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Differential metabolism of 4-hydroxynonenal in liver, lung and brain of mice and rats

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Abstract

The lipid peroxidation end-product 4-hydroxynonenal (4-HNE) is generated in tissues during oxidative stress. As a reactive aldehyde, it forms Michael adducts with nucleophiles, a process that disrupts cellular functioning. Liver, lung and brain are highly sensitive to xenobiotic-induced oxidative stress and readily generate 4-HNE. In the present studies, we compared 4-HNE metabolism in these tissues, a process that protects against tissue injury. 4-HNE was degraded slowly in total homogenates and S9 fractions of mouse liver, lung and brain. In liver, but not lung or brain, NAD(P)⁺ and NAD(P)H markedly stimulated 4-HNE metabolism. Similar results were observed in rat S9 fractions from these tissues. In liver, lung and brain S9 fractions, 4-HNE formed protein adducts. When NADH was used to stimulate 4-HNE metabolism, the formation of protein adducts was suppressed in liver, but not lung or brain. In both mouse and rat tissues, 4-HNE was also metabolized by glutathione S-transferases. The greatest activity was noted in livers of mice and in lungs of rats; relatively low glutathione S-transferase activity was detected in brain. In mouse hepatocytes, 4-HNE was rapidly taken up and metabolized. Simultaneously, 4-HNE-protein adducts were formed, suggesting that 4-HNE metabolism in intact cells does not prevent protein modifications. These data demonstrate that, in contrast to liver, lung and brain have a limited capacity to metabolize 4-HNE. The persistence of 4-HNE in these tissues may increase the likelihood of tissue injury during oxidative stress.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Keywords

4-hydroxynonenal; alcohol dehydrogenase; lipid peroxidation; reactive oxygen species; pulmonary toxicity; neurotoxicity

Introduction

It is well recognized that excessive production of reactive oxygen species (ROS) can lead to oxidative stress and tissue damage (Sinclair *et al.*, 1990; Hollan, 1995; Flora, 2007). ROS react with many cellular components including lipids, which can initiate lipid peroxidation (Bergamini *et al.*, 2004). Peroxidation of unsaturated fatty acids such as linoleic acid, arachidonic acid and docosahexaenoic acid generates a variety of water soluble short chain reactive carbonyl compounds as degradation products (Alary *et al.*, 2003b). One of these products is 4-hydroxynonenal (4-HNE), a relatively abundant reactive aldehyde derived from the peroxidation of omega-6-polyunsaturated fatty acids (Poli *et al.*, 2008a). 4-HNE forms Michael adducts with nucleophilic sites in cells including those in DNA, lipids and proteins (LoPachin *et al.*, 2009). These adducts can cause mutations, disrupt cell structures, and negatively modulate cellular metabolism (Poli *et al.*, 2008b; LoPachin *et al.*, 2009).

4-HNE is readily formed in liver, lung and brain in response to toxicants. A number of diseases and pathologies have been linked to the generation of 4-HNE in these tissues including alcoholic liver disease (Paradis *et al.*, 1997), chronic obstructive pulmonary disease, emphysema and asthma (Rahman *et al.*, 2002; Arunachalam *et al.*, 2010), as well as Alzheimer disease and Parkinson disease (Zarkovic, 2003). Detoxification of 4-HNE is an important process that has been shown to protect against tissue injury and disease progression (Hartley *et al.*, 1999; Terneus *et al.*, 2008; Galligan *et al.*, 2012). Distinct enzymes have been identified that detoxify 4-HNE, including alcohol dehydrogenase, aldehyde dehydrogenase, aldo-keto reductase, alkenal/one oxidoreductase, cytochrome P450's and various glutathione S-transferases (Hartley *et al.*, 1995; Srivastava *et al.*, 2000; Burczynski *et al.*, 2001; Dick *et al.*, 2001; Forman, 2010; Amunom *et al.*, 2011). Although metabolism of 4-HNE has been studied extensively in the liver, much less is known about its metabolism in lung and brain, and this represents the focus of the present studies. Using tissues from both mice and rats, we found rapid degradation of 4-HNE in liver fractions, a process that limited the formation of 4-HNE protein adducts. In contrast, degradation of 4-HNE was very slow in lung and brain. This resulted in extensive protein-adduct formation in these tissues. Low rates of degradation suggest that 4-HNE accumulates in greater amounts in lung and brain during oxidative stress; this may lead to increased susceptibility to tissue damage.

