Published in final edited form as:

Free Radic Biol Med. 2013 May ; 58: 35–44. doi:10.1016/j.freeradbiomed.2013.01.005.

HNE catabolism and formation of HNE adducts are modulated by beta oxidation of fatty acids in the isolated rat heart

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Abstract

We previously reported that a novel metabolic pathway functionally catabolizes 4-hydroxy-2-(*E*)-nonenal (HNE) via two parallel pathways, which rely heavily on β oxidation pathways. The hypothesis driving the present report is that perturbations of β oxidation will alter the catabolic disposal of HNE, favoring an increase in the concentrations of HNE and HNE modified proteins that may further exacerbate pathology. The current study employed Langendorff perfused hearts to investigate the impact of cardiac injury modeled by ischemia/reperfusion and, in a separate set of perfusions, the effects of elevated lipid (typically observed in obesity and type II diabetes) by perfusing with increased fatty acid concentrations (1 mM octanoate). During ischemia, HNE concentrations doubled and the glutathione-HNE adduct and 4-hydroxynonanoyl-CoA were increased by 7- and 10-fold, respectively. Under conditions of increased fatty acid, oxidation to 4-hydroxynonenoic acid was sustained, however, further catabolism through β oxidation was nearly abolished. The inhibition of HNE catabolism was not compensated by other disposal pathways of HNE, rather an increase in HNE-modified proteins was observed. Taken together, this study presents a mechanistic rationale for the accumulation of HNE and HNE-modified proteins in pathological conditions that involve alterations to β oxidation, such as myocardial ischemia, obesity and high fat diet induced diseases.

Keywords

4-hydroxy-2(*E*)-nonenal; catabolism; fatty acid (β) oxidation; heart; lipid peroxidation; mass isotopomer

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Introduction

4-Hydroxy-2(*E*)-nonenal (HNE) is one of the most abundant lipid peroxidation products derived from ω -6 polyunsaturated fatty acids (PUFA). HNE is elevated in oxidative stress-related diseases [1–5], such as Alzheimer's [6,7], atherosclerosis [8–10], ischemia/reperfusion [8,11–13], obesity and diabetes [8]. The strongly electrophilic nature of HNE is imparted through the α,β -unsaturated aldehyde, made further susceptible to Michael addition through the inductive effects of the C-4 hydroxyl [14]. Due to its highly reactive nature, HNE forms conjugates with cellular components such as DNA, phospholipids, and proteins, negatively impacting function [1,3].

Under normal physiological conditions, the concentration of HNE is maintained below 1 μ M through the action of several detoxification pathways. These detoxification pathways have been previously described [15–20] and are defined by: reduction to 1,4-dihydroxy-2(*E*)-nonene (DHN) [13], conjugation via Michael addition or Schiff base adduct [21], and oxidation to 4-hydroxynonenic acid (HNEA). More recently, we have reported an additional pathway of HNEA disposal characterized by β oxidation of the HNE-derived product (HNEA) via two parallel metabolic intermediate pathways (A and B in Figure 4 of Reference 23) to generate acetyl-CoA, propionyl-CoA, and formic acid [22,23]. Pathway A is an isomerization process followed by β oxidation. 4-Hydroxynonanoyl-CoA (HNA-CoA) is isomerized to 3-hydroxynonanoyl-CoA via the formation of a new class of acyl-CoA, i.e., 4-phosphonanoyl-CoA (4-P-nonanoyl-CoA). 3-Hydroxynonanoyl-CoA is a regular β oxidation intermediate that is metabolized to acetyl-CoA and propionyl-CoA. Pathway B starts with a first round of β oxidation followed by one α oxidation to form hexanoyl-CoA which is further catabolized by β -oxidation to acetyl-CoA.

As β oxidation plays a major role in the catabolic degradation of HNE, it is likely that any alteration to β oxidation would change the catabolic rate of HNE and consequently increase HNE concentrations to create a greater burden on the detoxifying pathways in the cell. This is suggested by our previous work [21], where we compared the concentration of HNE in the livers from rats fed with different types of diets, i.e. “western” style diets (high fat and high carbohydrate) and ketogenic diets (high fat and low carbohydrate), which adversely regulate fatty acid oxidation. In these studies, HNE is accumulated in the liver from rats fed the western diet, whereas the ketogenic diet resulted in lower hepatic HNE concentrations. The potential underlying mechanism is related to an up-regulation of β oxidation by the ketogenic diet to accelerate the catabolic disposal of HNE, while the high fat western diets decreases the catabolic disposal of HNE because of the down-regulated β oxidation. To further confirm our proposed mechanism, we devised two experimental strategies in perfused rat hearts to investigate HNE catabolism during 1) ischemia, a condition that abolishes aerobic metabolism, including β oxidation [24–27] to evaluate endogenous HNE, and 2) an increased fatty acid concentration to mimic hyperlipidemia by introducing a known competitor with endogenous β oxidation substrates, octanoate (1mM) [28] and evaluating HNE catabolism by isotope analysis using [5,5,6,6,7,7,8,8,9,9,9- $^2\text{H}_{11}$]-4-hydroxy-2-(*E*)-nonenal ([$^2\text{H}_{11}$]HNE).

Materials and methods

Materials

General chemicals, octanoate, glutathione, and acyl-coenzyme A were purchased from Sigma-Aldrich (St. Louis, MO). HNE and [$^2\text{H}_{11}$]HNE were synthesized according to previously published procedures [29]. 4-Oxononenal (ONE) and 1,4-dihydroxynonene (DHN), [3- ^{13}C]-4-hydroxynonanonic acid ([3- ^{13}C]HNA) were also synthesized in the laboratory according to previously published methodology [21]. Antibodies were purchased

from the following sources: anti-4-hydroxy2-(*E*)-nonenal (AbCam, Cambridge, MA), anti-HSC70 (Santa Cruz Biotechnology, Santa Cruz, CA). Protease inhibitor cocktail (Complete EDTA free™) was purchased from Roche (Indianapolis, IN).

