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Glutathionylated 4-hydroxy-2-(*E*)-alkenal enantiomers in rat organs and its contributions towards the disposal of 4-hydroxy-2-(*E*)-nonenal in rat liver

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Abstract

The major route for elimination of 4-hydroxy-2-(*E*)-nonenal (4-HNE) has long been considered to be through glutathionylation and eventual excretion as a mercapturic acid conjugate. To better quantitate the glutathionylation process, we developed a sensitive LC-MS/MS method for the detection of glutathione (GSH) conjugates of 4-hydroxy-2-(*E*)-alkenal enantiomers having carbon skeleton of C-5 to C-12. The newly developed method enabled us to quantify 4-hydroxy-2-(*E*)-alkenal-glutathione diastereomers in various organs, i.e. liver, heart and brain. We identified the addition of iodoacetic acid as a critical step during sample preparation to avoid an overestimation of glutathione-alkenal conjugation. Specifically, we found that in the absence of a quenching step reduced GSH and 4-hydroxy-2-(*E*)-alkenals react very rapidly during the extraction and concentration steps of sample preparation. Rat liver perfused with *d*₁₁-4-hydroxy-2-(*E*)-nonenal (*d*₁₁-4-HNE) revealed enantioselective conjugation with GSH and transportation out of the liver. In the *d*₁₁-4-HNE perfused rat livers, the amount of *d*₁₁-(*S*)-4-HNE-GSH released from the rat liver is higher than the *d*₁₁-(*R*)-4-HNE-GSH, and more *d*₁₁-(*R*)-4-HNE-GSH than *d*₁₁-(*S*)-4-HNE-GSH remained in the perfused liver tissues. Overall, the glutathionylation pathway was found to account for only 8.7% of the disposition of 4-HNE, whereas catabolism to acetyl-CoA, propionyl-CoA, and formate represented the major detoxification pathway.

Keywords

4-hydroxy-2-(*E*)-alkenal; 4-hydroxy-2-(*E*)-nonenal; rat organs; rat liver; LC-MS/MS; glutathione conjugate

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1. INTRODUCTION

Oxidative stress is a prevalent source of damage to bio-macromolecules including DNA, proteins, lipids and sugars within the cells. This fundamental process can best be described as an imbalance between the production of reactive oxygen/nitrogen species (ROS/RNS) and their consumption/detoxification. As lipid membranes represent the primary barrier for free diffusion of ROS/RNS into the cell, they often become the primary target of these biological oxidants, a process commonly known as lipid peroxidation. The peroxidation of polyunsaturated fatty acids (PUFAs) results in unsaturated lipid hydroperoxides that undergo fragmentation, giving rise to various reactive aldehydes, including malondialdehyde (MDA), 2-alkenals, and 4-hydroxy-2-(*E*)-alkenals (4-HAE) such as 4-hydroxy-2-(*E*)-nonenal (4-HNE), and 4-hydroxy-2-(*E*)-hexenal (4-HHE) [1–6]. Among these, 4-HNE and 4-HHE are known to be the major α,β -unsaturated aldehydes produced during the lipid oxidation of ω -6 and ω -3 polyunsaturated fatty acids (PUFAs), respectively. 4-HNE, derived from ω -6 PUFAs, such as linoleic acid and arachidonic acid, is a major end product of lipid peroxidation and has been widely accepted as an inducer of oxidative stress, and has been implicated in the pathogenesis of a number of degenerative diseases including Alzheimer's disease [7, 8], atherosclerosis [9–11], and cancer [12].

A central tenet of the 4-HAE fields is that the pathogenesis of these molecules is directly linked to their highly electrophilic nature that can subsequently react with the biological nucleophiles including the protein side chains [13–15], DNA/RNA base pairs, lipids, and sugars to modify or abrogate their ability to function normally. The electrophilic nature of 4-HAE is attributed from the conjugation of the π -electrons with the aldehyde functionality. Tautomeric equilibrium generates a regional electron deficiency at β -carbon and in addition the presence of γ -OH group promotes its electrophilicity by its electron withdrawing effect.

Glutathione *S*-transferases (GSTs) are hypothesized to play a key role in detoxifying highly reactive aldehydes including 4-HNE, 4-HHE and MDA formed during the oxidative insults. GSTs mediated conjugation of 4-HNE to glutathione (GSH), resulting in the formation of the GSH conjugate, is considered to be the major pathway for its metabolism in certain tissue types. GSH conjugates are known to be transported out of cells through an ATP-dependent primary active efflux mechanism by RLIP76 for eventual excretion via the kidneys [16, 17]. Glutathionylated 4-HNE can further be reduced by aldoketoreductases to glutathionyl-1,4-dihydroxynonene, which can also be exported by RLIP76 [18]. GSTs also catalyze the conjugation of cholesterol-5,6-oxide, epoxyeicosatrienoic acid, and 9,10-epoxystearic acid with GSH. Thus GSTs have been well accepted to provide the cell with protection against a range of harmful electrophiles produced during oxidative stress [19]. The metabolism of 4-HAE at least for 4-HNE is known to be enantioselective in different organs. For example, (*S*)-4-HNE is preferentially detoxified by GSTs mediated glutathionylation [20] while (*R*)-4-HNE is preferentially metabolized by NAD⁺-dependent oxidation mediated by aldehyde dehydrogenase (ALDH) [21].

Although different metabolic fates of 4-HNE have been discussed in the literature for years, the proportion of one over another in different physiological states has been largely ignored.

Before our work describing the catabolic fate of 4-HNE, the only substantive knowledge regarding the fate of 4-HNE dealt with the intact nine-carbon framework. To briefly summarize, the major transformations involve either the oxidation or reduction of the aldehyde functionality, saturation of the carbon-carbon double bond, and/or conjugation to glutathione. Additionally, the formation of a five-membered lactone has been reported [22–25]. Three studies examined the fate of radioactively labeled [4-³H]-4-HNE or [2-³H]-4-HNE in the rat [22, 26, 27]. In one study, only 5.9% of the recovered radioactivity could be attributed to a nine-carbon skeleton [27], while two others found between 27% and 44% of 4-HNE was metabolized and excreted to a molecule that maintained its nine-carbon framework. At the time of these reports, there was suggestion as to the possibility of degradation of the 4-HNE skeleton, but no data to support it.

In our previous study, we reported the catabolic fates of 4-HNE in perfused rat livers using a combination of metabolomics and mass isotopic analysis [28, 29]. A key finding of this work was that 4-HNE catabolism can proceed via two parallel pathways that involve (i) isomerization of 4-hydroxynonanoyl-CoA to 3-hydroxynonanoyl-CoA followed by 3 cycles of β -oxidation to form acetyl-CoA and propionyl-CoA; and (ii) β -oxidation/ α -oxidation sequence of 4-hydroxynonanoyl-CoA resulting in the production of acetyl-CoA and formate. As β -oxidation is the major pathway for the catabolic degradation of 4-HNE, any intervention/interruption to this pathway can alter its catabolism resulting in the accumulation of free 4-HNE or other secondary metabolites. We have observed elevated levels of the glutathionylation in rat liver and heart upon inhibiting the catabolism of 4-HNE [30]. Thus, we became interested in understanding the interplay between the glutathionylation and catabolism of 4-HNE as well as the other 4-HAEs. To accomplish with that we first need a sensitive analytical technique to measure artifact free glutathionylation of lipid aldehydes. Multiple analytical techniques have been developed over the years to measure 4-HNE or 4-HNE-GSH conjugates, but have a number of shortcomings. Derivatizing reagents such as 5, 5-dimethyl-1, 3-cyclohexanedione or dinitrophenylhydrazine have been used previously to derivatize 4-HNE and followed by analysis with LC with UV, fluorescence, MS/MS, or GC-MS [31–34]. However, these methods require extensive sample preparation for derivatization and multiple runs are often needed as 4-HNE and its metabolites cannot be measured in a single run. Direct LC–MS/MS is advantageous from this prospect as it does not require any extensive sample preparation and also employs mild condition.

Here we present the first study demonstrating a simple, rapid and sensitive analytical method to quantitatively profile the endogenous diastereomers of GSH conjugates of 4-hydroxyalkenals with from 5 carbons to 12 carbons chain lengths in rat organs. After a series of orientation experiments that initially showed poor precision in GSH conjugate quantification, iodoacetic acid was utilized to prevent the artificial data from the spontaneous chemical reaction between free glutathione and free 4-hydroxyalkenals during sample preparation. LC-MS/MS analyses were then performed to measure and compare the endogenous level of GSH conjugates of 4-hydroxyalkenal enantiomers in different rat organs. Finally, the rat livers were perfused with *d*₁₁-4-HNE to investigate the extent of

glutathionylation of exogenous 4-HNE and its release in the rat liver by measuring the conjugates in the perfusate as well as rat liver tissues.

2. EXPERIMENTAL SECTION

2.1 Materials and methods

General chemicals, reduced glutathione, iodoacetic acid (IAA), butylhydroxytoluene (BHT) were purchased from Sigma-Aldrich. All the standard solutions and buffers were prepared in distilled Milli-Q water.

2.2 Synthesis of standard 4-hydroxy-2-(*E*)-alkenal (4-HAE) series

4-Hydroxy-2-(*E*)-alkenal (4-HAE) derivatives (Figure 1, $n = 0-7$) were prepared by methods to be reported else-where. d_{11} -4-HNE was prepared following reported procedure [35] in the literature from our group as for 4-HNE, replacing $n\text{-C}_5\text{H}_{11}\text{Br}$ with its perdeuterated version. (*R*)- and (*S*)-4-HNE were synthesized following the modified literature procedure starting from commercially available oct-2-(*E*)-en-1-ol [36]. The purity of the synthesized compounds was verified by GC-MS, high resolution MS and NMR.

2.3 Preparation of 4-HAE-GSH conjugates

4-HAE-GSH conjugates were synthesized as described previously with minor modifications [37]. 100 μM 4-HAE-GSH conjugates were prepared by incubating 4-HAE with 1.1 equivalent of reduced glutathione in Milli-Q water at 4 °C for overnight. The conjugates were then analyzed by GC-MS and LC-MS/MS to make sure no free 4-HAE is present. Similarly 2 μM internal standard (d_{11} -4-HNE-GSH conjugate) was prepared using the d_{11} -4-HNE.

2.4 Liver perfusion

All rats were kept on a 12 h light/dark cycle with ad libitum access to food and water. All experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University. Livers from overnight fasted male Sprague-Dawley rats (160–180 g) were perfused with bicarbonate buffer containing 4 mM glucose in non-recirculating perfusions [29, 38]. The flow rate of perfusate was 30 ml/min. After equilibration, 0 to 50 μM of d_{11} -4-HNE was added to the perfusate. Three individual perfusions were carried out for each concentration of d_{11} -4-HNE. Inflow perfusate and the perfusate coming out of the liver at 18–19 min were collected and quick-frozen. Livers were quick-frozen in liquid nitrogen at the end of the experiments (20 minutes perfusion).

2.5 Sample collections

Control liver, brain and heart were collected from male Sprague-Dawley rats (160–180 g) following the regular laboratory procedure and stored at –80 °C until the analysis.

2.6 Sample preparations

Powder frozen control organs (~100 mg), spiked with 0.1 nmol of d_{11} -4-HNE-GSH conjugate as internal standard (to quantify d_{11} -4-HNE-GSH conjugate in the d_{11} -4-HNE perfused samples, 0.1 nmol of C10 4-HAE-GSH was used as internal standard.), and was extracted for 2 min with 2 ml of 100 mM IAA and 2 mM BHT in 10 mM ammonium bicarbonate buffer pH 9.8, 4 ml of acetonitrile and 2 ml of chloroform using a Polytron homogenizer. Then the homogenate was centrifuged for 30 min at 4 °C at 800 ×g and the aqueous part was dried with nitrogen gas, additional 10 times volume of acetonitrile was added to remove protein when sample was dried to ~200 µl. The sample was completely dried and stored at –80 °C until LC-MS/MS analysis. The dried residue was then dissolved in 100 µl Milli-Q water and analyzed by LC-MS/MS.

2.7 Recovery test

Recovery experiments were performed in the liver tissue homogenate (biological matrix) to validate the accuracy of the described analytical method. The biological matrix was prepared by homogenizing 100 mg of powdered frozen liver, spiked with 0.1 nmol of d_{11} -4-HNE-GSH conjugate as internal standard, followed by the extraction for 2 min with 2 ml of 100 mM IAA and 2 mM BHT in 10 mM ammonium bicarbonate buffer pH 9.8, 4 ml of acetonitrile and 2 ml of chloroform using a Polytron homogenizer. Then the homogenate was centrifuged for 30 min at 4 °C at 800 ×g and the aqueous part was dried with nitrogen gas. The dried residue was again dissolved in 500 µl Milli-Q water and 2 ml of acetonitrile to precipitate any remaining protein. The sample was again centrifuged for 30 min at 4 °C at 800 ×g and the aqueous part was dried with nitrogen gas and redissolved in 4 ml of Milli-Q water and stored as the biological matrix at –80 °C until further analysis. For the recovery test, 100 µl of the biological matrix was taken and known amounts of the standard 4-HAE-GSH mixtures were mixed followed by the LC-MS/MS analysis. The ratio of measured concentration versus the added amount was used to evaluate recovery.

2.8 LC-MS/MS assay for 4-HAE-GSH conjugate

After dissolving the dried sample in 100 µl of Milli-Q water, 40 µl were injected on a Thermo Scientific Hypersil GOLD C18 column (150 × 2.1 mm), protected by a guard column (Hypersil GOLD C18, 5 µm, 10 × 2.1 mm), in an Agilent 1100 liquid chromatograph. The chromatogram was developed at 0.2 ml/min (i) from 0 to 25 min with a 1–45% gradient of buffer B (95% acetonitrile, 2% water and 0.25% formic acid) in buffer A (98% water, 2% 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) for 10 min of stabilization with 99% buffer A before the next injection.

The liquid chromatograph was coupled to a 4000 QTrap mass spectrometer (Applied Biosystems, Foster City, CA) operated under positive ionization mode with the following source settings: turbo-ion-spray source at 600 °C under N₂ nebulization at 65 p.s.i., N₂ 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. The Analyst software (version 1.4.2; Applied Biosystems) was used for data registration.

Data acquisition was performed in multiple reaction monitoring (MRM) mode monitoring the transition of $[M+H]^+$ m/z 464 in Q1 to $[MH_2\ 156]^+$ m/z 308 (protonated GSH) in Q3 as quantifier and of $[M+H]^+$ m/z 464 in Q1 to $[MH_2\ 156\ 129]^+$ m/z 179 (protonated GSH-pyroglutamic acid) in Q3 as qualifier for 4-HNE-GSH conjugates. Similarly, the data acquisition were performed for other 4-HAE-GSH and d_{11} -4-HNE-GSH conjugates by changing the corresponding $[M+H]^+$ m/z values.

2.9 GC-MS and LC-MS/MS assays for d_{11} -4-HNE uptake and acyl-CoA

d_{11} -4-HNE uptake was measured by our reported GC-MS method [39]. Labeling and concentration of acyl-CoA in rat livers perfused with d_{11} -4-HNE were assayed by LC-MS/MS [29].

2.10 Calculations and statistics

Correction of raw mass isotopic profiles for natural enrichment at each mass was conducted using matrix correction method [40]. Statistical differences were tested using a paired Student's *t* test (GraphPad Prism version 3).

3. RESULTS AND DISCUSSIONS

3.1 Chromatographic resolution of GSH conjugates

The spontaneous reaction of racemic 4-HNE with free reduced GSH gives rise to four diastereomers as shown in Figure 1. Our first step for quantifying the various stereochemical conjugates was to develop a chromatographic method to resolve the conjugates. As shown in Figure 1, the LC-MS/MS chromatogram from 4-HNE-GSH conjugate contains three unresolved peaks. 4-HNE itself possesses a chiral center at C-4 and an additional chiral center is formed at C-3 when 4-HNE reacts with GSH. So, in the course of GSH conjugate formation four different diastereomers are formed as shown in Figure 1B. The (*S*)-4-HNE-GSH conjugates elute as unresolved middle peak (Figure 2), and other two peaks correspond to the (*R*)-4-HNE-GSH conjugates (Figure 2). All other 4-HAE-GSH conjugates showed same pattern in the LC-MS/MS trace except for 4-hydroxy-2-(*E*)-pentenal which gave only two peaks (Figure 3).