Materials and Methods

Reagents

Mouse monoclonal 4-HNE antibody (catalog #MAB3249) was purchased from R&D Systems (Minneapolis, MN) and goat anti-mouse horseradish peroxidase-conjugated secondary antibody from BioRad Laboratories (Hercules, CA). The Western Lightning

enhanced chemiluminescence kit (ECL) was from Perkin Elmer Life Sciences (Boston, MA) and Alamar Blue solution from Invitrogen (Grand Island, NY). 4-HNE was obtained from Calbiochem (La Jolla, CA). William's Medium E, glutamine and fetal bovine serum (FBS) were from Invitrogen Corp (Carlsbad, CA). 4-Methylpyrazole hydrochloride was from Santa Cruz (Santa Cruz, CA). NADH, NAD⁺, NADPH, NADP⁺, disulfiram, proteinase inhibitors and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Isolation and analysis of hepatocytes

In all experiments, animals received humane care in compliance with the institution's guidelines, as outlined in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Hepatocyte isolation was performed as described previously (Dragomir *et al.*, 2011). Briefly, C57BL/6J male mice (The Jackson Laboratory, Bar Harbor, ME) were euthanized with Nembutal (200 mg/kg). The liver was perfused through the portal vein with warm Ca²⁺/Mg²⁺-free Hank's balanced salt solution (pH 7.3) containing 25 mM HEPES and 0.5 mM EGTA, followed by Leibowitz L-15 medium containing HEPES and 0.2 U/ml Liberase 3 Blendzyme. The liver was then excised, disaggregated, and the resulting cell suspension filtered through a 220 µm nylon mesh. Hepatocytes were recovered by centrifugation at 50 × g, and cell viability, assessed by trypan blue dye exclusion, was greater than 90%. To characterize the formation of 4-HNE-protein adducts, hepatocytes (2 × 10⁶) were cultured on collagen I-coated 6-well plates in William's Medium E supplemented with 10% FBS, 1% penicillin-streptomycin, 1 mM sodium pyruvate, 1% insulin-transferrin-selenium, and 2 mM L-glutamine. Non-adherent cells were removed 3 h later by washing, the cells refed with fresh medium and cultured overnight at 37 °C in a 5% CO₂ incubator. Cells were then treated with vehicle control, 30 µM or 100 µM 4-HNE in 1.5 ml of serum-free medium. After 0-60 min, cells were washed with HBSS and lysed in 300 µl of buffer containing 1% SDS, 10 mM Tris-base, pH 7.6 and protease inhibitors. 4-HNE-protein adduct formation in lysates was analyzed by Western blotting as described below. Protein content in cell lysates was determined by the DC (Detergent Compatible) protein assay kit (BioRad Laboratories) using bovine serum albumin as the standard. To assay 4-HNE uptake and metabolism, freshly isolated hepatocytes (2 × 10⁶ cells/ml) were suspended in serum-free William's medium E in closed 1.5 ml Eppendorf tubes and incubated in a shaking water bath at 37 °C. 4-HNE (100 µM) was added to the vials with rapid shaking to initiate the reactions. After increasing periods of time (0-60 min), 200 µl of the cell suspension was withdrawn and centrifuged. Pelleted cells were washed with HBSS and extracted with 200 µl of acetonitrile/acetic acid (96:4) for analysis. Cell viability, assayed using Alamar blue, was greater than 95% after 2 h treatment with 100 µM 4-HNE.

Preparation of tissue fractions and metabolism studies

Liver, lung and brain were collected from male C57BL/6J mice (Jackson Labs, Bar Harbor, ME) and Long-Evans Hooded rats (Charles River Laboratories, Wilmington, MA), washed with ice-cold 0.9% NaCl, cut into 0.1-0.2 cm³ sections, and homogenized in 3~4 volumes of ice-cold buffer (50 mM Tris-HCl buffer containing 1.15% KCl, pH 7.4) using 20 strokes of a Potter-Elvehjem homogenizer. Tissue samples were then centrifuged at 1,500 × g, 4°C for 20 min to remove nuclei and cellular debris, and the supernatants used in metabolism assays

as total homogenates. S9 fractions were prepared by centrifuging homogenized tissues at $9,000 \times g$, 4°C for 20 min using an Eppendorf 5417R centrifuge and stored at -70°C until analysis. For 4-HNE metabolism assays, 100 μg of tissue protein was incubated in a 1.0 ml reaction mix containing 10 mM potassium phosphate buffer, pH 7.8, and 100 μM 4-HNE, with or without 1 mM concentrations of reduced or oxidized pyridine nucleotides, or enzyme inhibitors. Tissue proteins, heated for 5 min at 100°C , were used as controls. After 15-120 min, 200 μl of the reaction mixes were withdrawn. 4-HNE was extracted from the samples by the addition of an equal volume of acetonitrile/acetic acid (96:4, v/v). Samples were then centrifuged at $1,000 \times g$ at 4°C for 10 min and clear supernatants analyzed by HPLC as described by Hartley *et al.* (1995) using a Jasco HPLC system (Jasco Corporation, Tokyo, Japan) fitted with a Phenomenex 5 $\mu\text{C}18$ column (Luna (2), 250×2.00 mm). 4-HNE and its metabolites were separated using a mobile phase consisting of 70% 50 mM potassium phosphate buffer (pH 2.7) and 30% acetonitrile (v/v) at a flow rate of 0.25 ml/min and the HPLC effluent monitored at 224 nm.