Synthesis of HNEA

HNEA was synthesized from ethyl 4-hydroxy-(*E*)-non-2-enoate according to previous methods, with minor modifications [22]. Briefly, aqueous LiOH (0.14 g, 6.0 mmol, 3 ml) was added to a solution of 4-hydroxy-(*E*)-non-2-enoate (0.50 g, 2.5 mmol) in MeOH (10 ml). After stirring for 3 h at room temperature, the reaction mixture was acidified with 1M HCl. The mixture was then extracted with dichloromethane (3 × 15 ml), the combined organic layers were dried over Na₂SO₄, and the solvent was removed *in vacuo*. The crude product was purified by column chromatography over silica (hexane, 45% ethyl acetate) to give HNEA (0.33 g, 1.9 mmol, 76%). NMR results were consistent with the literature [30]. ¹H-NMR (400 MHz, CDCl₃): 0.89 (t, 3H, J = 5.9 Hz), 1.30-1.22 (m, 6H), 1.56 (m, 2H), 4.35 (dt, 1H, J = 5.1, 5.9 Hz), 6.00 (d, 1H, J = 15.6 Hz), 6.39 (br, 1H), 7.05 (dd, 1H, J = 4.6, 15.6 Hz); ¹³C-NMR (CDCl₃): 13.9, 22.7, 24.8, 31.6, 36.3, 71.1, 119.4, 152.7, 171.7.

Animals

Adult, male Sprague-Dawley rats (300–350 g) were fed *ad libitum* for 8–12 days with standard laboratory chow prior to experiments. All experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University.

Heart perfusion experiments

Fed rats were anesthetized with 5% isoflurane in an induction chamber, and maintained via facial mask at 2% during the surgical procedure. A median laparotomy was performed and a bolus of heparin (500 U/Kg) was given via the inferior vena cava. After incision of the diaphragm, the thorax was filled with 100 ml of ice-cold Krebs-Henseleit buffer to initiate cardioplegia. The heart was quickly excised and immersed on ice-cold Krebs-Henseleit buffer until complete cardioplegia. The aortic arch was carefully dissected and cannulated with an 11-Gauge, stainless steel cannula attached to a modified Langendorff apparatus. The hearts were perfused in retrograde fashion (Langendorff) with gassed (95% O₂, 5% CO₂) modified Krebs-Henseleit buffer (pH 7.4) containing: 119 mM NaCl, 4.8 mM KCl, 2.6 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 11 mM glucose, and 0.05 mM carnitine [31]. Perfusate was delivered at continuous flow (12 ml/min) with a RAININ Rabbit-Plus peristaltic pump (Woburn, MA) in non-recirculating mode with an inline 0.22 μm pore size filter (Millipore, MA). A temperature-controlled cabinet and a water-jacketed chamber were used to maintain temperature. The perfusion medium was passed through a Radnoti glass heating coil (Monrovia, CA) equipped with an integral classic bubble trap before entering the heart cannula to ensure a constant heart temperature of 37°C. The hearts were allowed to beat spontaneously throughout the perfusion. At the end of each perfusion, hearts were quick-frozen in liquid nitrogen and stored at –80 °C until further analysis.

Ischemia & reperfusion

To investigate the influence of ischemia and reperfusion on the endogenous HNE metabolism, isolated heart perfusions followed 4 different protocols (Fig. 1). Each protocol group consisted of 5 different rats (n=5 × 4). Group 1 (G1) was a 45 minute perfusion control. Group 2 (G2) was a 90 minute control perfusion. Group 3 (G3) was perfused for 15 minutes to allow for baseline equilibration, followed by 30 minutes of global no-flow ischemia. Group 4 (G4) consisted of 15 minutes of baseline perfusion, 30 minutes global no-

flow ischemia followed by 45 minutes reperfusion. Hearts were quick-frozen at the end of each protocol. The detailed ischemia/reperfusion heart perfusions are outlined in Fig. 1.

Heart perfusion with [$^2\text{H}_{11}$]HNE $\pm$ octanoate

The following heart perfusion protocols were designed to investigate the exogenous HNE metabolism in heart under the competition of exogenous fatty acid (octanoate). Two different groups (group 5 and 6 in Fig. 1) of heart perfusions with [$^2\text{H}_{11}$]HNE $\pm$ octanoate were performed. The Group 5 (G5) comprised of 5 control heart perfusions with 0–50 μM [$^2\text{H}_{11}$]HNE introduced into the perfusate via infusion pump during the time period of 80 to 90 minutes. Group 6 (G6) was also perfused with 0–50 μM [$^2\text{H}_{11}$]HNE from 80 to 90 minutes, but with 1 mM octanoate present throughout the perfusion (0 to 90 minutes). Cardiac effluent samples (12 ml) were collected at 89 minutes. The inflow samples (12 ml) were collected after the removal of hearts in the end of perfusions. Hearts were quick-frozen in liquid nitrogen after perfusion and stored at -80°C . The selection of 1 mM octanoate as exogenous competition to the fatty acid oxidation in the heart has the following reasons. First, long chain fatty acid needs albumin as a carrier because of solubility [32]. However, the addition of albumin in the perfusate complicates the investigation of HNE catabolism because of the conjugation of HNE with albumin. Second, the primary metabolism of octanoate in the heart is the β oxidation to generate acetyl-CoA that enters citric acid cycle and generates ATP [28].

