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Aldehyde stress and up-regulation of Nrf2-mediated antioxidant systems accompany functional adaptations in cardiac mitochondria from mice fed n-3 polyunsaturated fatty acids

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Abstract

Diets replete with n-3 poly-unsaturated fatty acids (n-3 PUFAs) are known to have therapeutic potential for the heart, although a specifically defined duration of n-3 PUFA diet required to achieve these effects remains unknown, as does their mechanism of action. This study was undertaken to establish whether adaptations in mitochondrial function and stress tolerance in the heart is evident following a short- (3 weeks) and long-term (14 weeks) dietary intervention of n-3 PUFAs, and to identify novel mechanisms by which these adaptations occur. Mitochondrial respiration (mO_2), H_2O_2 emission (mH_2O_2) and Ca^{2+} retention capacity (mCa^{2+}) were assessed in mouse hearts following dietary intervention. Mice fed n-3 PUFA's for 14 weeks showed significantly lower mH_2O_2 and greater mCa^{2+} compared to all other groups. However, no significant differences were observed after 3 weeks of n-3 PUFA diet, or in mice fed a high fat diet devoid of n-3 PUFAs for 14 weeks. Interestingly, at 14 weeks n-3 PUFA mice had significantly greater glutathione reductase activity, reflected by a substantially higher GSH/GSSG ratio. Levels of protein adducts of 4-hydroxyhexenal, an aldehyde formed from peroxidation of n-3 PUFAs, were significantly elevated in n-3 PUFA fed mice, even at 3 weeks. These findings demonstrate distinct time-dependent effects of n-3 PUFAs on mitochondrial function and stress tolerance in the heart. In addition, they are first to provide direct evidence that increases in non-enzymatic lipid oxidation products precede these mitochondrial and redox-mediated adaptations, thereby revealing a novel mechanism for n-3 PUFA action in heart.

Keywords

n-3 PUFAs; mitochondria; glutathione; permeability transition pore; aldehydes; lipid peroxidation

INTRODUCTION

Considerable interest in the relationship between dietary fatty acids and cardiac function has arisen from a number of recent reports showing that low-glycemic/high-fat diets preserve

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contractile force in experimental models of heart failure [1–3]. Notably, some of the most dramatic effects have been demonstrated by inclusion of n-3 poly-unsaturated fatty acids (PUFAs) to the diet [4, 5], findings that have been mirrored and compounded in importance recently by clinical studies showing that dietary supplementation with low-to-moderate doses of fish oil n-3 PUFA docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) improve left ventricular function [6] and decrease hospitalization rates and overall mortality [7] in patients with heart failure. However, despite the potential benefits and improved cardiac performance brought about by n-3 PUFAs in these studies, the proper dosage and duration of intake required to achieve these effects remain controversial, and their mechanisms remain obscure.

Mitochondria are of paramount importance in the heart for a multiplicity of reasons in addition to ATP production. Due to their role as critical loci of necrotic and apoptotic cell death programs within the cardiomyocyte, mitochondria have repeatedly been implicated to play significant roles in cardiomyocyte cell death, a singular feature of heart failure [8, 9]. Much focus has also been directed at cardiolipin, the phospholipid that is exclusive and crucial to mitochondrial inner-membrane functionality and integrity, as its decrease in concentration and peroxidation have been associated with increased cell death and mitochondrial dysfunction [10–12]. It has recently been shown that dietary supplementation with fish oil, particularly DHA, increases mitochondrial tolerance to Ca^{2+} overload (i.e. decreased necrotic susceptibility), and alters the cardiolipin composition of the mitochondrial inner-membrane [13, 14].