Glutathione S-transferase assays using 4-HNE as the substrate were performed as previously described (Alin *et al.*, 1985), except that enzyme activity was monitored by the disappearance of 4-HNE. Reactions were run for 3 min and contained 100 μM 4-HNE, 500 μM glutathione and 100-200 μg of S9 tissue fraction protein. Background degradation of 4-HNE by glutathione conjugation in the absence of tissue fractions was subtracted from measurements of enzyme activity.

To assess the effects of 4-methylpyrazole, disulfiram or glutathione on 4-HNE-protein adduct formation, S9 samples containing 10 μg of protein were incubated with 100 μM 4-HNE in 15 μl phosphate buffer in the absence or presence of 1 mM NADH or glutathione. After 0, 15, 30 and 60 min, reactions were stopped by the addition of an equal volume of $2 \times$ SDS gel loading buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, 5% mercaptoethanol), followed by heating at 95°C for 5 min. Adducts formed in tissue extracts were analyzed by Western blotting.

Western blotting

Proteins were analyzed by Western blotting as previously described (Zhenget *al.*, 2013). Briefly, 10 μg of protein were electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and blocked in Tris buffer supplemented with 5% milk for 1 h at room temperature. The blots were then washed and incubated overnight at 4°C with 4-HNE primary antibody (1:3000). This was followed by washing with tTBS (Tris-buffered saline supplement with 0.1% Tween 20), and incubation with horseradish peroxidase-conjugated secondary antibody (1: 10,000). After 1 h at room temperature, proteins were visualized by ECL chemiluminescence.

Statistical analysis

Data were evaluated using the two-way ANOVA. $p < 0.05$ was considered statistically significant.

Results

4-HNE metabolism in liver, lung and brain

In initial studies we compared 4-HNE metabolism in total homogenates and S9 fractions prepared from mouse liver, lung and brain tissues. A generally similar pattern of 4-HNE degradation was evident in the different preparations from these tissues (Figs. 1-3). Thus, in the absence of added pyridine nucleotide cofactors, 4-HNE was degraded slowly over time. After 30 min, approximately 20-30% of 4-HNE was degraded in the liver while 10-12% was degraded in lung and brain (Figs. 1 and 2). A similar slow degradation of 4-HNE was noted in S9 fractions from rat liver, lung and brain (Figs. 2 and 4). Earlier studies showed that pyridine nucleotide cofactor-dependent enzymes present in the liver, including aldehyde dehydrogenase and alcohol dehydrogenase, metabolize 4-HNE (Alary *et al.*, 2003b). Similarly, we found that both reduced and oxidized pyridine nucleotides markedly stimulated 4-HNE metabolism in total homogenates and S9 fractions from mouse liver, but not lung or brain (Figs. 1-3). A marked increase in 4-HNE metabolism was also observed in S9 fractions from rat liver, but not lung and brain following treatment with the pyridine nucleotides (Figs. 2 and 4). Greater amounts of 4-HNE were degraded in liver fractions from mice and rats treated with NADH and NAD⁺ compared to NADPH and NADP⁺. In mouse tissues, although the relative ratios of 4-HNE degradation with reduced and oxidized pyridine nucleotides were similar in total homogenates (Fig. 1) and S9 liver fractions (Fig. 3, upper panels), greater absolute amounts of 4-HNE degradation were noted in S9 fractions. This is likely due to increased amounts of proteins unrelated to metabolism in total homogenates of the liver, when compared to S9 fractions. To further characterize 4-HNE metabolism, we analyzed an S9 fraction from mouse liver treated with NADH. We found that the effects of the pyridine nucleotide were concentration- and time-dependent (Fig. 5, panels A and B). At least two metabolites (labeled a and b on the HPLC tracings) were detected during 4-HNE metabolism (Fig. 5, panels C and D). Based on the degradation of 4-HNE after 5 min in S9 fractions (Fig. 6), we estimate the rate of NADH-supported 4-HNE degradation as ~120 nmol/min/mg protein for mouse liver and ~180 nmol/min/mg protein for rat liver.

Enzymes mediating 4-HNE metabolism in S9 fractions

As indicated above, both alcohol dehydrogenase and aldehyde dehydrogenase have been shown to metabolize 4-HNE (Alary *et al.*, 2003b). We found that 200 μ M 4-methylpyrazole, an inhibitor of alcohol dehydrogenase, and to lesser extent, 100 μ M disulfiram, an inhibitor to aldehyde dehydrogenase, suppressed 4-HNE degradation in S9 fractions from mouse and rat liver (Figs. 6 and 7) (Dawidek-Pietryka *et al.*, 1998; Moreb *et al.*, 2008). These concentrations of inhibitors were found to maximally suppress 4-HNE metabolism (Fig. 8). The combination of these two inhibitors resulted in almost complete inhibition of 4-HNE metabolism (data not shown). These data indicate that 4-HNE is metabolized in liver S9 fractions largely by alcohol dehydrogenase, with a smaller contribution by aldehyde dehydrogenase. 4-Methylpyrazole and disulfiram had minimal effects on 4-HNE metabolism in mouse and rat lung and brain (Fig. 6).

