Published in final edited form as:

Methods Enzymol. 2010 ; 474: 297–313. doi:10.1016/S0076-6879(10)74018-0.

Synthesis, Quantification, Characterization, and Signaling Properties of Glutathionyl Conjugates of Enals

Sanjay Srivastava^{*}, Kota V. Ramana[†], Aruni Bhatnagar^{*}, and Satish K. Srivastava[†]

[†] Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, Texas, USA

^{*}Diabetes and Obesity Center, University of Louisville, Louisville, Kentucky, USA

Abstract

Oxidation of lipids generates large quantities of highly reactive α,β -unsaturated aldehydes (enals). Enals and their protein adducts accumulate in the tissues of several pathologies. *In vitro*, low concentrations of enals such as HNE (4-hydroxy *trans*-2-nonenal) affect cell signaling whereas high concentrations of enals are cytotoxic. Direct conjugation of the C2–C3 double bond of enals with the sulfhydryl group of GSH is a major route for the metabolism and detoxification of enals. Recently, we found that glutathionyl conjugate of HNE (GS-HNE) enhances the peritoneal leukocyte infiltration and stimulates the formation of proinflammatory lipid mediators. Moreover, the reduced form of the glutathione conjugate of HNE (GS-DHN) elicits strong mitogenic signaling in smooth muscle cells. In this chapter we discuss the methods to study the metabolism of enals and the redox signaling properties of glutathionyl conjugates of HNE.

1. Introduction

Oxidation of lipids generates a variety of bioactive molecules including lipid radicals, peroxides, isoprostanes, and epoxides. Of these, aldehydes, generated from the degradation of lipid hydroperoxides, are the major end products (Esterbauer *et al.*, 1991). Among the free aldehydes generated from lipid peroxidation, the α,β -unsaturated aldehydes (enals) are highly reactive. The α,β -unsaturation in aldehydes generates two reactive centers in the molecule: a polarized carbonyl that could participate in the formation of Schiff bases and an electrophilic δ carbon that undergoes Michael addition reactions (Esterbauer *et al.*, 1991). Consequently, enals are several orders of magnitude more reactive than corresponding saturated aldehydes or the aromatic aldehydes. Among the enals, the C9 unsaturated aldehyde 4-hydroxy *trans*-2-nonenal (HNE) is the most abundant. HNE is generated primarily from the peroxidation of ω -6 polyunsaturated fatty acids, and under some conditions, accounts for > 95% of the enals produced (Benedetti *et al.*, 1980). Normal HNE concentrations in tissue and plasma range from 0.8 to 2.8 μ M and the concentration of HNE in oxLDL has been estimated to be 150 mM (Esterbauer *et al.*, 1991). Apart from HNE, lipid peroxidation also generates highly reactive C3 enal, acrolein; C4 enal, crotonaldehyde; and the C6 enal, hexenal. Acrolein is also generated from myeloperoxidase, metabolism of anticancer drug cyclophosphamide and allyl amine, a dye extensively used in the dye industry ([Bhatnagar, 2006] and [Uchida *et al.*, 1998]). High concentrations of acrolein and crotonaldehyde are also present in automobile exhaust and cigarette smoke ([Bhatnagar, 2006] and [Uchida *et al.*, 1998]). Hexenal is one of the most abundant aldehydes present in fruits and vegetables and is also extensively used as a flavoring agent (Feron *et al.*, 1991).

Enals and their protein adducts accumulate in several diseased tissues such as atherosclerotic lesions ([Srivastava *et al.*, 2009] and [Uchida *et al.*, 1998]), inflamed arteries (Rittner *et al.*, 1999), ischemic hearts (Eaton *et al.*, 1999), failing hearts (Srivastava *et al.*, 2002), and

neuronal lesions associated with Parkinson (Yoritaka *et al.*, 1996) and Alzheimer (Montine *et al.*, 1997) disease. The accumulation of enals and their protein adducts is indicative of oxidative stress. *In vitro*, enals such as HNE affect multiple signaling pathways ([Dianzani, 1998], [Dianzani, 2003] and [Robino *et al.*, 2001]), including membrane conductances and signal transduction cascades ([Dianzani, 1998], [Dianzani, 2003] and [Robino *et al.*, 2001]). In several different cell types, exposure to HNE leads to the activation of JNK ([Dianzani, 1998], [Dianzani, 2003] and [Robino *et al.*, 2001]) and in stellate cells, HNE has been shown to covalently modify the p46 and p54 isoforms of JNK (Parola *et al.*, 1998). Data from our laboratory (Ruef *et al.*, 2000) showed that HNE is a potent mitogen for vascular smooth muscle cells (VSMC) and Ishii *et al.* (2004) found that HNE induces the expression of scavenger receptors in VSMC. However, the mechanisms by which enals such as HNE exert cell signaling are not quite clear.

Our studies show that enals such as HNE are metabolized either by direct conjugation with glutathione or by oxidative or reductive transformations ([Hill *et al.*, 2009], [Srivastava *et al.*, 1998b] and [Srivastava *et al.*, 2001]). The conjugation of unsaturated aldehydes to glutathione is catalyzed by the glutathione *S*-transferase (GSTs) isoforms, whereas oxidation of HNE to 4-hydroxy-nonanoic acid (HNA) is catalyzed by aldehyde dehydrogenases. Both free and glutathiolated HNE are efficient substrates of aldose reductase (AR), and reduction via AR appears to be a significant fate of enals and their glutathione conjugates *in vivo*.

Although enals such as HNE readily form glutathione conjugates, the formation of GS-HNE may not by itself be sufficient for detoxification. We found that in VSMC, GS-HNE activates PKC and stimulates NF- κ B- and AP-1-dependent gene transcription and smooth muscle cell proliferation (Ramana *et al.*, 2006). Our recent studies show that GS-HNE stimulates the formation of superoxide radical and induces the expression of CD11b in polymorphonuclear leukocytes (PMNs) *in vitro*, and intraperitoneal injection with GS-HNE significantly increases the peritoneal leukocyte infiltration and stimulates the formation of proinflammatory lipid mediators (Spite *et al.*, 2009). Similarly, the glutathionyl conjugate of acrolein has been shown to increase superoxide formation (Adams and Klaidman, 1993) and glutathione conjugates of enals have been shown to be markedly toxic and can induce DNA damage or stimulate the production of reactive oxygen species ([Dittberner *et al.*, 1995] and [Horvath *et al.*, 1992]). Initially, it was thought that the reduction of aldehyde-glutathione conjugate by AR would annul the reactivity and therefore the toxicity of the conjugate. However, on contrary to this expectation, we found that the reduced form of the glutathione conjugate of HNE (GS-DHN) elicits strong mitogenic signaling in smooth muscle cells (Ramana *et al.*, 2006). Incubation of cell permeable esters of GS-HNE and GS-DHN with VSMC stimulated protein kinase C, NF- κ B, AP-1, and promoted cell growth (Ramana *et al.*, 2006). Inhibition of AR, the protein which catalyzes the reduction of GS-HNE to GS-DHN, prevented the GS-HNE-induced protein kinase C, NF- κ B, AP-1 activation and cell proliferation, but had no effect on GS-DHN-induced cell signaling and growth. Pretreatment of the cells with antibodies raised against the transporters of glutathione conjugates—RLIP (Ral-binding protein) or multidrug resistance protein-2, promotes the mitogenic effects of GS-DHN. Similarly, inhibition of RLIP by siRNA, enhances the cell growth in response to glutathionyl conjugates of HNE, whereas overexpression of RLIP diminishes the glutathionyl conjugates-induced cell proliferation. Moreover, RLIP-knockout mice show higher levels of GS-HNE in the liver (Warnke *et al.*, 2008). Here, we briefly describe the methods for the formation, characterization, and quantification of glutathione conjugates of enals and their signaling properties.

2. Synthesis, Quantification, and Characterization of Reagent Glutathionyl Conjugates of HNE

2.1. GS-HNE

HNE and other α,β -unsaturated aldehydes are strong electrophiles that react spontaneously with reduced glutathione. The reaction is stoichiometric, and is accelerated by inorganic phosphate (Esterbauer *et al.*, 1991). Other nucleophiles such as histidine, lysine, and carnosine also react with these aldehydes but the rate of conjugate formation with these nucleophiles is usually an order of magnitude lower than with GSH. The GS-DHN is stable at acidic pH and the conjugate, once prepared, could be stored either in an acidic or an aprotic (acetonitrile) medium.

2.1.1. Synthesis—Reagent HNE and 4-(^3H)-HNE (specific activity of 175 mCi/mmol) were synthesized as described before (Srivastava *et al.*, 1998b). Because of their high reactivity, several unsaturated aldehydes are synthesized and stored as esters. Before the experiments, the free aldehyde is released by the acid hydrolysis of the ester (Srivastava *et al.*, 1998b).

Described below briefly is the method for the synthesis of GS-HNE:

1. Dissolve 100 nmol of nonradioactive HNE and 100 nmol of radiolabeled HNE containing 100,000 cpm 4-(^3H)-HNE in 990 μl of 0.1 M potassium phosphate, pH 7.0 in a quartz cuvette.
2. Add 10 μl of 50 mM GSH, prepared in ice-cold water. Incubate the mixture at 37 $^{\circ}\text{C}$ in a temperature-controlled water bath.
3. Monitor the reaction by following the consumption of HNE at 224 nm (molar extinction coefficient = 13,750). Formation of the glutathione conjugates results in a decrease in the absorbance at 224 due to saturation of the conjugated diene chromophore. Typically, under these experimental conditions, > 99% HNE is consumed in 30 min.

A similar procedure could be used to synthesize conjugates of other enals. Short chain enals react faster with the sulfhydryl group of GSH than the longer chain enals. The rate of reaction of acrolein with GSH is 25-fold faster than crotonaldehyde and the rate of reaction of enals decreases further with an increase in hydrocarbon chain length. Presence of a hydroxyl group at the fourth carbon of medium chain enals (C5–C10) increases the rate of reaction of enals with the sulfhydryl group of GSH by four- to eightfold, whereas introduction of an additional double bond between the C4 and C5 of hydrocarbon chain completely abolishes their reactivity with GSH (Srivastava *et al.*, 1999).

2.1.2. Purification and quantification—For the synthesis of GS-HNE, HNE is incubated with an excess of GSH. To remove unreacted GSH, the conjugate has to be purified by HPLC. This is best accomplished by using reverse-phase (RP) HPLC. In our laboratory, we typically use a Varian RP ODS C₁₈ column preequilibrated with 0.1% aqueous trifluoroacetic acid. The column is eluted using a gradient consisting of solvent A (0.1% aqueous trifluoroacetic acid) and solvent B (100% acetonitrile) at a flow rate of 1 ml min⁻¹. The gradient is established such that solvent B reaches 24% in 20 min, 26% in 30 min and is held at this value for 10 min. In the next 10 min, solvent B reaches 60%, and in an additional 5 min it reaches 100% and is held at this value for 10 min. Samples are collected every 1 min and 50 μl aliquots are used to count the radioactivity on a scintillation counter. As shown in Fig. 1A, under these conditions, GS-HNE elutes with a retention time

of 22 min. Typically, we recover $86 \pm 6\%$ radioactivity applied to the column by this protocol.

2.1.3. Characterization of GS-HNE—The chemical identity of reagent GS-HNE purified by HPLC is established by electrospray ionization mass spectrometry (ESI-MS). We use a MicroMass ZMD 2000 mass spectrometer (Waters-Micromass, Milford, MA). The ESI⁺-MS operating parameters are as follows: capillary voltage, 3.0 kV; cone voltage, 13 V; extractor voltage, 9 V; source block temperature, 100 °C; and dissolution temperature, 200 °C. Nitrogen at 3 p.s.i. is used as nebulizer gas. Samples (50 µl) are mixed 1:1 with 50 µl of acetonitrile/water/acetic acid (100/100/0.2) (v/v/v) and applied to the mass spectrophotometer using a Harvard syringe pump at a rate of 5 µl/min. Spectra are acquired at the rate of 200 a.m.u./s over the range of 20–2000 a.m.u.. As shown in Fig. 1B, the ESI⁺-MS of HPLC peak I, eluting at 22 min, shows a strong molecular ion with an m/z value of 464.3, consistent with $[M + H]^+$ of GS-HNE. Another strong ion is generated with an m/z ratio of 446.3, corresponding to the dehydrated daughter ion $[M - H_2O]^+$ of GS-HNE. That the m/z 446 ion is derived from GS-HNE could be verified by changing the repeller voltage of the electrospray source. An increase in the repeller voltage should increase fragmentation and dehydration leading to an increase in the m/z 446 ion. For quantification, the intensity of the 446 and the 464 m/z ions is added together.

2.2. Synthesis, purification, and characterization of reagent GS-DHN

The GS-HNE conjugate could be reduced to generate GS-1,4-dihydroxy-nonanol (GS-DHN). Our studies show that the AR catalyzes the reduction of the conjugate (Srivastava *et al.*, 1998a) and that the reduced conjugate is biologically active (Ramana *et al.*, 2006). The GS-HNE conjugate could be reduced enzymatically, using recombinant AR (Abanova, Walnut, CA). Alternatively, the conjugate could be reduced by sodium borohydride.

1. Incubate 10 µg of human recombinant AR in 940 µl of 0.1 M potassium phosphate, pH 6.0, for 5 min at 37 °C in a cuvette.
2. Add 10 µl of 7.5 mM NADPH prepared in ice-cold water.
3. Add 50 µl of GS-[4-(³H)]-HNE, reconstituted in 0.1 M potassium phosphate at a concentration of 200 µM.
4. Monitor the reaction by following the consumption of NADPH at 340 nm. Typically, under these experimental conditions, > 99% GS-[4-(³H)]-HNE is reduced in 3 h.
5. Alternatively, to a GS-HNE solution in methanol add 4 M excess of NaBH₄. Stir the mixture for 60 min at room temperature and then add 0.1 M HCl dropwise to the reaction mixture to bring the pH to 2.0. Leave the mixture in acidic conditions for 30 min to decompose unreacted NaBH₄ and extract the conjugate three times with dichloromethane and purify by HPLC as described above. Under the experimental conditions described, the GS-DHN conjugate coelutes with GS-HNE at the retention time of 22 min.
6. Chemical identity of reagent GS-DHN is established by ESI⁺-MS as described above. As shown in Fig. 1C, upon ESI-MS GS-DHN forms a strong molecular ion with m/z value of 466.3.

2.3. Synthesis and characterization of esterified glutathionyl conjugates

To examine the effect of GS-HNE and GS-DHN on cell signaling, cell permeable esterified forms of their glutathionyl conjugates are used (Ramana *et al.*, 2006). They are synthesized as described below:

1. Incubate 4- (^3H) -HNE (100 nmol) with 5 M excess of glutathione monoethyl ester and monitor the consumption of HNE at 224 nm as described above.
2. For the synthesis of the reduced form of the esterified glutathione-HNE conjugate (GS-DHN-ester), incubate aliquots of the GS-HNE-ester with AR and NADPH as described above.
3. Esterified glutathionyl conjugates are purified on HPLC as described above. Unlike the nonesterified conjugates, the GS-DHN-ester and GS-HNE-ester are nicely separated and elute with the retention time of 28 and 31 min, respectively (
4. Chemical identity of the esterified glutathionyl conjugates is established by ESI⁺-MS. ESI⁺-MS of HPLC peak I shows a molecular ion with an m/z value of 494.3, consistent with the $[\text{M} + \text{H}]^+$ of GS-DHN-ester and ESI⁺-MS of HPLC peak II shows strong ions with m/z values of 492.2 and 274.2 corresponding to $[\text{M} + \text{H}]^+$ of GS-HNE-ester and its dehydrated daughter ion, respectively (Fig. 2B). The in-source dehydration of GS-HNE-ester could be completely prevented by either decreasing the cone voltage or by lowering the block temperature; however, this results in a 10-fold decrease in signal intensity and thereby decreases the signal-to-noise ratio (data not shown).

3. Metabolism of HNE

3.1. Cellular metabolism of HNE

In most cells, HNE is rapidly metabolized. Cellular metabolism of HNE generates several products that result from the enzymatic reduction or oxidation of the aldehyde and the formation of a glutathione conjugate (GS-HNE), which undergoes reductive metabolism to generate GS-DHN. Glutathionyl conjugates of HNE account for 40–60% of the total metabolites in cardiovascular tissues ([Hill et al., 2009], [Srivastava et al., 1998b] and [Srivastava et al., 2001]). Most of these metabolites including the glutathionyl conjugates are extruded out of the cell and < 1% of the enal is retained inside the cells or are bound to intracellular proteins. Here, we describe briefly the method for the quantification and characterization of HNE metabolites in PC12 cells. For quantification, we have found it necessary to use radiolabeled [4- (^3H) -HNE] HNE.

1. Culture cells in 10 cm² dishes to 80% confluency.
2. Prior to the experiment, rinse the cells with prewarmed (37 °C) Hank's balanced salt solution (HBSS).
3. Incubate the cells with 5 μM 4- (^3H) -HNE (100,000 cpm) in 2.5 ml HBSS for 30 min.
4. At the end of the incubation, remove the medium and separate the radioactivity by HPLC on an RP C₁₈ column (Fig. 3A). HPLC conditions are identical to those described for the purification of reagent glutathionyl conjugates. On the basis of the retention time of the synthetic metabolites four major peaks in the eluate are identified to be glutathionyl conjugates of HNE (peak I), 1,4-dihydroxynonene (DHN; peak II), HNA (peak III), and unmetabolized HNE (peak IV). The chemical identity of these peaks is established by ESI⁺-MS or gas chromatography–mass spectrometry (GC–MS). Two other minor peaks (peaks V and VI) are also observed. Chemical identity of these peaks has yet not been established.
5. As shown in Fig. 3B, ESI⁺-MS of HPLC peak I shows strong molecular ions with m/z value of 466.3 and 464.3, consistent with $[\text{M} + \text{H}]^+$ GS-DHN and GS-HNE. These data are consistent with our previous studies showing that glutathionyl conjugates of HNE are the major metabolites of HNE in cardiovascular tissues

([Hill et al., 2009], [Srivastava et al., 1998b] and [Srivastava et al., 2001]). Sensitivity for the detection of glutathionyl conjugates of enals in tissues could be significantly increased by using ESI⁺ and select ion monitoring (Warnke *et al.*, 2008).

3.2. Systemic metabolism of HNE

In this method, we describe, how enals such as HNE are metabolized *in vivo*. It is currently believed that glutathionyl conjugates of HNE and related enals are actively extruded into the circulation from the tissues in which they are generated. The conjugates appearing the circulation are taken up by the kidney and further processed and excreted in the urine as mercapturates. Of the total metabolites of HNE recovered in the urine, upon intravenous injection of 4-(³H)-HNE to mouse, *N*-acetylcysteine-DHN (NAC-DHN), formed from GS-DHN, is the major metabolite and it accounts for 34 ± 4% of radioactivity recovered in the urine. These observations suggest glutathione conjugate formation and its subsequent reduction is the major pathway for the metabolism of HNE *in vivo*. NAC-DHN has also been detected as the physiological component of human and rat urine (Alary *et al.*, 1998). NAC-DHN concentration in the urine could also be used as a biomarker of oxidative stress *in vivo* (Kuiper *et al.*, 2008). Described below is the method for the quantification and characterization of NAC-DHN in the urine of mice exposed to HNE. For identification and quantification, radioactive HNE is required for these experiments.

1. Inject 4-(³H)-HNE (1 mg/kg in PBS; 1.0 × 10⁶ cpm) in the tail vein of C57BL/6 mice (25 ± 3 g). House mice in metabolic cages and provide water and food *ad libitum*.
2. Collect urine for 8 h
3. Aspirate an aliquot of urine (40 µl) in a glass tube and mix it with an equal volume of trichloroacetic acid (20%, w/v) and remove the precipitated proteins by centrifugation.
4. Separate the radioactivity in the supernatant by HPLC (

3.3. Systemic metabolism of acrolein

Similar to HNE, exposure of mice to the C3 enal acrolein results in the formation of the formation of glutathionyl conjugates which are subsequently metabolized and excreted in the urine as mercapturic acids. Hydroxypropyl mercapturic acid (HPMA) is the major metabolite recovered in the urine of mice exposed to acrolein. The following procedure is used for the quantification and characterization of HPMA in urine. Because acrolein is highly reactive and volatile, it is difficult to synthesize radiolabeled acrolein. Hence, a ¹³C-standard is required to quantify acrolein-mercapturates by GC-MS. ¹³C-Acrolein was custom synthesized (Sigma Chemical Co., St. Louis, MO). To study acrolein metabolism:

- 1 Inject the mice with saline (0.1 ml, i.p.) or acrolein (2 mg/kg) and collect the urine as described above.
- 2a Synthesize the internal standard, [¹³C₃]3-HPMA, by incubating [¹³C₃]3-acrolein with a 10-fold excess of NAC in 0.1 M K⁺-phosphate, pH 7.4, for 1 h at room temperature. Purify the NAC-propanal generated from this reaction by RP HPLC on an ODS C₁₈ column. Reduce the NAC-propanal by incubating with AR or sodium borohydride as described above and purify the product [¹³C₃]3-HPMA by HPLC. Establish the chemical identity by ESI-MS (Conklin *et al.*, 2009).
- 2b To quantify HPMA in the urine of control and acrolein exposed mice, mix the internal standard [¹³C₃]3-HPMA (10 nmol) to urine and subject the urine to solid-

phase extraction (Conklin *et al.*, 2009). After conditioning the cartridge, apply the urine on the cartridge, and wash the cartridge with 2% NH_4OH (6 ml) followed by methanol (6 ml). Dry the cartridge under N_2 and wash with 2% formic acid (6 ml, aq.). Elute the samples with 30% MeOH/2% formic acid and dry them under vacuum. Separate the mercapturates by RP HPLC and collect the peak corresponding to the retention time of reagent HPMA. Derivatize the samples with bistrimethylsilyltrifluoroacetamide for 1 h at 70 °C. Inject samples (1 μl) into an Agilent Technologies (Santa Clara, CA) 6890 N gas chromatograph equipped with an HP-5 capillary column (50 m $\times$ 0.2 mm i.d. $\times$ 0.5- μm phase thickness) coupled to a 5973 detector operated in the positive chemical ionization mode with ammonia as the reagent gas. Quantify the ion with m/z value of 366, using the ion with m/z value of 369 ($^{13}\text{C}_3$]3-HPMA) as an internal standard (Conklin *et al.*, 2009).

4. Signaling Properties of Glutathionyl Conjugates of HNE

Unsaturated aldehydes are highly reactive. Thus, when generated in cells or delivered exogenously, they induce a variety of stress responses and activate inflammatory signaling. At high concentrations, these aldehydes cause nonspecific toxicity (Esterbauer *et al.*, 1991). In contrast, the glutathionyl conjugates of enals such as HNE, though less reactive than the parent aldehyde, elicit more selective signaling pathways. GS-DHN is a mitogen for VSMC (Ramana *et al.*, 2006) and its formation is required for stimulation of the NF- κB pathway by HNE. GS-HNE, displays strong proinflammatory properties (Spite *et al.*, 2009). Described below are methods to examine the inflammatory properties of GS-HNE in acute peritonitis. Other glutathione conjugates of enals may also have similar properties, but these remain to be studied. Similar studies could be performed to examine the biological effects of other conjugates of HNE and related enals.

4.1. Effect of glutathionyl conjugates of HNE on acute peritonitis

1. Inject saline, GS-HNE, intraperitoneally (each 10 μg) to 6- to 8-week-old male FVB mice.
2. After 4 h, lavage the peritonium with phosphate buffer saline.
3. Count total PMNs by differential analysis using Wright–Giemsa staining and by FACS analysis using PE-conjugated anti-Gr-1 antibodies.
4. For the assessment of vascular permeability, inject Evan's blue dye (1%, i.v.) prior to the intraperitoneal injection of GS-HNE and determine the absorbance ($\text{OD}_{610\text{ nm}}$) of the peritoneal lavage after 15 min.
5. For the quantification of lipid mediators, process the samples as following:
 - a. Lavage the peritonium with phosphate buffer saline, 4 h after the i.p injection of saline or GS-HNE.
 - b. Mix the exudates with 2 volumes of cold methanol, containing 400 pg of d4-PGE₂ as an internal standard.
 - c. Cool the samples to $-20\text{ }^\circ\text{C}$ for 30 min to allow protein precipitation.
 - d. Centrifuge the samples at $850\times g$ for 20 min ($4\text{ }^\circ\text{C}$) and perform the solid-phase extraction of the supernatant, using C₁₈ SEP-PAK cartridges (Alltech, Deerfield, IL) as per manufacturer's instructions.
 - e. Dry the methyl formate fractions under N_2 and resuspend the samples in methanol and perform the LC/MS/MS analysis for the quantification of proinflammatory lipid mediators LTB₄, PGE₂ and thromboxane B₂

(TXB₂), and monohydroxy fatty acids—5-, 12-, and 15-HETE, and 17-HDHA.

6. For the quantification of cysteinyl leukotriene formation, perform the solid-phase extraction on exudates (5 ml), dry the methanol fractions under N₂ and resuspend the samples in extraction buffer. Quantify the cysteinyl leukotriene formation by ELISA.

5. Conclusions

Our studies show that conjugation of enals with glutathione and subsequent reduction of the glutathionyl conjugates of enals is a major pathway for the cellular metabolism of enals. NAC-DHN and HPMA, the reduced form of the mercapturic acids of HNE and acrolein, respectively, are the major metabolites recovered in the urine of mice exposed to acrolein and DHN. NAC-DHN has also been shown to be present in the urine of healthy human subjects. GS-HNE promotes ROS formation in PMNs and causes acute peritonitis in mice. Glutathionyl conjugates of HNE mediate redox signaling and promote smooth muscle cell proliferation, and the mitogenic effect of glutathionyl conjugates is regulated by glutathione conjugate transporter, RLIP (Fig. 5). In summary, glutathionyl conjugates of enals could be critical regulators of redox signaling. Careful employment of methods to synthesize and study the effects of glutathione conjugates could lead to a better understanding of their role in regulating cell physiology and function and the role of oxidative stress in pharmacology and toxicology.

Acknowledgments

This work was supported in parts by NIH grants HL95593, ES17260, ES11594, ES11860, HL59378, RR 24489, GM71036, and DK36118.

References

- Adams JD Jr, Klaidman LK. Acrolein-induced oxygen radical formation. *Free Radic Biol Med* 1993;15:187–193. [PubMed: 8397144]
- Alary J, Debrauwer L, Fernandez Y, Cravedi JP, Rao D, Bories G. 1,4-Dihydroxynonene mercapturic acid, the major end metabolite of exogenous 4-hydroxy-2-nonenal, is a physiological component of rat and human urine. *Chem Res Toxicol* 1998;11:130–135. [PubMed: 9511904]
- Benedetti A, Comporti M, Esterbauer H. Identification of 4-hydroxynonenal as a cytotoxic product originating from the peroxidation of liver microsomal lipids. *Biochim Biophys Acta* 1980;620:281–296. [PubMed: 6254573]
- Bhatnagar A. Environmental cardiology: Studying mechanistic links between pollution and heart disease. *Circ Res* 2006;99:692–705. [PubMed: 17008598]
- Conklin DJ, Habertzell P, Lesgards JF, Prough RA, Srivastava S, Bhatnagar A. Increased sensitivity of glutathione S-transferase P-null mice to cyclophosphamide-induced urinary bladder toxicity. *J Pharmacol Exp Ther* 2009;331:456–469. [PubMed: 19696094]
- Dianzani MU. 4-Hydroxynonenal and cell signalling. *Free Radic Res* 1998;28:553–560. [PubMed: 9736307]
- Dianzani MU. 4-Hydroxynonenal from pathology to physiology. *Mol Aspects Med* 2003;24:263–272. [PubMed: 12893004]
- Dittberner U, Eisenbrand G, Zankl H. Genotoxic effects of the alpha, beta-unsaturated aldehydes 2-trans-buteneal, 2-trans-hexenal and 2-trans, 6-cis-nonadienal. *Mutat Res* 1995;335:259–265. [PubMed: 8524341]
- Eaton P, Li JM, Hearse DJ, Shattock MJ. Formation of 4-hydroxy-2-nonenal-modified proteins in ischemic rat heart. *Am J Physiol* 1999;276:H935–H943. [PubMed: 10070077]

- Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 1991;11:81–128. [PubMed: 1937131]
- Feron VJ, Til HP, de VF, Woutersen RA, Cassee FR, van Bladeren PJ. Aldehydes: Occurrence, carcinogenic potential, mechanism of action and risk assessment. *Mutat Res* 1991;259:363–385. [PubMed: 2017217]
- Hill BG, Awe SO, Vladyskovskaya E, Ahmed Y, Liu SQ, Bhatnagar A, Srivastava S. Myocardial ischaemia inhibits mitochondrial metabolism of 4-hydroxy-trans-2-nonenal. *Biochem J* 2009;417:513–524. [PubMed: 18800966]
- Horvath JJ, Witmer CM, Witz G. Nephrotoxicity of the 1:1 acrolein-glutathione adduct in the rat. *Toxicol Appl Pharmacol* 1992;117:200–207. [PubMed: 1471152]
- Ishii T, Itoh K, Ruiz E, Leake DS, Unoki H, Yamamoto M, Mann GE. Role of Nrf2 in the regulation of CD36 and stress protein expression in murine macrophages: Activation by oxidatively modified LDL and 4-hydroxynonenal. *Circ Res* 2004;94:609–616. [PubMed: 14752028]
- Kuiper HC, Miranda CL, Sowell JD, Stevens JF. Mercapturic acid conjugates of 4-hydroxy-2-nonenal and 4-oxo-2-nonenal metabolites are in vivo markers of oxidative stress. *J Biol Chem* 2008;283:17131–17138. [PubMed: 18442969]
- Montine KS, Kim PJ, Olson SJ, Markesbery WR, Montine TJ. 4-Hydroxy-2-nonenal pyrrole adducts in human neurodegenerative disease. *J Neuropathol Exp Neurol* 1997;56:866–871. [PubMed: 9258256]
- Parola M, Robino G, Marra F, Pinzani M, Bellomo G, Leonarduzzi G, Chiarugi P, Camandola S, Poli G, Waeg G, Gentilini P, Dianzani MU. HNE interacts directly with JNK isoforms in human hepatic stellate cells. *J Clin Invest* 1998;102:1942–1950. [PubMed: 9835619]
- Ramana KV, Bhatnagar A, Srivastava S, Yadav UC, Awasthi S, Awasthi YC, Srivastava SK. Mitogenic responses of vascular smooth muscle cells to lipid peroxidation-derived aldehyde 4-hydroxy-trans-2-nonenal (HNE): Role of aldose reductase-catalyzed reduction of the HNE-glutathione conjugates in regulating cell growth. *J Biol Chem* 2006;281:17652–17660. [PubMed: 16648138]
- Rittner HL, Hafner V, Klimiuk PA, Szweda LI, Goronzy JJ, Weyand CM. Aldose reductase functions as a detoxification system for lipid peroxidation products in vasculitis. *J Clin Invest* 1999;103:1007–1013. [PubMed: 10194473]
- Robino G, Zamara E, Novo E, Dianzani MU, Parola M. 4-Hydroxy-2, 3-alkenals as signal molecules modulating proliferative and adaptive cell responses. *Biofactors* 2001;15:103–106. [PubMed: 12016336]
- Ruef J, Liu SQ, Bode C, Tocchi M, Srivastava S, Runge MS, Bhatnagar A. Involvement of aldose reductase in vascular smooth muscle cell growth and lesion formation after arterial injury. *Arterioscler Thromb Vasc Biol* 2000;20:1745–1752. [PubMed: 10894812]
- Spite M, Summers L, Porter TF, Srivastava S, Bhatnagar A, Serhan CN. Resolvin D1 controls inflammation initiated by glutathione-lipid conjugates formed during oxidative stress. *Br J Pharmacol* 2009;158:1062–1073. [PubMed: 19422383]
- Srivastava S, Chandra A, Ansari NH, Srivastava SK, Bhatnagar A. Identification of cardiac oxidoreductase(s) involved in the metabolism of the lipid peroxidation-derived aldehyde-4-hydroxynonenal. *Biochem J* 1998a;329:469–475. [PubMed: 9445372]
- Srivastava S, Chandra A, Wang LF, Seifert WE Jr, Dague BB, Ansari NH, Srivastava SK, Bhatnagar A. Metabolism of the lipid peroxidation product, 4-hydroxy-trans-2-nonenal, in isolated perfused rat heart. *J Biol Chem* 1998b;273:10893–10900. [PubMed: 9556565]
- Srivastava S, Watowich SJ, Petrash JM, Srivastava SK, Bhatnagar A. Structural and kinetic determinants of aldehyde reduction by aldose reductase. *Biochemistry* 1999;38:42–54. [PubMed: 9890881]
- Srivastava S, Conklin DJ, Liu SQ, Prakash N, Boor PJ, Srivastava SK, Bhatnagar A. Identification of biochemical pathways for the metabolism of oxidized low-density lipoprotein derived aldehyde-4-hydroxy trans-2-nonenal in vascular smooth muscle cells. *Atherosclerosis* 2001;158:339–350. [PubMed: 11583712]

- Srivastava S, Chandrasekar B, Bhatnagar A, Prabhu SD. Lipid peroxidation-derived aldehydes and oxidative stress in the failing heart: Role of aldose reductase. *Am J Physiol Heart Circ Physiol* 2002;283:H2612–H2619. [PubMed: 12388223]
- Srivastava S, Vladyskovskaya E, Barski OA, Spite M, Kaiserova K, Petrash JM, Chung SS, Hunt G, Dawn B, Bhatnagar A. Aldose reductase protects against early atherosclerotic lesion formation in apolipoprotein E-null mice. *Circ Res* 2009;105:793–802. [PubMed: 19729598]
- Uchida K, Kanematsu M, Morimitsu Y, Osawa T, Noguchi N, Niki E. Acrolein is a product of lipid peroxidation reaction. Formation of free acrolein and its conjugate with lysine residues in oxidized low density lipoproteins. *J Biol Chem* 1998;273:16058–16066. [PubMed: 9632657]
- Warnke MM, Wanigasekara E, Singhal SS, Singhal J, Awasthi S, Armstrong DW. The determination of glutathione-4-hydroxynonenal (GSHNE), E-4-hydroxynonenal (HNE), and E-1-hydroxynon-2-en-4-one (HNO) in mouse liver tissue by LC-ESI-MS. *Anal Bioanal Chem* 2008;392:1325–1333. [PubMed: 18815773]
- Yoritaka A, Hattori N, Uchida K, Tanaka M, Stadtman ER, Mizuno Y. Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *Proc Natl Acad Sci USA* 1996;93:2696–2701. [PubMed: 8610103]

Figure 1.

Purification and characterization of radiolabeled GS-HNE and GS-DHN. GS-HNE is purified by reverse-phase HPLC on an ODS C₁₈ column. One milliliter fractions are collected and the radioactivity is quantified on a scintillation counter (A). The HPLC-purified peak I is analyzed by ESI⁺-MS (B). Ions with m/z values of 464.3 and 446.3 represent, $[M + H]^+$ of GS-HNE and its dehydrated daughter ion, respectively. Panel C shows the ESI⁺-MS analysis of reagent GS-DHN. Retention time of the reagent GS-DHN on HPLC is identical to that of reagent GS-HNE. ESI⁺-MS of the HPLC peak corresponding to reagent GS-DHN shows the molecular ion with m/z 466.3 corresponds to $[M + H]^+$ of GS-DHN.

Figure 2.

Purification and characterization of GS-HNE-ester and GS-DHN-ester. Reagentesterified glutathionyl conjugates of HNE are separated by reverse-phase HPLC on an ODS C₁₈ column (A). Chemical identities of GS-HNE-ester and GS-DHN-ester are established by ESI⁺-MS. ESI⁺-MS of the HPLC peak I shows strong molecular ion with *m/z* value of 494.3 corresponding to the [M + H]⁺ of GS-DHN-ester (B). ESI⁺-MS of the HPLC- peak II shows strong ions with *m/z* values of 492.2 and 474.2; consistent with the [M + H]⁺ of GS-HNE-ester and its dehydrated daughter ion, respectively (C).

Figure 3.

Metabolism of HNE in PC12 Cells. PC12 Cells are incubated with 4- $(^3\text{H})\text{-HNE}$ ($5\text{ }\mu\text{M}$) in 2.5 ml HBSS for 30 min at $37\text{ }^\circ\text{C}$. After the incubation, radioactivity in the incubation medium is separated by HPLC on an ODS C_{18} reverse-phase HPLC column. Panel A shows the HPLC profile of radiolabeled metabolites of $[^3\text{H}]\text{-HNE}$. Peaks I–IV correspond to the retention time of reagent glutathione conjugates of HNE (GS-HNE and GS-DHN), DHN, HNA, and unmetabolized HNE, respectively. Chemical identities of peaks V and VI have yet not been established. Panel B shows the ESI⁺-MS chromatogram of HPLC peak I. Molecular ions with m/z value of 464.3 and 466.3 correspond to $[\text{M} + \text{H}]^+$ of GS-HNE and GS-DHN, respectively. Ion with m/z ratio of 446.3 corresponds to the dehydrated daughter ion of GS-HNE.

Figure 4.

Systemic metabolism of HNE. Male C57 are injected with 4-(^3H)-HNE (1 mg/kg in PBS) in the tail vein and urine is collected for 8 h. Fifty microliters urine was mixed with an equal volume of trichloroacetic acid (20%, w/v) and the proteins that are precipitated are removed by centrifugation. Radioactivity in the supernatant is separated by HPLC on an ODS C_{18} reverse-phase HPLC column as described in Fig. 1. Panel A shows the HPLC profile of radiolabeled metabolites of [^3H]-HNE in the urine. Chemical identity of the radiolabeled peak eluting at 26 min, corresponding to the retention time of reagent NAC-DHN, is established by ESI $^-$ -MS. As shown in Panel B, ESI $^-$ -MS of these peak shows a strong molecular ion with m/z value of 320.1, consistent with $[\text{M} - \text{H}]^-$ of NAC-DHN.

Figure 5. Effect of HNE and its glutathionyl conjugates on redox signaling