3.2 Calibration curve and limit of detection (LOD) and limit of quantitation (LOQ)

We then sought to generate the calibration curves for each 4-HAE-GSH conjugate to identify and quantitate them in different tissues. An LC-MS/MS method using MRM was then developed for each separate 4-HAE-GSH isomers (inclusive of C5-C12, each diastereomer). The calibration curves were acquired in MRM mode using the precursor ion of protonated 4-HAE-GSH conjugate and the daughter ion of m/z 308 (protonated glutathione). Calibration curves were subsequently determined for the GSH conjugate of each individual 4-HAE enantiomers i.e. for (*R*)-4-HAE-GSH and (*S*)-4-HAE-GSH and were linear ($R^2 > 0.99$) over the range from 1.3 pmol-1.3 nmol. LOQ and LOD were determined to be 2.6 pmol and 1.3 pmol, respectively (Table S1 in the Supplementary Information).

3.3 Recovery test

Recoveries were determined by spiking different 4-HAE-GSH conjugates at low, medium and high concentrations and determining the mean, and standard deviation for intra-day as well as inter-day assays. As summarized in Table S2 in the Supplementary Information, recoveries for different 4-HAE-GSH conjugates at different concentrations range from 82 to 124% demonstrate the accuracy of the present method for 4-HAE-GSH conjugates.

3.4 Effect of IAA and BHT in the sample preparation

During sample handling process free radical induced lipid peroxidation can further increase the concentration of 4-HNE in the biological samples and may result in erroneous results. More importantly, 4-HNE can also readily react with free reduced GSH to give the 4-HNE-GSH conjugate as a result of an in vitro reaction, which can also produce the overestimated value of the conjugates. This becomes extremely important during sample preparation, as this in vitro reaction can be greatly accelerated by the transient, but massive increase in concentration of both free GSH and 4-HNE during sample drying. We hypothesized that our initial lack of precision in orientation experiments was due to a combination of these factors. In order to better assess the in vivo concentration of 4-HNE-GSH conjugates (or, any 4-HAE-GSH conjugate), one must ensure that both the in vitro reaction as well as the ex vivo lipid peroxidation is minimized as much as possible during sample preparation. We accomplished this through the addition of two factors during sample preparation. The lipid peroxidation during the sample preparation was prevented by adding BHT as an antioxidant. Additionally, we tested the effect of IAA and found that when IAA is not used in the sample preparation the quantified level of 4-HNE-GSH conjugate is significantly higher (more than 200 fold in rat liver; see Figure 4 C9-GSH). This artificially high amount of 4-HNE-GSH conjugate is attributed to the chemical reaction between free 4-HNE and reduced GSH present in the tissue. The presence of IAA impairs the free GSH and hence prevents any overestimated 4-HNE-GSH (as a result of in vitro chemical reaction) conjugates in the samples. The amounts of 4-HAE-GSH conjugates in different tissues have been estimated with and without IAA (Figure 4).

One major concern about the IAA effect is whether glutathione conjugates of 4-hydroxyalkenals are stable under the condition of IAA reaction. We incubated 4-hydroxyalkenals glutathione conjugates with 100 mM IAA at the same conditions (10 mM ammonium carbonate buffer, pH 9.8), and no degradation of 4-hydroxyalkenals glutathione conjugates was observed (data not shown). This excluded the possibility that the lower amount of glutathione conjugates of 4-hydroxyalkenals in the presence of IAA compared to no IAA is the degradation of glutathione conjugates by IAA.

3.5 Perfusion of *d*₁₁-4-HNE in rat liver

Although lipid peroxidation leads to the formation of both enantiomer of 4-HNE, Glutathione *S*-transferase A4-4 shows a substrate selectivity towards the (*S*)-4-HNE in presence of both enantiomers [20]. In the perfused rat liver we found the higher amount of (*S*)-4-HNE-GSH conjugate while it's exactly opposite in the perfusate where the (*R*)-4-HNE-GSH is predominant (Figure 5). The 4-HNE-GSH conjugate can be formed in different organs and tissues including liver. The downstream metabolism of the released 4-

HNE-GSH conjugate occurs in the kidney where it can further be oxidized to 4-HNE-mercapturic acid conjugates and secreted into urine. The release of (*S*)-4-HNE-GSH into perfusate by liver is higher than (*R*)-4-HNE-GSH. This possibly attributes to the selectivity of GSTs on (*S*)-4-HNE and the enantioselectivity of 4-HNE-GSH conjugate transporter on (*S*)-4-HNE-GSH conjugate. Chemical reaction of GSH with 4-HNE is still very fast but relatively slower than the GSTs mediated enzymatic reaction. This explains the lower abundance of the (*R*)-4-HNE-GSH in biological samples. (*R*)-4-HNE-GSH was accumulated in the perfused rat liver tissue probably because of its lower efficiency of transportation compared to (*S*)-4-HNE-GSH conjugate. Based on our *d*₁₁-4-HNE liver perfusion data, (*R*)-4-HNE could be more harmful to the liver.