Conjugation of 4-HNE with glutathione is also an important detoxification pathway; this occurs by the direct reaction of 4-HNE with glutathione and via several glutathione S-transferase enzymes (Alin *et al.*, 1985; Singhal *et al.*, 1994; Cheng *et al.*, 2001; Engle *et al.*, 2004). Glutathione S-transferase mediated conjugation of 4-HNE with glutathione is known to be significantly more rapid than direct 4-HNE-glutathione conjugation (Spitz *et al.*, 1991). In further studies we measured the activity of glutathione S-transferase in S9 fractions from the liver, lung and brain using 4-HNE as the substrate (Table 1). In mouse tissues, the greatest glutathione S-transferase activity was detected in liver fractions; significantly less enzyme activity was observed in lung and brain. In the rat, the highest activity was noted in lung followed by liver and brain. These data are consistent with the idea that there is differential metabolism of 4-HNE in liver, lung and brain from mice and rats.

Binding of 4-HNE to liver, lung and brain proteins

The α , β -unsaturated bond of 4-HNE is known to form adducts with proteins by reacting with cysteine, histidine and lysine residues through Michael additions (Vila *et al.*, 2008). We found that proteins in S9 fractions of liver, lung and brain from mice were modified by 4-HNE in a time-dependent manner, as detected by Western blotting using anti-4-HNE antibodies (Fig. 9). Over 20 distinct bands with molecular weights ranging from 20-200 kDa were modified by 4-HNE in each of the tissues. The addition of NADH to reaction mixes to stimulate alcohol dehydrogenase activity and 4-HNE metabolism, resulted in suppression of 4-HNE protein adduct formation in the liver (Fig. 9, upper panel). 4-Methylpyrazole, but not disulfiram, restored the ability of 4-HNE to modify proteins in NADH-stimulated S9 liver fractions. These data are in accord with our findings that the alcohol dehydrogenase inhibitor was highly effective in inhibiting 4-HNE metabolism in the liver (Figs. 6 and 7). In contrast, NADH treatment of S9 fractions from lung and brain had minimal effects on 4-HNE protein adduct formation (Fig. 9, middle and lower panels), a finding consistent with the fact that NADH had no significant effect on 4-HNE metabolism in these tissue. As expected, neither 4-methylpyrazole nor disulfiram altered 4-HNE protein adduct formation in the lung and brain; only a small increase in 4-HNE-modified proteins was evident in these tissues 30 min after treatment with the inhibitors. In further studies we analyzed the effects of glutathione on the formation of 4-HNE-protein adducts. In liver, lung and brain, glutathione was found to readily prevent 4-HNE adduct formation (Fig. 10).

4-HNE metabolism in isolated mouse hepatocytes

We next analyzed 4-HNE metabolism in mouse hepatocytes to determine if cells that actively degrade this reactive aldehyde also form 4-HNE protein adducts. 4-HNE was found to be readily taken up by mouse hepatocytes (Fig. 11, panels A and B). Accumulation of 4-HNE in the cells was maximal within 15 min, declining thereafter. This appeared to be due to 4-HNE metabolism. A time-dependent increase in the appearance of three metabolites (labeled c, d and e on the HPLC tracing) was observed in the cells (Fig. 11, panels A). 4-HNE was found to form protein adducts in a time- and concentration-dependent manner (Fig. 11, panel C). After 15 min, two major bands (MW = 100,000 and 150,000) were detected in Western blots suggesting that 4-HNE selectively reacts with specific proteins in

the cells. After 60 min, these proteins, along with a number of additional proteins of higher and lower molecular weights, were also modified by 4-HNE (Fig. 11, panel C).