GC-MS assay of HNE, ONE, DHN, HNEA and HNA

HNE, ONE (HNE analog) and DHN (reduction product of HNE) were assayed using our previously described methods [21]. HNEA (oxidation product of HNE) and HNA (saturated oxidation product of HNE) in the effluent perfusate were assayed by GC-MS. One nmol of [$3\text{-}^{13}\text{C}$]-HNA was added to 200 μl of sample perfusates to serve as an internal standard. Protein was removed by precipitation with 1 ml of acetonitrile and centrifugation for 25 minutes at $800 \times g$. The supernatant was dried under nitrogen and the resulting residue was derivatized with 60 μl of N,O-bis(trimethylsilyl) trifluoroacetamide at 70°C for 1 hour. After derivatization, 1 μl of each sample was analyzed by GC/MS using an Agilent 6890 gas chromatograph and Agilent 5973 mass spectrometer equipped with an Agilent VF-5MS capillary column ($60\text{ m} \times 0.32\text{ mm} \times 0.25\text{ }\mu\text{m}$). The carrier gas was helium (1 ml/min) with a pulse pressure of 20.9 psi. The injection was in splitless mode. The temperature for both inlet and transfer line was set at 290 and 300°C , respectively. The ion source and quadrupole temperature were set at 230 and 150°C . The GC temperature program was as follows: start at 100°C , hold for 1 min, and increase by $10^\circ\text{C}/\text{min}$ to 240°C followed by $30^\circ\text{C}/\text{min}$ to 300°C , finally remaining at 300°C for 10 min. The m/z values monitored were for HNEA, 301; [$^2\text{H}_{11}$]HNEA, 312; HNA, 303; and internal standard, 304. All the masses were measured in electron impact (EI) ionization and selective ion (SIM) mode. The retention times of HNEA and HNA were 14.3 and 14.2 minutes, respectively.

LC-MS/MS assay of acyl-CoAs

Acyl-CoAs in heart tissues were assayed by our previously reported method [23,28].

LC-MS/MS assay of GS-HNE conjugates

GS-4-hydroxydecanenal, GS-HNE, and GS- $[\text{}^2\text{H}_{11}]$ HNE Michael addition adduct were assayed from our previously published methods, with modification [21,33]. Briefly, powdered, frozen heart tissue ($\sim 100\text{ mg}$) spiked with 0.1 nmol of GS-4-hydroxydecanenal (internal standard) was extracted for 2 min with 2 ml of 100 mM iodoacetic acid in 10 mM ammonium bicarbonate buffer (pH 9.8), 4 ml of acetonitrile, and 2 ml of chloroform using a Polytron homogenizer. The top layer was separated and dried under N_2 gas. After dissolving

the dried sample in 100 μ l of Milli-Q water, 20 μ l was injected on a Thermo Scientific Hypersil GOLD C18 column (150 $\times$ 2.1 mm) with a guard column (Hypersil GOLD C18 5 μ m, 10 $\times$ 2.1 mm) into an Dionex-UltiMate 3000 HPLC combined with a 4000 Qtrap mass spectrometer (Applied Biosystems, Foster City, CA). The chromatographic method was developed at 0.2 ml/min (i) from 0 to 25 min with a 1–45% gradient of buffer B (95% acetonitrile, 5% water and 0.25% formic acid) in buffer A (95% water, 5% acetonitrile and 0.25% formic acid), (ii) from 25 to 26 min with a 45–90% gradient of buffer B in buffer A, (iii) from 26 to 31 min with 90% buffer B in buffer A, (iv) from 31 to 32 min with a 90–1% gradient of buffer B in buffer A, and (v) 10 min of equilibration with 99% buffer A before the next injection. The 4000 QTrap mass spectrometer was operated under positive ionization mode with the following source settings: turbo-ion-spray source at 600 $^{\circ}$ C, N_2 nebulization at 65 p.s.i., N_2 heater gas at 55 p.s.i., curtain gas at 30 p.s.i., collision-activated dissociation gas pressure held at high, turbo ion-spray voltage at 5,500 V, declustering potential at 90 V, entrance potential at 10 V, collision energy at 50 V, and collision cell exit potential at 10 V. Data acquisition was performed in Multiple Reaction Monitoring (MRM) mode monitoring the transition of $[M+H]^+$ m/z 464 in Q1 to $[MH-156]^+$ m/z 308 (protonated GSH) in Q3 as quantifier. The internal standard precursor ion and product ion are at m/z 475 and 308, respectively.

LC-MS/MS assay of GSH and GSS-R

The concentrations of GSH and GSS-R in the perfused heart tissues were assayed by our previously reported LC-MS/MS method [21,34].

Western blot assay of HNE-modified proteins

A separate set of control, 50 μ M HNE, and 50 μ M HNE + 1 mM octanoate, perfused hearts were used for Western blotting (n=5). Perfusion protocols were identical to groups 2, 5 and 6 (Fig. 1), except that HNE was unlabeled. Powdered, frozen heart tissue (100 mg) was homogenized in 10 volumes of lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mmol/L NaCl, 1 mmol/L EDTA, supplemented with 0.1 mM protease inhibitor cocktail, 1 mmol/L vanadate, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 1 μ M butylated hydroxytoluene, 2 μ M diethylenetriaminepentaacetic acid, using an Omni General Laboratory homogenizer. Homogenates were centrifuged at 3000 $\times g$ for 30 minutes at 4 $^{\circ}$ C to remove insoluble material. Protein concentrations were measured using a BCA kit according to manufacturer's suggestions (Pierce, IL). Samples were prepared in standard Laemmli buffer and boiled for 5 minutes at 100 $^{\circ}$ C. Samples were then separated by 4–20% SDS-PAGE (Biorad, CA) and transferred to PVDF membranes (90V for 50 min) for western blotting. Membranes were blocked for one hour using 5% BSA in Tris-buffered saline with 0.01% Tween (TBST) followed by incubation with specific antibodies against 4-HNE protein adducts (1:1000 dilution) or HSC70 (1:5000, used as a loading control). Membranes were then washed with TBST and incubated for 1 hour in secondary antibodies conjugated with horseradish peroxidase. Immunoreactivity was detected by chemiluminescence (Supersignal, Pierce, IL) and detected on autoradiography film. Developed films were scanned on an Epson P4490 scanner (transmittance mode) at 720 dpi and the digital images were used for densitometric measures with Image J software in the histogram analysis mode to determine the area under the curve for the gel lane (NIH, MD). Densitometric ratios were derived from sequential probing of membranes stripped between incubations with RestoreTM Western blot stripping buffer (Pierce, IL).

Calculation and statistics

The results are presented as mean values $\pm$ standard deviation from five hearts in each group. The statistical significance among the different groups was determined by ANOVA analysis that was verified by a Bartlett test. Tukey's Post Hoc test was used to determine

significant differences between each group after ANOVA analysis. The results of Tukey's Post Hoc test for all analytes were shown in the respective figures.

Results

Concentration of endogenous HNE, HNE analogs, and GS-HNE conjugates in the heart during ischemia and reperfusion

The concentrations of free HNE, ONE, DHN and GS-HNE in heart tissues were measured by GC-MS and LC-MS/MS, and are shown in Fig. 2. HNE was ~2 fold higher in hearts that were subjected to 30 minutes global ischemia (G3) as compared to control, non-ischemic hearts (G1 and G2). However, in hearts that underwent ischemia followed by reperfusion (G4), the concentration of free HNE returned to the level of controls (Fig. 2A). ONE concentration in heart tissue followed the same trend as HNE, where a significant increase in ONE concentration was apparent following ischemia alone (Fig. 2B), and no difference in ONE concentration after reperfusion (G1, G2 and G4) (Fig. 2B). It is interesting to note that the concentration of ONE in heart tissue is similar to HNE under the conditions tested, ranging from 2 nmol/g under control conditions and 4 nmol/g during the ischemic insult. The concentration of DHN was 10-fold lower than either HNE or ONE, and was not significantly altered by ischemia or ischemia followed by reperfusion (Fig. 2D).

The conjugation of HNE with GSH in the hearts was also quantitated under ischemia and reperfusion conditions. Ischemia alone induced approximately a 7-fold increase of GS-HNE (2.7 ± 0.4 nmol/g) in perfused hearts as compared to controls ($0.3\sim 0.4$ nmol/g, $p < 0.05$, Fig. 2D). In ischemia followed by reperfusion, GS-HNE was significantly lower than during the ischemic period (0.9 ± 0.2 nmol/g), however, it was still elevated as compared to control hearts (Fig. 2D).

Acyl-CoAs profile in the control, ischemic and ischemia/reperfusion hearts

In order to investigate the relation between fatty acid β oxidation and the catabolism of HNE in the heart, we performed a tissue profile of acyl-CoAs including the two HNE-derived intermediates, HNA-CoA and 4-P-nonanoyl CoA. The level of HNA-CoA was found to be increased by ~10 times in ischemic hearts (G3) when compared with control hearts, but returned to basal level after 45 minutes reperfusion (Fig. 3A). Unfortunately, 4-P-nonanoyl-CoA was below detection for all the conditions tested. Acyl-CoAs related to fatty acid oxidation were also measured to probe the global status of fatty acid oxidation. The quantity of stearoyl-CoA and oleoyl-CoA was significantly increased in ischemic hearts (G3) as compared to control perfusions (G1 and G2), in addition to ischemia/reperfusion hearts (G4) (Figs. 3B and C). Similar data were acquired for other long chain acyl-CoAs assayed, including palmitoyl-CoA. Further, other intermediates of fatty acid oxidation, such as butyryl-CoA, were also found to be significantly elevated during ischemia with a return to baseline following reperfusion (Fig. 3D). In contrast to the literature [27], the level of acetyl-CoA was higher following both ischemia (G3) and ischemia followed by reperfusion in heart tissues (G4) when compared to controls (Fig. 3E). The concentration of free CoA appeared slightly lower in G3 and G4 hearts when compared to control heart tissues (G1 and G2), but this difference was not significant (Fig. 3F). Levels of 3-hydroxybutyryl-CoA (BHB-CoA) in the ischemic G3 hearts were 30 times higher than controls (G1 and G2). However, levels of acetoacetyl-CoA (AcAc-CoA) were found to be the same in all groups. The BHB-CoA/AcAc-CoA ratio therefore increased 35 times in ischemic hearts (Fig. 3G). Malonyl-CoA, which regulates the fatty acid synthesis and inhibits fatty acid oxidation, was not significantly different among the hearts from all groups (Fig. 3H).

Oxidation state of glutathione in perfused hearts

The concentrations of reduced glutathione (GSH) and bound glutathione (GSS-R; which includes all DTT-reducible forms, primarily glutathione disulfide and other glutathione conjugates) were measured in heart tissues. GSH did not show dramatic changes in the ischemic hearts (G3) as compared to controls (G1 and 2). However, GSH was 50% lower in the reperfused hearts (G4) when compared to the other groups (Fig. 4A). GSS-R was significantly higher in the ischemic hearts when compared to controls and reperfused hearts (Fig. 4B).

Metabolism of [$^2\text{H}_{11}$]HNE in hearts perfused with an increased fatty acid concentration

To investigate the catabolism of HNE in the heart exposed to an increase in fatty acid concentrations, octanoate was added to perfusions at the final concentration of 1mM with [$^2\text{H}_{11}$]-labeled HNE to track the catabolism of HNE. The labeling of propionyl-CoA in the form of M3 and/or M5 served as an accurate marker of HNE catabolism. The M3 and M5 enrichment of propionyl-CoA in [$^2\text{H}_{11}$]HNE perfused hearts reached 25 and 22%, respectively (Fig. 5). In contrast, in the presence of octanoate, there was negligible labeling of propionyl-CoA (Fig. 5), indicating inhibition of HNE catabolism. As the metabolism of HNE to propionyl-CoA is characterized by two major steps; (i) oxidation to HNEA [13,18,30,35–38] and (ii) catabolism of HNEA to propionyl-CoA and acetyl-CoA [22,23], we sought to define which upstream reaction(s) octanoate inhibits to diminish the production of propionyl-CoA. Several intermediates were quantified in the [$^2\text{H}_{11}$]HNE perfused hearts including HNEA, HNEA-CoA, HNA-CoA, and 4-P-nonanoyl CoA. As shown in Fig 6, the production of [$^2\text{H}_{11}$]HNEA was significantly increased at all [$^2\text{H}_{11}$]HNE concentrations tested with the addition of octanoate to the perfusion medium (Fig. 6A). The negligible quantity of HNA (the saturated oxidation product of HNE) detected suggests that HNA is primarily the result of HNA-CoA hydrolysis and it is likely that HNA-CoA is a product of the reversible reaction of HNEA-CoA catalyzed by acyl-CoA dehydrogenase. Acyl-CoAs associated with HNE catabolism were also quantified. [$^2\text{H}_{11}$]HNEA-CoA, [$^2\text{H}_{11}$]HNA-CoA and 4-P-[$^2\text{H}_{11}$]nonanoyl-CoA in the perfused heart increased linearly with increasing [$^2\text{H}_{11}$]HNE controls (Figs. 6B, C and D), however the production of all three acyl-CoAs were almost completely blocked by 1 mM octanoate (Figs. 6B, C and D). The extent to which octanoate was oxidized in perfused hearts was indicated by the profile of non-labeled acyl-CoAs detailed on Fig. 7. Octanoyl-CoA accumulated in hearts perfused with octanoate, but not in controls (Fig. 7A). BHB-CoA and acetyl-CoA, the intermediate and product of octanoate oxidation, were also dramatically increased compared to control perfused hearts (Figs. 7B and C). The elevated concentration of acetyl-CoA from the oxidation of octanoate also dramatically increased the synthesis of malonyl-CoA in the perfused hearts (Fig. 7D).

HNE uptake and production of detoxification intermediates in the heart with increased fatty acid concentrations

The inhibition of HNE catabolism by exogenous octanoate in perfused hearts could be due to the inhibition of [$^2\text{H}_{11}$]HNE uptake, or the acceleration metabolic disposal by octanoate. Therefore, the rate of [$^2\text{H}_{11}$]HNE uptake and quantities of primary intermediates involved in HNE disposal were measured. The uptake of [$^2\text{H}_{11}$]HNE into perfused hearts was positively related to [$^2\text{H}_{11}$]HNE concentration in the perfusate (Supplementary Fig. S1A), with 80–95% of the [$^2\text{H}_{11}$]HNE taken up at all concentrations. The rate of [$^2\text{H}_{11}$]HNE uptake was not significantly different between the hearts perfused with and without octanoate (Supplementary Fig. S1A). As HNE uptake was not affected by the presence of octanoate, we subsequently evaluated whether DHN, the reduction product of HNE, represented the primary metabolic fate of HNE, particularly since the redox ratio (NADH/NAD^+) is known to be elevated with octanoate perfusion in the heart [39]. The release of [$^2\text{H}_{11}$]DHN into the

perfusate was measured (Supplementary Fig. S1B), and found to be similar in both groups with or without 1 mM octanoate.

Additionally, the conjugate of HNE with GSH was also investigated. Supplementary Figs. S1C and D showed that the presence of 1 mM octanoate in the perfusate did not affect the production of either GS-R-[²H₁₁]HNE or GS-S-[²H₁₁]HNE, however, it is interesting to note that GS-S-[²H₁₁]HNE concentrations are much higher than GS-R-[²H₁₁]HNE in [²H₁₁]HNE perfused hearts with or without 1 mM octanoate. Since elevated GS-[²H₁₁]HNE production could possibly lead to a depletion of endogenous glutathione, the concentration of GSH and GSS-R in the perfused heart tissue was analyzed (Supplementary Figs. S1E and F). With increasing concentrations of [²H₁₁]HNE, GSS-R increased, while GSH was slightly decreased. The addition of octanoate to the perfusion did not significantly impact GSH concentration.

HNE modified proteins in the heart perfused with [²H₁₁]HNE ± octanoate

It has been well-established that HNE-modified proteins in the heart increase with increasing ischemia time [11]. Therefore, the HNE-modified protein profile in the ischemic and ischemic/reperfusion heart is not reported here. We measured the HNE-modified protein in the heart tissues from control and HNE ± octanoate by western blot (Supplementary Fig. S2). Compared to controls, HNE-modified proteins were increased slightly (though not significantly) in [²H₁₁]HNE perfused hearts, but were significantly elevated in hearts perfused with [²H₁₁]HNE plus 1 mM octanoate. Interestingly, the quantity of acetyl-CoA was increased in 50 μM HNE perfused hearts compared to controls and was the highest in hearts perfused with 50 μM HNE + 1 mM octanoate (data was not shown). The reverse was true with the succinyl-CoA, with the lowest quantity present in the HNE + octanoate (data was not shown). The alterations of acetyl-CoA and succinyl-CoA indicate the possibility that enzymes involved in metabolic steps from acetyl-CoA to succinyl-CoA are modified and functionally impaired by HNE.

Discussion

HNE, the primary product of lipid peroxidation, is used as a biomarker of oxidative stress and has been found to be elevated in a number of chronic degenerative diseases [40]. The toxicity of HNE is directly linked to its high reactivity with various cellular components, predominantly the end-products formed via reaction with proteins and DNA. According to the established knowledge, the detoxification pathways of HNE include: (i) conjugation with glutathione, (ii) reduction to DHN, (iii) oxidation to HNEA. More recently, we have characterized the catabolic pathway of HNE metabolism after its oxidation to HNEA. Our previous work showed that HNEA can be fully catabolized in the liver via two main pathways (pathway A and B, Fig. 8). Pathway A involves an isomerization via a new class of acyl-CoA, i.e. 4-P-nonanoyl-CoA followed by β-oxidation to form acetyl-CoA and propionyl-CoA. Pathway B starts with one round of β-oxidation to form 2-hydroxyheptanoyl-CoA, which is further broken down via one α-oxidation to form hexanoyl-CoA, which is consumed by β-oxidation [22,23] and formic acid.

The current investigation was designed to evaluate the effect of conditions that alter β oxidation in the heart on the accumulation of HNE and HNE-modified protein.

Using the Langendorff perfused heart model, we have demonstrated HNE accumulates in the ischemic heart, nearly 2 fold over baseline (control) conditions, but is resolved after reperfusion. It is of interest to note that in addition to HNE, ONE (another lipid peroxidation product) was present in similar concentrations. Given that ONE possesses greater reactivity than HNE, and the majority of the literature focuses on HNE alone, the disposal pathways

for this reactive chemical species, including catabolism, warrants investigation in our future work.

It is well-known that glutathione conjugation to HNE (GS-HNE) is a prominent mode of disposal which allows excretion via the urine. Indeed, the increase of the conjugate with glutathione is even higher than the increase of HNE in the ischemia hearts (Fig. 2D). However, during reperfusion, GS-HNE conjugate remained elevated compared to control, which is likely due to the GS-HNE remaining resident in the tissues, while HNE can freely diffuse during the reperfusion. GS-HNE may also remain elevated in reperfused hearts as a number of studies have established the occurrence of reactive oxygen species resulting from the burst of oxygen during reperfusion [41]. Eaton et al. found that HNE modified protein in the rat heart accumulated with increasing ischemia over 30 minutes [11]. They proposed that the increased level of protein adduction by HNE is probably due to the depletion of glutathione during the ischemia [11]. The oxidized and conjugated glutathione (GSS-R concentrations in Fig. 4B) was indeed increased by two-fold. However, we did not find a significant change in reduced glutathione during ischemia compared to control hearts (Fig. 4A). The unchanged concentration of reduced glutathione in the ischemic heart is likely due to the large pool size (~400 nmol/g) and the relatively small increase in reactive aldehydes (< 10 nmol/g). However, a significant change in GSS-R is observable implying GSH oxidation and conjugation with other reactive aldehydes is occurring. Therefore, the increased GS-HNE and unchanged available GSH could not explain the accumulation of HNE (ONE) in the ischemic hearts.

Other detoxification reactions of HNE include reduction to DHN. However, the concentration of DHN did not differ with ischemia or reperfusion suggesting this pathway is not sensitive to changes in HNE production and, as compared to GS-HNE production, does not constitute a major product of HNE disposal in the perfused heart. It should be noted that others have reported DHN concentration in the heart, but our data is consistent with our previous work in liver along with others [18,21].

The accumulated HNE in the ischemic heart tissues is unlikely the result of additional lipid peroxidation since the ischemic heart lacks oxygen. Therefore, HNE accumulation in the ischemia is likely due to diminished rates of β oxidation, decreasing HNE catabolic disposal. A block in β oxidation in the ischemic hearts is evidenced by the accumulation of acyl-CoAs, such as stearoyl-CoA, oleoyl-CoA, and butyryl-CoA (Figs. 3B, C and D). This rationale is in line with the suspended β oxidation of fatty acids in the ischemic hearts inducing acyl-CoA accumulation as reported by Lerch et al. [42] and Whitmer et al. [27]. The significantly increased concentration of HNA-CoA in the ischemic hearts supports the notion that HNE catabolism is inhibited (Fig. 3A). Inhibition may be mediated, in part, by the reduction in [ATP] during ischemia [43] that limits the phosphorylation of HNA-CoA to prohibit catabolism via pathway A (Fig. 8). Moreover, the pathway A serves as the predominant catabolic pathway of HNE compared to pathway B [23]. However, confirmation of this was not possible as 4-P-nonanoyl-CoA was undetectable in our samples. The return of HNA-CoA concentrations after reperfusion in the heart (Fig. 3A) is likely due to an increased clearance rate of HNE as β oxidation resumes in the presence of oxygen [44]. The ratio of BHB-CoA/AcAc-CoA in ischemic heart tissues is 35-fold higher than control and reperfused hearts. The high ratio of BHB-CoA/AcAc-CoA is indicative of an elevated ratio of NAD(P)H/NAD(P)⁺, which has been previously reported to increase 20–40 fold during the ischemia [13]. The excess of NAD(P)H accumulated during ischemia is a direct consequence of blocking aerobic respiration and the accumulated NAD(P)H, in turn, down-regulates fatty acid β oxidation. The fatty acid oxidation decreased by the reduction of coronary flow and oxygen consumption was also reported by Whitmer et. al. [27]. They also found the acetyl-CoA and free CoA decreased in the ischemic hearts because of CoA

trapping. We found free CoA was slightly decreased during ischemia, but the difference was not significant (Fig. 3F). However, we found acetyl-CoA was higher in the ischemic heart (Fig. 3E) than the control heart. The slightly lower level of free CoA found in this work is likely because of CoA trapping by the accumulated acyl-CoAs [45,46]. The reason acetyl-CoA is higher in the ischemic heart is probably attributable to the attenuated rate of the citric acid cycle. The higher amount of acetyl-CoA in reperfused hearts reflects the accelerated fatty acid oxidation (G4 in Fig. 3E). From the above discussions, we can conclude that HNE accumulation in the ischemic hearts is because of its inhibited catabolic disposal.

To directly investigate the influence of fatty acid oxidation on HNE disposal, isotope-labeled HNE ($[^2\text{H}_{11}]\text{HNE}$) and 1 mM octanoate, a competitive substrate for β oxidation, was included in a separate set of perfusions. The oxidation of octanoate in the heart was demonstrated by a significant increase in octanoyl-CoA, BHB-CoA, acetyl-CoA, and malonyl-CoA in octanoate perfused hearts (Figs. 7A, B, C and D). The high concentration of malonyl-CoA in the hearts perfused with octanoate in turn down-regulates fatty acid oxidation [47]. The competition and down regulation of fatty acid oxidation by octanoate makes it a perfect candidate to investigate the effect of β oxidation intervention on HNE catabolic disposal in the heart. The catabolism of HNE generates formic acid, acetyl-CoA and propionyl-CoA. Only labeled propionyl-CoA is detected in $[^2\text{H}_{11}]\text{HNE}$ perfused hearts as it retains the deuterium from C-8 and 9 of $[^2\text{H}_{11}]\text{HNE}$ and dilution by low amounts of endogenous propionyl-CoA is negligible. Propionyl-CoA labeling (M3 and M5) occurred at all concentrations of $[^2\text{H}_{11}]\text{HNE}$ tested, reaching 20–25% enrichment with 50 μM $[^2\text{H}_{11}]\text{HNE}$. M3 propionyl-CoA most likely results from the loss of two deuterium in the keto-enyl tautomerism of 3-keto-pentanoyl-CoA. However, in contrast, labeling of propionyl-CoA was negligible in the presence of 1mM octanoate (dashed lines in Fig. 5) indicating that elevated lipid concentrations inhibit HNE catabolism in the heart. Although it is possible that reduced labeling of propionyl-CoA results from dilution with endogenous (unlabeled) propionyl-CoA, it is unlikely that octanoate stimulates endogenous propionyl-CoA production as there is a greater competition for free CoA, resulting in a smaller pool [28]. As the inhibition of HNE catabolism could occur in reactions prior to propionyl-CoA formation, therefore, upstream intermediates were also assessed. The concentration of HNEA was greatly elevated when octanoate was introduced into the perfusion (Fig. 6A). This is strong evidence that inhibition of HNE by octanoate does not include the oxidation step. The accumulated HNEA in the perfusate of HNE and octanoic acid is probably due to the blockage of the downstream of HNEA metabolism through β oxidation which is also supported by our data on the concentration of other catabolic intermediates of HNE in the heart. In the control hearts perfused only with increasing amounts of $[^2\text{H}_{11}]\text{HNE}$, the concentrations of $[^2\text{H}_{11}]\text{HNEA-CoA}$, $[^2\text{H}_{11}]\text{HNA-CoA}$, and $[^2\text{H}_{11}]\text{-4-P-nonanoyl-CoA}$ were increased (Figs. 6B, C and D). When 1 mM octanoate was introduced, the production of all HNE-related acyl-CoAs was inhibited (Figs. 6B, C and D).

The activity of other disposal pathways of $[^2\text{H}_{11}]\text{HNE}$ in the heart were observed in the increasing quantities of $[^2\text{H}_{11}]\text{DHN}$, glutathione conjugate, GSS-R, along with the decreased GSH (See Supplementary Figs. 1A, B, C and D) perfused with increasing $[^2\text{H}_{11}]\text{HNE} \pm$ octanoate. However, the dramatically inhibited HNE catabolism by octanoate did not induce an increase of other disposal pathways of HNE. The consequence of non-metabolized HNE is possibly an increase in HNE-modified proteins, lipoprotein, and DNA to increase cellular damage as supported by our observations that HNE-protein conjugation is increased in hearts perfused with HNE (Supplementary Fig. S2). The increase in modified protein is also indicated by the decreased succinyl-CoA concentration in HNE or HNE + octanoate perfused hearts (data not shown), although acetyl-CoA in both groups of hearts is higher compared to control group (data not shown). From the metabolic pathway of acetyl-CoA to succinyl-CoA in the citric acid cycle, there are several enzymes that could be

impaired by HNE modification, such as citrate synthase [48], aconitase [49], isocitrate dehydrogenase [2], α -ketoglutarate dehydrogenase [50]. The oxidative modification of enzymes by HNE may alter the metabolic flux from acetyl-CoA to succinyl-CoA. Further work is needed to investigate the HNE modification of these enzymes and the resulting influence on metabolic flux.

Taken together, by using ischemic rat heart and the rat hearts perfused with exogenous isotope labeled HNE together with 1 mM octanoate, the present work provides a direct link between fatty acid oxidation and the disposal of HNE. The results clearly showed that β oxidation pathway plays a major role in the catabolic disposal of HNE. Inhibition of β oxidation decreases HNE catabolism and increases HNE, and the accumulated HNE leads to enhanced protein modification by HNE (see the summary scheme in Fig. 8). The mechanism described in the present work provides better understanding of HNE accumulation in some diseases, such as ischemia and reperfusion injuries, and diet-induced obesity. The accumulated HNE leads to more protein, lipoprotein, and DNA modifications, which worsens the diseases. And the HNE catabolism through β oxidation may have a role in the pathology of disease states that exhibit changes in fatty acid metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING

This publication was made possible by the Case Western Reserve University/Cleveland Clinic CTSA Grant Number UL1 RR024989 from the National Center for Research Resources (NCRR) to G.F.Z, a component of the National Institutes of Health and NIH roadmap for Medical Research and AHA award 12GRNT12050453 to G.F.Z. This work was supported, in whole or in part, by National Institutes of Health Roadmap Grant R33DK070291 and Grant R01ES013925 (to Henri Brunengraber, Nutrition Dept, Case Western Reserve University), NIH R01CA157735 and NSF MCB-0844801 to G.P.T, and NIH 5R37AA011876 to L.E.N. This work was also supported by a grant from the Cleveland Mt. Sinai Health Care Foundation.

Abbreviations

HNE	4-hydroxy-2(<i>E</i>)-nonenal
ONE	4-oxo-2(<i>E</i>)-nonenal
DHN	1,4-dihydroxy-2(<i>E</i>)-nonene
HNEA	4-hydroxy-(<i>E</i>)-nonenoic acid
HNA	4-hydroxynonanoic acid
ROS	reactive oxygen species
PUFA	polyunsaturated fatty acid
HNEA-CoA	4-hydroxynoenoyl-CoA
HNA-CoA	4-hydroxynonanoyl-CoA
4-P-nonanoyl-CoA	4-phosphonanoyl-CoA
[²H₁₁]HNE	[5,5,6,6,7,7,8,8,9,9,9- ² H ₁₁]-4-hydroxy-2(<i>E</i>)-nonenal
ECL	enhanced chemiluminescence
GS-HNE	glutathione-HNE conjugate

GSH	reduced glutathione
GSS-R	bound glutathione
TBST	Tris-buffered saline with 0.01% Tween

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Highlights

- HNE catabolism is inhibited in ischemic rat heart and by exogenous fatty acids.
- Altered beta oxidation regulates HNE catabolism.
- Inhibited catabolism of HNE increases protein modification.

Figure 1.

Perfusion protocols used to model ischemia/reperfusion and increased fatty acid concentrations. The detailed perfusion protocol for each group is: group 1: control perfusion with Krebs-Henseleit buffer for 45 minutes (n=5); group 2: control perfusion with Krebs-Henseleit buffer for 90 minutes (n=5); group 3: a 15 minutes perfusion followed by 30 minute ischemia (n=5); group 4: group 3 followed by 45 minutes reperfusion (n=5); group 5: 90 minutes control perfusion with $[^2\text{H}_{11}]\text{HNE}$ (0–50 μM) in the last 10 minutes (n=5); group 6: group 5 with 1 mM octanoate in the perfusate (n=5).

Figure 2.

Concentrations of HNE, ONE, DHN and GS-HNE in Langedorff perfused rat hearts during ischemia and/or reperfusion. G1, G2, G3, and G4 represent perfusions of groups 1, 2, 3, and 4 illustrated in Fig 1. Panel A: HNE concentration in the perfused hearts; Panel B: ONE concentration in the perfused hearts; Panel C: DHN concentration in the perfused hearts; Panel D: GS-HNE concentration in the perfused hearts (** $p < 0.01$ and * $p < 0.05$, $n = 5$). Data was normalized to the wet weight of heart tissue.

Figure 3.

Acyl-CoA concentrations in the perfused heart during ischemia and/or reperfusion. G1, G2, G3, and G4 represent perfusions of groups 1, 2, 3, and 4 illustrated in Fig 1. Panel A: HNA-CoA concentration in the perfused hearts; Panel B: stearoyl-CoA in the perfused hearts; Panel C: oleoyl-CoA concentration in the perfused hearts; Panel D: butyryl-CoA concentration in the perfused hearts; Panel E: acetyl-CoA concentration in the perfused hearts; Panel F: free coenzyme A concentration in the perfused hearts; Panel G: the ratio of BHB-CoA/AcAc-CoA (acetoacetyl-CoA) in the perfused hearts; Panel H: malonyl-CoA concentration in the perfused hearts (** $P < 0.01$ and * $P < 0.05$, $n=5$). Data was normalized to tissue wet weight.

Figure 4. Concentration of GSH and GSS-R during ischemia and/or reperfusion. G1, G2, G3, and G4 represent perfusions of groups 1, 2, 3, and 4 illustrated in Fig 1. Panel A: GSSH concentration in the perfused hearts; Panel B: GSS-R concentration in the perfused hearts (** $p < 0.01$ and * $p < 0.05$, $n=5$). Data was normalized to tissue wet weight.

Figure 5.

Mass isotopomer enrichment of M3 and M5 propionyl-CoA at various [²H₁₁]HNE concentrations (0–50 μM) ± 1 mM octanoate. –▲–: M3 propionyl-CoA enrichment in heart perfused with [²H₁₁]HNE (0–50 μM); –■–: M5 propionyl-CoA enrichment in heart perfused with [²H₁₁]HNE (0–50 μM); --▲--: M3 propionyl-CoA enrichment in the heart perfused with [²H₁₁]HNE (0–50 μM) and 1 mM octanoate; --■--: M5 propionyl-CoA enrichment in the heart perfused with [²H₁₁]HNE (0–50 μM) and 1 mM octanoate.

Figure 6.

HNEA and HNE-related acyl-CoA content of hearts perfused with $[^2\text{H}_{11}]$ HNE (0–50 μM) 1 mM octanoate. Panels A, B, C, and D are the concentrations of $[^2\text{H}_{11}]$ HNEA, $[^2\text{H}_{11}]$ HNEA-CoA, $[^2\text{H}_{11}]$ HNA-CoA, and $[^2\text{H}_{11}]$ -4-P-nonanoyl-CoA in the perfusates (HNEA) or rat heart tissues (acyl-CoAs). —■—: $[^2\text{H}_{11}]$ HNE (0–50 μM) control perfusions. --▲--: $[^2\text{H}_{11}]$ HNE (0–50 μM) perfusions with 1mM octanoate. Data was normalized to heart tissue wet weight.

Figure 7.

Octanoate-derived acyl-CoAs in hearts perfused with elevated fatty acid at varying concentrations of [²H₁₁]HNE (0–50 μM) ± 1 mM octanoate. Panels A, B, C, and D are the concentrations of octanoyl-CoA, BHB-CoA, acetyl-CoA and malonyl-CoA in the rat heart tissues. —■—: perfusions from [²H₁₁]HNE (0–50 μM) without octanoate; --▲--: perfusions from [²H₁₁]HNE (0–50 μM) with octanoate. Data was normalized to heart tissue wet weight.

Figure 8.

Proposed model of HNE disposal pathways. In addition to the well-established detoxification pathways for HNE, catabolism through fatty acid β oxidation serves as an important downstream pathway of HNE disposal. The catabolism of HNE is blocked under conditions where fatty acid β oxidation is inhibited under pathological conditions such as ischemia/reperfusion or when the heart is exposed to elevated concentrations of lipid. Diminished catabolism of HNE through β oxidation leads to a greater burden of disposal through detoxification pathways and an enhanced accumulation of HNE-modified protein, lipid and DNA. CAC; citric acid cycle.