Aside from the direct effects of the n-3 PUFAs on mitochondria and other targets in the heart, indirect mechanisms may also be at work in these systems and must be considered. The high degree of unsaturation in all PUFAs makes them particularly vulnerable to lipid peroxidation, and cardiomyocytes have a number of adaptive processes with which to prevent lipid peroxidation and counteract the products formed from these reactions [15–17]. Indeed, the α,β -unsaturated hydroxyalkenal 4-hydroxynonenal (HNE) is a well-known and widely studied product of n-6 PUFA peroxidation. As a result of its chemical stability, lipophilic structure and electrophilic reactivity, HNE readily reacts with proteins and phospholipids to trigger alterations in cellular processes and enzymatic activities [18]. In contrast to HNE, far less is known about 4-hydroxyhexenal (HHE), the aldehyde formed from non-enzymatic peroxidation of n-3 PUFAs [19].

Since n-3 PUFA treatment in the heart has been repeatedly shown to cause increased tolerance to stress, we speculated that this may be due to a low-level, sub-toxic stress imposed by n-3 PUFAs on the heart which may induce protective adaptations over time (i.e. hormesis). The present study was undertaken to establish whether adaptations in mitochondrial function and improved tolerance to stress in the heart is evident following a short- (3 weeks) and long-term (14 weeks) dietary intervention of n-3 PUFAs and to determine the mechanism by which these adaptations occur. We provide evidence that long-term dietary intervention with n-3 PUFAs causes a substantial decrease in mitochondrial reactive oxygen species (ROS) emission, combined with improved tolerance to Ca^{2+} overload. Intriguingly, these alterations are accompanied by increased activity of key enzymes in the glutathione redox-balance system. We also provide completely novel data regarding the formation of HHE through dietary n-3 PUFAs, and how HHE-protein adduct formation in heart precedes these mitochondria and redox-mediated adaptations during the course of the diet.

EXPERIMENTAL

Animals, reagents and diet

Chemicals used in this study were all obtained from Sigma-Aldrich, with the exception of Amplex Red and Calcium Green 5-N, which were purchased from Invitrogen. Mice used for this study were male C57BL/6 (Charles River), aged 4–8 weeks at onset of the diet (Harlan-Teklad, Madison, WI). The composition of these diets was previously described in detail [20]. Briefly, the standard chow diet (Std Chow) consisted of 5% fat by weight and n-3 PUFA diet consisted of 20% fat by weight, representing ~13% and ~42% total kcal/day from fat, respectively. The sources of fat in these diets are of mixed composition: of the 13% kcal/day in Std Chow, 2% is saturated fatty acids (SFA), 3% mono-unsaturated fatty acids (MUFA), and 8% mixed PUFAs. Of the 42% in n-3 PUFA diet, 8% is SFA, 9% MUFA, and 24% PUFA. The PUFA composition breakdown consists of 2% kcal/day from DHA, 3% EPA, 11% linolenic acid, and ~25% mixed n-6 PUFAs. An additional set of mice were placed on a 42% kcal/day high-fat diet completely devoid of n-3 PUFA's, to serve as a high fat control (HFC) diet. Of the 42% kcal/day in HFC, 11% was SFA, 23% hydrogenated fatty acids (vegetable shortening), and 7% n-6 PUFA's. Mice were fed ad libitum for 3 weeks or 14 weeks as indicated, at which time they were euthanized (in fed state) and hearts removed, rinsed in saline and a portion of left ventricle (LV) used for mitochondrial experiments. The remaining LV and right ventricle (RV) were snap-frozen in liquid N₂ for biochemistry.

Preparation of permeabilized cardiac myofibers

This technique has been described by our group [21] and others [22] in detail. For this study, small portions (<30 mg) of LV tissue were dissected and placed in ice-cold *buffer X*, containing (in mM) 50 MES, 7.23 K₂EGTA, 2.77 CaK₂EGTA, 20 imidazole, 0.5 DTT, 20 taurine, 5.7 ATP, 14.3 PCr, and 6.56 MgCl₂·6 H₂O (pH 7.1, 290 mOsm). The muscle was trimmed of connective and vascular tissue. Five or six small muscle bundles (~4–6 mm in length, 1.0–2.5 mg wet weight) were prepared from each animal. These bundles were gently separated along their longitudinal axis with a pair of needle-tipped forceps under magnification (MX6 Stereoscope, Leica Microsystems, Inc., Wetzlar, DE). Cardiac fiber bundles were then treated with 50 µg/ml saponin in ice-cold buffer X, and incubated on a rotor for 30 min at 4°C. Following permeabilization, the fibers used for mitochondrial O₂ (mO₂) consumption experiments were placed in buffer Z containing (in mM) 105 K-MES, 30 KCl, 1 EGTA, 10 K₂HPO₄, and 5 MgCl₂·6H₂O, 0.005 glutamate, and 0.002 malate with 5.0 mg/ml BSA (pH 7.4, 290 mOsm). The fibers used for mitochondrial H₂O₂ (mH₂O₂) and Ca²⁺ uptake (mCa²⁺) were placed in ice-cold Buffer Y containing (in mM) 250 Sucrose, 10 Tris-HCl, 20 Tris base, 10 KH₂PO₄, 2 MgCl₂·6H₂O, and 0.5 mg/ml BSA). Permeabilized fibers remained in buffer Z on a rotator at 4°C until analysis (<100 min).

Measurement of mO₂, mH₂O₂ and mCa²⁺ in permeabilized cardiac myofibers

All mitochondrial measurements were performed at 30°C. The Oroboros O₂K Oxygraph system (Oroboros Instruments, Innsbruck, Austria) was used for all mO₂ consumption measurements. The mH₂O₂ and mCa²⁺ measurements were obtained using a spectrofluorometer (Photon Technology Instruments, Birmingham, NJ), equipped with a thermo-jacketed cuvette chamber. The mO₂ experiments were performed in Buffer Z. During the course of the experiments, substrates, nucleotides and respiratory inhibitors were provided as indicated in the Figure legends. Both mCa²⁺ and mH₂O₂ experiments in this study were all performed in Buffer Y with 100 µM ADP, 5 mM glucose and 1 U/ml hexokinase present to keep the mitochondria in a permanent, submaximal phosphorylating state. For mH₂O₂ measurements, Buffer Y contained 10 µM Amplex Red (Invitrogen, Carlsbad, CA), 1 U/ml horseradish peroxidase, 5 mM pyruvate, 2 mM malate, 5 mM Succinate, and mH₂O₂ emission was calculated as outlined in [21]. For mCa²⁺

measurements, Buffer Y contained 1 μM Calcium Green 5-N (Invitrogen) and 5 mM pyruvate, 2mM malate. At start of mCa^{2+} experiments, 1 μM Thapsigargin was added to inhibit SERCA, and 40 μM EGTA added to chelate residual Ca^{2+} and to establish F_{min} . Pulses of 4 nmols Ca^{2+} (CaCl_2) were sequentially added and Ca^{2+} uptake followed until mPTP opening as in [23]. At end of experiment, 1 mM CaCl_2 was added to saturate the probe and establish F_{max} . Changes in free Ca^{2+} in cuvette during mCa^{2+} uptake were then calculated using the known K_d for Calcium Green 5-N and the equations established by Tsien's group for calculating free ion concentrations using ion-sensitive fluorophores [24]. At conclusion of all experiments, fibers were rinsed in ddH₂O, lyophilized in a freeze-dryer (Labconco, Kansas City, MO) for > 2 hours and weighed on a micro-scale. Data are expressed as $\text{pmol}\cdot\text{min}^{-1}\cdot\text{mg dry wt.}^{-1}$ (mH_2O_2), or $\text{nmol}\cdot\text{mg dry wt.}^{-1}$ (mCa^{2+}).

Glutathione (GSH/GSSG) and related enzyme activity measurements

Myocardial samples frozen in liquid N₂ were homogenized in 10X (wt./vol) TEE buffer containing (in mM: 10 Tris base, 1 EDTA, 1 EGTA), and 0.5% Tween-20, using a glass grinder (Kimble Chase, Vineland, NJ), and protein samples prepared for glutathione measurements as described previously [25] using a modified Tietze method [26]. For measurements of GSSG, samples were prepared in presence of 1-methyl-2-vinylpyridium triflate. Glutathione reductase (GR) activity in myocardial tissue was determined in TEE buffer containing 1 mM GSSG and 0.5 mM NADPH, where activity was calculated from the linear decrease in NADPH absorbance with time [27]. Glutathione peroxidase (GPx) activity was determined in TEE buffer containing 1 mM GSH, 100 mU/ml glutathione reductase enzyme, 0.5 mM NADPH. The reaction is initiated with a nominal amount of tert-Butyl-Hydroperoxide and the activity of GPx was calculated from the linear decrease in NADPH absorbance with time [28].

Immunoblot analysis of myocardial protein

Samples of myocardium were homogenized in 10X (wt./vol) TEE buffer, to which Complete™ Protease inhibitor cocktail (Roche, Mannheim, Germany) was added. Protein was then separated using SDS-PAGE, transferred to PVDF membrane and subjected to immunoblot using Mito OxPhos antibody cocktail™ (Mitosciences, Eugene, OR), which recognizes discrete subunits of mitochondrial OxPhos complexes I (~20 kDa), II (~30 kDa), III (~39 kDa), IV (~45 kDa), V (~50 kDa), in the electron transport system, and anti-GAPDH (Advanced Immunochemical, Long Beach, CA). For immunoblot analysis of aldehyde protein adducts, monoclonal antibodies to HHE-adducts (Genox, Baltimore, MD) and HNE-adducts (Percipio Biosciences, Inc, Foster City, CA) were used. Densitometry was performed using Image J software [29]. Quantification of total HHE- and HNE-adduct levels were normalized to GAPDH for each group.

Statistics

Data are presented as means $\pm$ SEM. Data were normally distributed which allowed for the use of parametric statistics. Therefore, interval variables between groups were compared using two-tailed student's *t*-test or 1-way ANOVA with post-hoc Dunnett's *t*-test for significance (GraphPad Prism, La Jolla, CA), as appropriate. Differences between groups was considered statistically significant for $P < 0.05$.

RESULTS

The body weights were identical between Std Chow and n-3 PUFA groups at 3 weeks, but by 14 weeks, animals had gained more weight on the n-3 PUFA diet than Std Chow (Table 1). Cardiac mass was not significantly affected by diet (not shown). In a previous report

using these same cohorts of mice, we demonstrated that levels of n-3 PUFAs in tissues were substantially increased as a result of this diet [20], so we have not reported them here.

Time-dependent effects of n-3 PUFA diet on mO_2 , mH_2O_2 and mCa^{2+} in mouse heart

To keep cardiac mitochondria permanently in a submaximal phosphorylating state (most physiological), we used 100 μ M ADP + glucose/hexokinase in our mH_2O_2 and mCa^{2+} experiments [23]. Mitochondria in permeabilized cardiac myofibers from mice fed n-3 PUFA diet for 3 weeks showed no significant differences in mH_2O_2 when supported by pyruvate, malate and succinate, or mCa^{2+} when supported by pyruvate, malate (Fig. 1A+B, left panel). However, under identical experimental conditions, cardiac mitochondria from mice fed n-3 PUFA diet for 14 weeks showed significant changes reflected by decreased rates of mH_2O_2 (Fig. 1A, right panel) and increased mCa^{2+} retention capacity (Fig. 1B, right panel). Both basal and maximal ADP-stimulated rates of mO_2 supported by pyruvate, malate, glutamate and succinate in permeabilized cardiac myofibers from mice fed n-3 PUFA diet for both 3- and 14-weeks were not significantly increased, though directionality towards an increase was observed (Table 2). Basal and maximal ADP-stimulated mO_2 supported by palmitoyl-L-carnitine and malate were also unchanged. Importantly, no significant differences were observed in respiratory control ratio (RCR), an indicator of mitochondrial coupling, between Std Chow and n-3 PUFA at either 3- or 14-weeks (Table 2), regardless of whether pyruvate/malate or palmitoyl-L-carnitine were used. Further, immunoblot analysis of complexes I – V in mitochondrial electron transport system from Std Chow and n-3 PUFA diet for 3- and 14-weeks showed no differences in protein content (not shown). To establish that the differences observed in cardiac mH_2O_2 and mCa^{2+} after 14 weeks of n-3 PUFA diet were due to n-3 PUFA and not simply a reflection of a high fat diet, an additional set of mice fed a high fat ‘control’ diet (HFC) comprised of a mixture of fatty acids was studied under identical experimental conditions. Mice fed HFC diet for 14 weeks were found to have no significant differences, compared to Std Chow, in any parameter of cardiac mitochondrial function measured (Fig. 1 + Table 1).

Effects of n-3 PUFA diet on cardiac glutathione and its related enzymes

We speculated that the decreased mH_2O_2 and increased mCa^{2+} tolerance observed in the n-3 PUFA fed mouse hearts might be due to increased antioxidant capacity (e.g. glutathione) in the tissue, as other groups have shown that the rates of mH_2O_2 efflux and opening of mitochondrial permeability transition pore (mPTP) can be greatly affected by glutathione status [30–32]. No significant differences were observed in levels of total (GSHt) or oxidized glutathione (GSSG) following 3- or 14-weeks of n-3 PUFA diet, or 14-weeks of HFC diet (Fig. 2A+B). However, cardiac GSH/GSSG ratio was significantly higher in the 14-week n-3 PUFA mice compared to Std Chow (Fig. 2C). To determine whether the differences in GSH/GSSG ratio in n-3 PUFA group at 14 weeks were due to altered activity of the enzymes responsible for maintaining this ratio, we measured activities of glutathione peroxidase (GPx) and reductase (GR) in cardiac tissue from these mice. As shown in Fig. 3A, no significant differences were observed in cardiac GPx activity at either 3- or 14 weeks, but cardiac GR activity was nearly 2-fold greater in mice fed n-3 PUFA diet for 14 weeks, though unchanged at 3 weeks (Fig. 3B). Both GPx and GR activity remained similar in cardiac tissue from mice fed HFC diet for 14 weeks, as compared to Std Chow (Fig. 3).

Aldehyde stress in myocardium of mice fed n-3 PUFA diet

Since aldehydes have been shown to trigger compensatory increases in glutathione synthesis in heart [17, 33, 34], we sought to ascertain whether the increases in GR and GSH/GSSG after 14 weeks of n-3 PUFA diet were a result of increased aldehyde stress throughout the course of the diet. Interestingly, the levels of HHE-adduct formation were already substantially higher in hearts of n-3 PUFA mice at 3 weeks compared to Std Chow. At 14

weeks, levels of HHE-adducts in n-3 PUFA mice were still significantly greater than Std Chow, and no significant differences existed between Std Chow and HFC diet for 14 weeks (Fig. 4A + B). Unlike HHE, however, levels of HNE- adducts did not significantly change under any of the dietary treatments examined (Fig. 4C).

DISCUSSION

Therapeutic application of dietary n-3 PUFAs in cardiac disease remains controversial despite years of experimental and clinical work in this area. The origin of much of this controversy can be attributed to the wide disparity in approaches used by investigators with respect to source of n-3 PUFA (fish vs. flaxseed oil) and dose required to achieve the observed effects. Even less is known about the time-dependent kinetics of these effects. In this context, the novelty of the study presented here is that 1) hearts from mice fed n-3 PUFA diet have decreased mitochondrial H_2O_2 emission and increased Ca^{2+} -retention capacity; 2) these mitochondrial adaptations are dependent upon time; 3) these mitochondrial adaptations are accompanied by increased glutathione redox status and increased activity of GR; and 4) accumulation of HHE, a lesser known and under-studied aldehyde produced exclusively from lipid peroxidation of n-3 PUFAs, precedes these adaptations in mitochondrial function and glutathione redox chemistry during the course of the diet. These results show a unique and important chronological sequence of biochemical events which may explain, at least in part, a mechanistic basis of n-3 PUFA action in heart, and establish a role for non-enzymatic lipid oxidation products of n-3 PUFA's in this process.

Increased capacity of cardiac mitochondria to buffer Ca^{2+} would be desirable for a variety of reasons, ranging from prevention of arrhythmia [35], increased activation of mitochondrial dehydrogenases and ATP production [36], and decreased susceptibility to Ca^{2+} -induced opening of mPTP and cell death [37, 38]. In the present study, a number of factors may explain the observed increase in mitochondrial Ca^{2+} -retention capacity in hearts of n-3 PUFA mice, consistent with reports from other labs. Similar studies using *in vitro* preparations of isolated cardiac mitochondria from fish oil-treated rats showed increased Ca^{2+} -retention capacity [13, 14] before mPTP opening, and global ischemia/reperfusion injury in *ex vivo* rat hearts show that hearts from fish oil-fed animals have increased recovery of contractile force and decreased infarct sizes compared with chow-fed or n-6 PUFA-fed animals [39, 40]. The authors of these studies have largely focused on how n-3 PUFAs may be altering mitochondrial phospholipid fatty acid composition, particularly in cardiolipin, and the impact that this remodeling may have on the biophysical/biochemical interactions between cardiolipin and key proteins involved in mitochondrial Ca^{2+} uptake and retention capacity.

An invariable link in all of these previous studies on n-3 PUFA treatment is that the levels of n-6 PUFAs, particularly arachidonic acid (AA), were shown to decrease in cell membranes following n-3 PUFA treatment, and levels of DHA increased. Importantly, the characteristic decrease in AA and increase in DHA following fish oil treatment has been shown to be most pronounced in mitochondrial membranes [41], reflected even by alterations in cardiolipin composition [13]. While no studies to date have shown specifically how altering cardiolipin composition may affect mitochondrial Ca^{2+} uptake and retention capacity, there is good evidence that decreased AA levels may be important in this process given that AA has been shown to trigger increased susceptibility to Ca^{2+} -induced mitochondrial swelling and cell death [42, 43]. Given these previous findings, it is plausible that the increased mitochondrial Ca^{2+} retention capacity and delayed mPTP observed in the present study is a result of mitochondrial phospholipid remodeling, although these parameters were not measured here. However, even if these parameters had been measured in this study, it would not have added

much to the field given that these effects are already well-established, and at present no clear biophysical/biochemical mechanism exists as to how changing the composition of mitochondrial fatty acids and/or cardiolipin would change the structure/function of mitochondrial inner-membrane proteins and enzymes.

We decided to direct our investigation towards other potential novel mediators of Ca^{2+} -induced mPTP to see if additional pathways and/or factors were also changing in response to n-3 PUFA diet. Given the substantial decrease in mitochondrial H_2O_2 emission in hearts from n-3 PUFA diet, another interesting and novel finding in this study, we decided to focus on glutathione and activity of its related enzymes. Our rationale for this was predicated on previous studies showing that oxidative stress and lipid peroxidation specifically alter key components of mPTP such as adenine nucleotide translocase [44, 45], cyclophilin D [46], and cardiolipin [47, 48]. If levels of glutathione were increased, or activities of glutathione-related enzymes GPx or GR were increased following n-3 PUFA diet, then theoretically the reactive thiols within these mPTP components would be preserved in a reduced state for longer periods of time, thereby decreasing the mPTP sensitivity to Ca^{2+} . We found that hearts from n-3 PUFA fed mice at 14 weeks had significantly higher GSH/GSSG ratio, due in part to the significantly increased activity of GR. This increase in GR activity following n-3 PUFA treatment is an unprecedented finding and would partly explain the changes in mitochondrial H_2O_2 emission and Ca^{2+} retention seen in hearts of these animals [49]. Whether these adaptations in GSH/GSSG and GR activity in cardiac homogenate, as it was assessed in this study, is reflective of these levels in mitochondria is not completely known. However, because mitochondria cannot synthesize their own glutathione pools and must rely on cytosolic stores for their own supply [50], and because mitochondrial matrix represents such a large proportion of the total volume of mouse heart, it is reasonable to conclude that the values obtained in homogenate do reflect, to a large extent, that of the mitochondria.

In consideration of the mechanism by which n-3 PUFA diet may be inducing these adaptations in the heart, it becomes necessary to consider the chemical nature of PUFAs. Because of their structure and chemical composition, PUFA's are highly susceptible to electrophilic attack and peroxidation, particularly at the methylene group located between the C=C bonds. Peroxidation of PUFAs is a process that can proceed through both enzymatic and spontaneous, non-enzymatic pathways. Enzymatic peroxidation of PUFAs, controlled largely by cyclo-oxygenases (COX) and lipoxygenases (LOX), result in production of eicosanoids. These pathways have been a well-known and intensely studied component of the systemic immune and inflammatory response for decades [51]. Non-enzymatic oxidation of PUFAs results in formation of a number of bioactive intermediates, and these intermediates, if left unchecked, ultimately form chemically stable and highly electrophilic aldehydes called 4-hydroxyalkenals [52]. The aldehyde formed in this manner that has received the most scrutiny from investigators is HNE, a nine-carbon aldehyde formed from peroxidation and degradation of n-6 PUFAs such as AA and linoleic acid [18]. Like many electrophiles, HNE exerts bi-directionality in its effect on cells and tissues. At low, sub-toxic levels, HNE is cardioprotective and induces up-regulation of many enzymes responsible for ROS de-toxification and cell survival, but is cytotoxic at high levels [53, 54]. Non-enzymatic peroxidation of n-3 PUFAs such as DHA, EPA and α -linolenic acid results in formation of HHE, a 6-carbon aldehyde with many similarities to HNE but slightly less electrophilic [19]. In contrast to HNE, almost nothing is known about HHE and its role in cell signaling and cytotoxicity.

Both HNE and HHE covalently react with proteins to form adducts. In this study, we have demonstrated for the first time that levels of HHE-adducts are increased in hearts of mice fed n-3 PUFA diet. Conversely, no significant changes in HNE-adducts were observed. These findings are significant because they unlock a potential clue as to the underlying

signals driving the increased GSH/GSSG, GR, and mitochondrial adaptations at 14 weeks. One possibility is that chronic increases in lipid peroxidation products from n-3 PUFAs, including HHE, up-regulate the expression and activity of glutathione-related enzymes through an NF-E2-Related Factor 2 (Nrf2)-dependent mechanism. Indeed, both HNE [15] and HHE [34] have been shown to activate Nrf2 in cardiovascular cell types. Alternatively, an elegant and comprehensive study by Fukuda's group has recently outlined a novel signaling pathway whereby aldehyde stress in the heart increases glutathione synthesis and enzyme activity through eukaryotic initiation factor (eIF) 2⁻-activating transcription factor 4 (Atf4) pathway [17]. Whether such pathways explain the data presented in this study remains to be determined, though it is interesting to note that increases in HHE-adducts are already present at 3 weeks, preceding the up-regulation of GSH/GSSG, GR and mitochondrial adaptations in chronological order (Fig. 4). This supports a causal role for HHE in this process.

To conclude, the findings of this study represent a completely novel paradigm regarding n-3 PUFA action in heart, in that it outlines a time-dependent framework of adaptive processes that occur in myocardium at the mitochondrial and tissue level during the course of n-3 PUFA treatment. Furthermore, it illustrates a role for n-3 PUFA lipid peroxidation products as potential mediators of n-3 PUFA cardioprotection, and lays a foundation for future work into the role of non-enzymatic lipid peroxidation of PUFAs in cardiac health and disease.

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Abbreviations used

PUFA	poly-unsaturated fatty acids
EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid
ADP	adenosine diphosphate
ATP	adenosine triphosphate
AA	arachidonic acid
HNE	4-hydroxynonenal
HHE	4-hydroxyhexenal
mPTP	mitochondrial permeability transition pore

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Figure 1.

Dietary n-3 PUFA intake causes time-dependent adaptations in mitochondrial function in mouse heart, resulting in decreased mH₂O₂ emission (A) and increased tolerance to mCa²⁺ overload (B) by 14 weeks. Shown are mean $\pm$ S.E.M. in N = 7 for 3 week diet and N = 5 for 14 week diet; * P<0.05 vs. Std Chow

Figure 2. Time-dependent effects of dietary n-3 PUFAs on levels of total glutathione GSH_t (A), oxidized glutathione GSSG (B) and GSH/GSSG ratio (C) in mouse hearts. Data are mean $\pm$ S.E.M. in N = 7 for 3 week diet and N = 6 for 14 week diet; * P<0.05 vs. Std Chow

Figure 3. Rates of glutathione peroxidase (A) and glutathione reductase (B) activity in mouse heart after dietary n-3 PUFA treatment for 3 and 14 weeks. Data are mean $\pm$ S.E.M. in N = 7 for 3 week diet and N = 6 for 14 week diet; * P < 0.05 vs. Std Chow

Figure 4. Immunoblots of protein adducts with 4-hydroxyhexenal, a n-3 PUFA-derived aldehyde, in mouse heart following n-3 PUFA diet are shown in representative blots (A) and quantified by densitometry (B). Shown in (C) are quantified levels of protein adducts with 4-hydroxynonenal, a n-6 PUFA-derived aldehyde. Data are mean $\pm$ S.E.M. in N = 6 for 3 week diet and N = 4 for 14 week diet; * P<0.05 vs. Std Chow

Table 1

Diet	Duration (wks)	Terminal Body Wt. (g)	Wt. Gain (g)
Std Chow	3	22.8 ± 0.8	7.0 ± 0.8
n-3 PUFA	3	23.5 ± 0.5	6.9 ± 1.2
Std Chow	14	31.6 ± 1.5	15.8 ± 2.1
n-3 PUFA	14	39.1 ± 0.8 *	22.6 ± 1.6 *
HFC	14	35.9 ± 1.3	19.8 ± 0.9

Data shown are mean ± S.E.M. in N = 6;

*
P<0.05

Table 2

Diet	Duration (wks)	Respiratory Substrates/Conditions						
		P/M	P/M _{ADP}	P/M/G/S _{ADP}	P/M/G/S _{ADP} + Rot	RCR	PC/M	PC/M _{ADP} RCR
Std Chow	3	190.5 ± 13.6	819.1 ± 70.3	1437 ± 122.5	947.5 ± 72.1	5.0 ± 0.5	127 ± 19.1	506.3 ± 67.6 3.8 ± 0.3
n-3 PUFA	3	215.7 ± 11.6	1097 ± 123.9	2044 ± 271.8	1546 ± 169.6	5.1 ± 0.3	153 ± 19.5	611.7 ± 82.3 4.1 ± 0.5
Std Chow	14	240.2 ± 36.7	1302 ± 200.7	1671 ± 259.3	969.4 ± 119.2	5.4 ± 0.5	164.6 ± 19.9	625.3 ± 93.6 3.6 ± 0.6
n-3 PUFA	14	347.6 ± 63.3	2248 ± 530.8	3301 ± 736.3	1832 ± 337.7	6.3 ± 0.6	205.5 ± 39.6	606.4 ± 103.9 3.1 ± 0.3
HFC	14	402.5 ± 152.6	2537 ± 732.8	3134 ± 759.8	1966 ± 444.2	6.2 ± 0.7	261.2 ± 57.2	856.9 ± 168.7 3.3 ± 0.4

Data shown are mean ± S.E.M. in N = 7 for 3 week n-3 PUFA diet and N = 5 for 14 week n-3 PUFA diet. High Fat Control diet (HFC). Rates of respiration are in presence of 5 mM pyruvate/2 mM malate (P/M); P/M + 5 mM ADP (P/M_{ADP}); P/M_{ADP} + 5 mM glutamate, 5 mM succinate (P/M/G/S_{ADP}); P/M/G/S_{ADP} + 1 μM Rotenone (P/M/G/S_{ADP} + Rot); 100 μM palmitoyl-L-carnitine/2 mM malate (PC/M); PC/M + 5 mM ADP (PC/M_{ADP}); Respiratory Control Ratio (RCR)