Discussion

Metabolism is key to the detoxification of cytotoxic lipid peroxidation products like 4-HNE (Poli *et al.*, 2008b; Roede *et al.*, 2010). In hepatocytes, 4-HNE has been shown to be metabolized by both oxidative and reductive pathways (Alary *et al.*, 2003b). Enzymatic and non-enzymatic conjugation reactions including those mediated by glutathione S-transferases, glutathione and various amino acids are also important in the detoxification process (Esterbauer *et al.*, 1975; Mitchell and Petersen, 1987; Hartley *et al.*, 1995; Cheng *et al.*, 2001; Alary *et al.*, 2003a). The present studies demonstrate that 4-HNE is rapidly degraded in total homogenates and S9 fractions of liver from mice and rats and that both reduced and oxidized pyridine nucleotide cofactors stimulated this response. This may be due, in part, to overlapping cofactor specificity for some of the enzymes mediating 4-HNE degradation. For example, both NAD⁺ and NADP⁺ can mediate aldehyde dehydrogenases activity (Leonarduzzi *et al.*, 1995). Alternatively, additional pyridine cofactor-dependent enzymes that have not been characterized may contribute to 4-HNE degradation. In our studies, although NADH and NAD⁺ were the preferred co-substrates for 4-HNE metabolism, NADPH and NADP⁺ displayed 80-90% activity. Using rat liver homogenates, Esterbauer *et al.* (1985) reported that 4-HNE metabolism was largely supported by NADH; thus NADPH mediated metabolism represented only 4-5% of the activity of NADH. Differences between these early studies and ours may reflect differences in the strains of animals used, and/or the subcellular fractions evaluated in the metabolism studies. Esterbauer *et al.* (1985) also identified alcohol dehydrogenase as an important mediator of 4-HNE metabolism in rat liver homogenates. Consistent with this is our findings that the alcohol dehydrogenase inhibitor, 4-methylpyrazole, effectively inhibited 4-HNE metabolism in both mouse and rat liver S9 fractions. We also found that the aldehyde dehydrogenase inhibitor, disulfiram, reduced 4-HNE metabolism, although not as effectively as 4-methylpyrazole. In this regard, previous studies have demonstrated that rat liver aldehyde dehydrogenase effectively metabolizes 4-HNE (Mitchell and Petersen, 1987). Taken together, these data indicate that multiple enzymes mediate 4-HNE metabolism in mouse and rat liver; they are also consistent with 4-HNE metabolism studies in rat hepatocytes in which both oxidative and reductive 4-HNE metabolites were identified (Ullrich *et al.*, 1994; Hartley *et al.*, 1995). In contrast to our findings, only limited metabolism of 4-HNE via alcohol dehydrogenase was observed in rat hepatocytes and rat liver precision cut sections (Hartley *et al.*, 1995; Siems *et al.*, 1997; Laurent *et al.*, 2000). This apparent disparity may be due to differences in the regulation of 4-HNE degradation in viable cells and tissues when compared to liver tissue homogenates and S9 fractions.

In contrast to the liver, 4-HNE degradation in S9 fractions from lung and brain was limited, presumably because of low levels of enzymes capable of metabolizing the reactive aldehyde (Crabb *et al.*, 2004). 4-HNE is formed in both lung and brain tissues following oxidative stress, a process linked to a number of pathologies and diseases (Kirichenko *et al.*, 1996; Rahman *et al.*, 2002). These data indicate that with limited metabolism, 4-HNE can persist in lung and brain resulting in increased reaction with cellular components and tissue injury.

Since 4-HNE is diffusible, surrounding cells and tissues are also at risk from 4-HNE-induced damage (Bennaars-Eiden *et al.*, 2002). Our data are in accord with earlier studies by Esterbauer *et al.* (1985) showing that rat lung and brain homogenates contain 0.2 to 3% of the 4-HNE metabolizing activity of rat liver. Similar low levels of 4-HNE metabolizing activity have also been described in rat heart, muscle, fat pads, spleen, small intestine and kidneys (Esterbauer *et al.*, 1985).

It is well recognized that 4-HNE is detoxified by its conjugation to glutathione which occurs directly and enzymatically via several glutathione S-transferases (Alin *et al.*, 1985; Danielson *et al.*, 1987; Roede *et al.*, 2010). In many tissues including the liver, glutathione conjugation is thought to be a predominant 4-HNE detoxification pathway (Poli *et al.*, 2008b; Roede *et al.*, 2010). In isolated rat hepatocytes, 50-60% of 4-HNE degradation has been attributed to glutathione conjugation (Hartley *et al.*, 1995). The present studies show that in S9 fractions from both mouse and rat tissues, 4-HNE stimulated glutathione S-transferase activity. In the mouse, liver contained the greatest GST activity, followed by lung and brain. These data are in accord with earlier studies on 4-HNE-glutathione conjugation in mouse liver, lung and brain (Engle *et al.*, 2004). Assuming that the values for the liver represent 50-60% of the total 4-HNE detoxification, then significantly less 4-HNE detoxification occurs via 4-HNE-glutathione conjugation in mouse lung and brain. The fact that neither lung nor brain exhibited significant reductive or oxidative metabolism of 4-HNE over the time course studied, further supports the idea that 4-HNE can persist in these tissues. In rats, S9 fractions of lung contained the greatest activity followed by liver, with much lower levels in brain. As observed with mouse liver, rat liver metabolized 4-HNE via reductive and oxidative pathways which, together with 4-HNE-glutathione conjugation, can markedly reduce intracellular concentrations of reactive aldehydes such as 4-HNE. Although rat lung may detoxify significant amounts of 4-HNE via glutathione conjugation, low levels of reductive and oxidative metabolism may allow greater amounts of 4-HNE to persist in the tissue. Low levels of 4-HNE-glutathione conjugation in rat and mouse brain are consistent with earlier studies showing limited 4-HNE metabolism via the mercapturic acid pathway in rat and human cerebrum (Sidell *et al.*, 2003).

A question arises as to the role of mitochondria in the metabolism of 4-HNE in liver, lung and brain. Mitochondria isolated from various tissues have been shown to metabolize 4-HNE via oxidative, reductive and GSH conjugative pathways (Chen and Yu, 1994; Ullrich *et al.*, 1994; Murphy *et al.*, 2003; Meyer *et al.*, 2004; Honzatko *et al.*, 2005). Mitochondria are also enriched with enzymes that detoxify 4-HNE (Mitchell and Petersen, 1991; Murphy *et al.*, 2003; Chen *et al.*, 2014). Detoxification of 4-HNE and related aldehydes by these pathways likely contributes to protecting mitochondria and other cellular compartments from oxidative stress. In our studies comparing 4-HNE degradation in total homogenates, which contain mitochondria, with mitochondrial free S9 fractions from mouse liver, lung and brain, no major differences in overall metabolism were noted. These data indicate that, although mitochondria can metabolize 4-HNE, their overall contribution to its metabolism in intact tissues is limited. This is supported by reports of relatively low rates of 4-HNE metabolism (~10-20 nmol/min/mg protein) in rat brain and liver mitochondria (Hartley and Petersen, 1993; Murphy *et al.*, 2003; Meyer *et al.*, 2004).

In metabolism studies with NADH-treated S9 fractions from liver, but not lung or brain, we detected several 4-HNE metabolites, which is consistent with our findings that 4-HNE is rapidly degraded in S9 fractions from liver, but not lung or brain. Earlier studies identified both oxidative and reductive metabolites of 4-HNE, including 4-hydroxy-2-nonenic acid and 1,4-dihydroxynonene, respectively, as well as glutathione conjugates in rat liver homogenates and rat liver-derived cells (Hartley *et al.*, 1995; Tjalkens *et al.*, 1999). Our observation that metabolism of 4-HNE in liver lysates is almost completely inhibited by 4-methylpyrazole, but only partially inhibited by disulfiram, suggests that reductive metabolites of 4-HNE derived via alcohol dehydrogenase are predominantly generated, with a smaller number of oxidative metabolites derived via aldehyde dehydrogenase. These findings are generally in line with studies showing that 4-HNE largely undergoes reductive metabolism in rat liver cytosolic fractions (Esterbauer *et al.*, 1985), and both oxidative and reductive metabolism in rat hepatocytes (Hartley *et al.*, 1995). It should be noted, however, that our results are biased towards reductive 4-HNE metabolism by our use of NADH. In future studies it will be of interest to compare 4-HNE metabolism using both oxidized and reduced pyridine nucleotides, as each will stimulate enzymes mediating different 4-HNE degradative pathways (Roede *et al.*, 2010).

In S9 fractions of mouse liver, lung and brain, 4-HNE caused a time-dependent modification of a number of proteins, which varied with the tissue. Using proteomic approaches, various protein targets of 4-HNE in different tissues have been identified, including growth factors and their receptors, cytokines, and enzymes mediating the detoxification of ROS (Vila *et al.*, 2008; Perluigi *et al.*, 2009; Smathers *et al.*, 2011; Galligan *et al.*, 2012). It remains to be determined if these proteins are modified in mouse liver, lung or brain fractions. Of interest is our finding that protein modifications were markedly inhibited by NADH in S9 fractions of liver, but not lung or brain. These results are in accord with our findings that NADH rapidly and selectively stimulates 4-HNE metabolism only in liver fractions. Thus, NADH stimulated enzymatic degradation of 4-HNE in mouse liver, preventing it from modifying proteins. The fact that this was not evident in lung or brain, suggests that once 4-HNE or other reactive aldehydes are formed in these tissues, they remain available to react with cellular components including proteins and cause tissue damage. We also found that inhibition of 4-HNE degradation in liver fractions with 4-methylpyrazole resulted in the generation of 4-HNE-protein adducts. In contrast, partial inhibition of 4-HNE degradation with disulfiram did not result in 4-HNE-protein adducts. Presumably, sufficient metabolism of 4-HNE takes place in liver fractions in the presence of the aldehyde dehydrogenase inhibitor preventing 4-HNE from reacting with hepatic proteins. As noted above with our metabolism studies, it will be of interest to compare the ability of different enzymes in the S9 liver fractions stimulated with either the oxidized or reduced pyridine nucleotides to modulate 4-HNE-induced protein modifications. As aldehyde dehydrogenase appears to be less effective in metabolizing 4-HNE, it may be that stimulating these enzymes with NAD(P)⁺ in the S9 liver fraction will be less effective in inhibiting the formation of 4-HNE protein adducts.

Our data also showed that glutathione suppressed the formation of 4-HNE protein adducts in S9 fractions from liver, lung and brain. This is likely due to both metabolism of 4-HNE by

glutathione S-transferase activity in the tissue fractions as well as degradation of 4-HNE due to its chemical reactivity with glutathione (Spitz et al., 1991). Since 4-HNE is rapidly metabolized and/or degraded in pyridine nucleotide- and glutathione-stimulated S9 liver fractions, and this can effectively suppress 4-HNE-protein adduct formation, we determined if 4-HNE-protein adducts could be formed in isolated hepatocytes containing physiological levels of reduced pyridine nucleotides and glutathione. 4-HNE was found to be rapidly metabolized in mouse hepatocytes; intracellular concentrations of 4-HNE in treated cells were maximal within 15 min declining thereafter. This was associated with the appearance of 4-HNE-derived metabolites in the cells. Rapid rates of 4-HNE metabolism have been described previously in isolated rat hepatocytes, hepatoma cell lines and several other cell types (Esterbauer *et al.*, 1985; Canuto *et al.*, 1994; Hartley *et al.*, 1995; Leonarduzzi *et al.*, 1995; Tjalkens *et al.*, 1999; Luckey and Petersen, 2001; Siems and Grune, 2003). Our studies also revealed that 4-HNE protein adducts increased in hepatocytes over time, and that this was coordinate with decreased levels of 4-HNE. This suggests that sustained maximal levels of 4-HNE are not required for 4-HNE-protein adduct formation, and that a sufficient concentration of the reactive aldehyde is available for time-dependent protein modifications. Previous studies using rat hepatocytes showed that approximately 3-6% of 4-HNE forms protein adducts (Siems and Grune, 2003). This relatively low level of adduct formation is presumably due to limited availability of 4-HNE due to metabolism. It is generally thought that glutathione conjugates of 4-HNE predominate in rat hepatocytes (Tjalkens *et al.*, 1999). At the present time, the identity of the 4-HNE-protein adducts formed in mouse hepatocytes is not known. Further studies are required to determine if they are similar to those formed in mouse liver lysates (Houglum *et al.*, 1990; Petersen and Doorn, 2004).

Lipid peroxidation generates highly toxic reactive aldehydes such as 4-HNE. Detoxification of reactive aldehydes is key for preventing tissue injury. The present studies demonstrate that homogenates and S9 fractions of lung and brain from mice, and S9 fractions from rats, display only a limited capacity to metabolize and detoxify 4-HNE, when compared to liver fractions. Thus, once formed during disease processes or oxidative stress, 4-HNE and related electrophiles, are likely to persist in lung and brain and contribute to tissue damage. Increasing 4-HNE degradation or protecting against the deleterious effects of 4-HNE may be effective in suppressing ROS-induced tissue injury.

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References

- Alary J, Fernandez Y, Debrauwer L, Perdu E, Gueraud F. Identification of intermediate pathways of 4-hydroxynonenal metabolism in the rat. *Chem Res Toxicol.* 2003a; 16:320–327. [PubMed: 12641432]
- Alary J, Gueraud F, Cravedi JP. Fate of 4-hydroxynonenal in vivo: disposition and metabolic pathways. *Mol Aspects Med.* 2003b; 24:177–187. [PubMed: 12892995]
- Alin P, Danielson UH, Mannervik B. 4-Hydroxyalk-2-enals are substrates for glutathione transferase. *FEBS Lett.* 1985; 179:267–270. [PubMed: 3838159]

- Amunom I, Dieter LJ, Tamasi V, Cai J, Conklin DJ, Srivastava S, Martin MV, Guengerich FP, Prough RA. Cytochromes P450 catalyze the reduction of alpha,beta-unsaturated aldehydes. *Chem Res Toxicol.* 2011; 24:1223–1230. [PubMed: 21766881]
- Arunachalam G, Sundar IK, Hwang JW, Yao H, Rahman I. Emphysema is associated with increased inflammation in lungs of atherosclerosis-prone mice by cigarette smoke: implications in comorbidities of COPD. *J Inflamm (Lond).* 2010; 7:34. [PubMed: 20663150]
- Bennaars-Eiden A, Higgins L, Hertzel AV, Kapphahn RJ, Ferrington DA, Bernlohr DA. Covalent modification of epithelial fatty acid-binding protein by 4-hydroxynonenal in vitro and in vivo. Evidence for a role in antioxidant biology. *J Biol Chem.* 2002; 277:50693–50702. [PubMed: 12386159]
- Bergamini CM, Gambetti S, Dondi A, Cervellati C. Oxygen, reactive oxygen species and tissue damage. *Curr Pharm Des.* 2004; 10:1611–1626. [PubMed: 15134560]
- Burczynski ME, Sridhar GR, Palackal NT, Penning TM. The reactive oxygen species--and Michael acceptor-inducible human aldo-keto reductase AKR1C1 reduces the alpha,beta-unsaturated aldehyde 4-hydroxy-2-nonenal to 1,4-dihydroxy-2-nonenal. *J Biol Chem.* 2001; 276:2890–2897. [PubMed: 11060293]
- Canuto RA, Ferro M, Muzio G, Bassi AM, Leonarduzzi G, Maggiora M, Adamo D, Poli G, Lindahl R. Role of aldehyde metabolizing enzymes in mediating effects of aldehyde products of lipid peroxidation in liver cells. *Carcinogenesis.* 1994; 15:1359–1364. [PubMed: 8033312]
- Chen CH, Ferreira JC, Gross ER, Mochly-Rosen D. Targeting aldehyde dehydrogenase 2: new therapeutic opportunities. *Physiol Rev.* 2014; 94:1–34. [PubMed: 24382882]
- Chen JJ, Yu BP. Alterations in mitochondrial membrane fluidity by lipid peroxidation products. *Free Radic Biol Med.* 1994; 17:411–418. [PubMed: 7835747]
- Cheng JZ, Yang Y, Singh SP, Singhal SS, Awasthi S, Pan SS, Singh SV, Zimniak P, Awasthi YC. Two distinct 4-hydroxynonenal metabolizing glutathione S-transferase isozymes are differentially expressed in human tissues. *Biochem Biophys Res Commun.* 2001; 282:1268–1274. [PubMed: 11302754]
- Crabb DW, Matsumoto M, Chang D, You M. Overview of the role of alcohol dehydrogenase and aldehyde dehydrogenase and their variants in the genesis of alcohol-related pathology. *Proc Nutr Soc.* 2004; 63:49–63. [PubMed: 15099407]
- Danielson UH, Esterbauer H, Mannervik B. Structure-activity relationships of 4-hydroxyalkenals in the conjugation catalysed by mammalian glutathione transferases. *Biochem J.* 1987; 247:707–713. [PubMed: 3426557]
- Dawidek-Pietryka K, Szczepaniak S, Dudka J, Mazur M. In vitro studies of human alcohol dehydrogenase inhibition in the process of methanol and ethylene glycol oxidation. *Arch Toxicol.* 1998; 72:604–607. [PubMed: 9806434]
- Dick RA, Kwak MK, Sutter TR, Kensler TW. Antioxidative function and substrate specificity of NAD(P)H-dependent alkenal/one oxidoreductase. A new role for leukotriene B₄ 12-hydroxydehydrogenase/15-oxoprostaglandin 13-reductase. *J Biol Chem.* 2001; 276:40803–40810. [PubMed: 11524419]
- Dragomir AC, Laskin JD, Laskin DL. Macrophage activation by factors released from acetaminophen-injured hepatocytes: potential role of HMGB1. *Toxicol Appl Pharmacol.* 2011; 253:170–177. [PubMed: 21513726]
- Engle MR, Singh SP, Czernik PJ, Gaddy D, Montague DC, Ceci JD, Yang Y, Awasthi S, Awasthi YC, Zimniak P. Physiological role of mGSTA4-4, a glutathione S-transferase metabolizing 4-hydroxynonenal: generation and analysis of mGsta4 null mouse. *Toxicol Appl Pharmacol.* 2004; 194:296–308. [PubMed: 14761685]
- Esterbauer H, Zollner H, Lang J. Metabolism of the lipid peroxidation product 4-hydroxynonenal by isolated hepatocytes and by liver cytosolic fractions. *Biochem J.* 1985; 228:363–373. [PubMed: 3160340]
- Esterbauer H, Zollner H, Scholz N. Reaction of glutathione with conjugated carbonyls. *Z Naturforsch C.* 1975; 30:466–473. [PubMed: 241172]
- Flora SJ. Role of free radicals and antioxidants in health and disease. *Cell Mol Biol (Noisy-le-grand).* 2007; 53:1–2. [PubMed: 17535753]

- Forman HJ. Reactive oxygen species and alpha,beta-unsaturated aldehydes as second messengers in signal transduction. *Ann N Y Acad Sci.* 2010; 1203:35–44. [PubMed: 20716281]
- Galligan JJ, Smathers RL, Fritz KS, Epperson LE, Hunter LE, Petersen DR. Protein carbonylation in a murine model for early alcoholic liver disease. *Chem Res Toxicol.* 2012; 25:1012–1021. [PubMed: 22502949]
- Hartley DP, Kolaja KL, Reichard J, Petersen DR. 4-Hydroxynonenal and malondialdehyde hepatic protein adducts in rats treated with carbon tetrachloride: immunochemical detection and lobular localization. *Toxicol Appl Pharmacol.* 1999; 161:23–33. [PubMed: 10558920]
- Hartley DP, