic constriction throughout the experimental period in both groups. Data are means ± SEM, n = 4-8/group. **P < 0.01 vs. Sham and Sham+SC. *P < 0.05 for TAC+SC vs. Sham.
Figure S4. Total CL content and representative mass spectra of CL molecular species from cardiac mitochondria.

Quantitation of the 18 most abundant CL species revealed no significant change in the amount of CL present in cardiac mitochondria (above left). Representative mass spectra obtained via electrospray ionization mass spectrometry show the relative abundance of CL molecular species obtained from cardiac mitochondria isolated from TAC and HF rats with and without D6D inhibition (SC-26196). X axis unit is mass/charge ratio (m/z), which is identical molecular weight, thus the peak at m/z 1448 is \( \text{L}_4\text{CL} \), and peaks at higher m/z values contain various combinations of AA and/or DHA (see Sparagna et al. J Lipid Res 46:1196, 2005). Y axis units are arbitrary relative peak intensities representing the relative abundance of CL species for each spectra.
Figure S5. D6D pathway expression

(A) Relative mean (+/- SEM) data for qRT-PCR of mRNA encoding D6D (fads2), delta-5 destaurase (fads1), and elongase-5 (elovl5) in the rat and human heart. *P < 0.05 vs. NF and DCM/HF; n = 4-6/group. (B) Representative blots and mean (+/- SEM) data for D6D protein by immunoblotting in rat and human tissues (n = 6/group).
Figure S6. Representative blots from Figures 6B (top) and 7D (bottom).

Blots are representative chemiluminescent images from several blotting experiments comparing all or some of the experimental groups. Data presented in Figures 6 and 7 were derived from several blotting experiments with various combinations of samples normalized to coomassie or ponceau staining for total protein, expressed relative to sham samples on each gel.
Figure S7. Semi-quantitative histological analysis of myocardial fibrosis

Semi-quantitative analysis of myocardial fibrosis was performed on Masson’s trichrome stained sections (10 µm thick) derived from 20-35 mg paraffin-embedded sections of LV free wall tissue fixed in 4% paraformaldehyde in PBS. The percent of interstitial collagen deposition was determined by measuring the total stained (blue) area relative total tissue area in at least 4 separate 1 X 1.4 mm light microscopy images of sections obtained from 2-4 rats per group using ImageJ software (NIH). Data are mean values of multiple sections from 2-4 rats per group ± SE. * P < 0.05 vs. Sham control. † P < 0.05 vs. untreated.
Supplemental References