In the effluent of perfusates with 1 to 50 μ M *d*₁₁-4-HNE, we could not find any trace amount of free *d*₁₁-4-HNE (Figure 6A). This suggested the fast and complete uptake of 1–50 μ M *d*₁₁-4-HNE by rat livers. Such fast uptake of *d*₁₁-4-HNE indicates the rapid metabolism of *d*₁₁-4-HNE and therefore the uneven distribution of *d*₁₁-4-HNE across the whole rat liver. This uneven distribution of *d*₁₁-4-HNE may lead to the zonation of 4-HNE metabolism in rat liver [41]. However, compared to uptake of *d*₁₁-4-HNE by the perfused liver, only 8.7% of *d*₁₁-4-HNE formed the GSH conjugate found in the perfusate and liver tissue. That prompted us to look into the labeling of other metabolites related to the 4-HNE metabolism to account for its metabolism. We found that propionyl-CoA was substantially labeled with M3 (from 7.0 to 26.7%) and M5 (from 1.8 to 33.9%) from 1 to 50 μ M *d*₁₁-4-HNE in the liver perfusion experiments (Figure 6B). This redirects to our previously reported catabolism of 4-HNE via two parallel pathways to generate acetyl-CoA, propionyl-CoA, and formic acid. The pathways also involve the production of 4-hydroxy-nonanoyl-CoA and 4-phospho-nonanoyl-CoA as the metabolic intermediates [28, 29]. We further looked for *d*₁₁-4-phospho-nonanoyl-CoA and *d*₁₁-4-hydroxy-nonanoyl-CoA in rat livers perfused with *d*₁₁-4-HNE to confirm the involvement of the catabolic pathways and indeed found increasing amount of those two intermediates with increasing amount of *d*₁₁-4-HNE in the perfusate (Figure 6C). This strongly suggests that catabolism of 4-HNE is the major metabolic pathway of 4-HNE in the liver. 1,4-Dihydroxynonene, another 4-HNE metabolite generated via the reduction of the later by aldoketo reductases, was even in less amount compared to the glutathione conjugates (data not shown).

3.6 4-HAE-GSH conjugates level in rat liver, brain and heart samples

With the developed method, we could show 4-HAE-GSH conjugates levels in various rat organs for the first time (Figure 4). The following interesting findings can be extracted out from that data: (I) 4-HAEs (from C5 to C12) exist in all these organs except the C12 4-HAE (n=6, Figure 1) which is undetectable in the heart. Without the IAA treatment 4-HAEs-GSH level is much higher than the ones with IAA; (II) 4-HNE-GSH and 4-HHE-GSH are the most abundant 4-HAE-GSH conjugates in all organs, 4-HNE-GSH being the highest one. This data also matches the concentrations of free 4-HNE and 4-HHE in the organs; (III) There are no detectable 4-HAE-GSH conjugates (with IAA) in all organs except the C6, C8 and C9 4-HAE-GSH conjugates. C6, C8 and C9 4-HAE-GSH conjugates distributions in various organs are different, liver usually has higher of these three 4-HAE-GSH conjugates than heart and brain, and (IV) (*R*)-4-HHE-GSH and (*R*)-4-HNE-GSH in liver and heart

tissues are higher than their (*S*)-forms (Figure 4), and this agrees well with the d_{11} -4-HNE liver perfusion data (Figure 5). Surprisingly, (*R*)- and (*S*)- forms of 4-HHE-GSH and 4-HNE-GSH levels are not significantly different in the brain tissues. This might be related to nonenantioselective metabolism of 4-HAE in brain tissues reported by Honzatko *et al* [42].

4. CONCLUSIONS

The 4-HAE-GSH conjugates in rat liver, heart and brain have been profiled with the present developed method. Our work first demonstrated the presence of all 4-HAE from C5 to C12 and some detectable 4-HAE-GSH conjugates (C6, C8 and C9) in these organs. C9 and C6 4-HAE-GSH conjugates are the most abundant. In exogenous HNE perfused rat liver, enzymatically formed (*S*)-4-HNE-GSH was released from the liver, while (*R*)-4-HNE-GSH was accumulated in liver tissue. Under these basal conditions, conjugation via glutathionylation represents only a small portion of exogenous HNE disposal by rat liver. Catabolism of HNE to form acetyl-CoA and propionyl-CoA entering citric acid cycle is by far the major disposal pathway of HNE in the rat liver.

Apart from developing analytical techniques to accurately measure the extent of glutathionylation of 4-hydroxyalkenals in vivo, our findings also shed light into the fate of 4-HNE during normal physiology. Our present studies show that in the normal “normal state”, 4-HNE primarily undergoes the catabolism and a small fraction (8.7%) is conjugated to glutathione for excretion. Other metabolites with intact nine-carbon skeleton can be considered trace, and do not substantially contribute to the overall fate of these lipid peroxidation products.

We have further reported that the catabolism of 4-HNE is also highly robust in the heart [30]. One of the key findings of this work was that during conditions of impaired β -oxidation (such as hypoxia and competition with saturated fatty acids) the catabolic fate of 4-HNE shifts, favoring the accumulation of 4-HNE. The inhibition of 4-HNE catabolism was not compensated by other disposal pathways. Rather, an increase in 4-HNE-modified proteins and glutathionylation was observed. So now putting the puzzle together, a mechanistic rationale can be drawn for the interplay of catabolism vs. glutathionylation vs. protein adduction with 4-HNE as shown in Figure 7. Under normal conditions (as shown in the current study) catabolism is the primary route for detoxification of 4-HNE in liver with 8.7% conjugated to GSH (green). Here we postulate that under conditions of moderate oxidative stress, the glutathionylation pathway becomes more prevalent, in essence acting as a ‘spill over’ pathway for detoxification of 4-HNE (yellow). Under times of severe oxidative stress, when β -oxidation is impaired the catabolic pathway is no longer functional and glutathionylation is the only route for detoxification (red). This situation would be indicative of the times when 4-HNE would be causative for etiology of disease, as indicated by increased adduction of proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- 4-Hydroxyalkenal (C5 – C12)-glutathione conjugates in rat organs were profiled.
- Conjugation with glutathione is around 9% of 4-hydroxynonenal metabolism in liver.
- Enzymatic conjugation of 4-hydroxynonenal with glutathione has stereoselectivity.

Figure 1.

(A) Structure of 4-hydroxy-2-(*E*)-alkenal (4-HAE) and 4-HAE-GSH conjugate; (B) Structure of different diastereomers of 4-HAE-GSH conjugate; (C) LC-MS/MS trace of 4-HAE-GSH conjugate (n = 4).

Figure 2. LC-MS/MS chromatogram of racemic-4-HNE-, (*S*)-4-HNE- and (*R*)-4-HNE-GSH conjugates.

Figure 3.

LC-MS trace of different 4-HAE-GSH conjugates. (Inset: LC-MS trace of racemic-4-HNE-GSH conjugate showing the peaks corresponding to (R)-4-HNE- and (S)-4-HNE-GSH).

Figure 4. Quantitation of GSH conjugates of (*R*)- and (*S*)-4-HAE in liver, heart and brain tissues (N = 3).

Figure 5.

Diastereomeric contents of (R)- and (S)- d_{11} -4-HNE-GSH conjugates in (A) the perfused rat liver and (B) perfusate. N is 3 for each d_{11} -4-HNE concentration.

Figure 6.

(A) Uptake of d_{11} -4-HNE by rat liver. The uptake of HNE was determined by the difference of HNE concentration in influent and effluent. (B) Mass isotopomer enrichment of propionyl-CoA in rat livers perfused with d_{11} -4-HNE. (C) Concentrations of acyl-CoAs catabolized from d_{11} -4-HNE in rat liver. N is 3 for each d_{11} -4-HNE concentration.

Figure 7.
Hypothesis for the interplay of catabolism vs. glutathionylation vs. protein adduction with 4-HNE in different conditions.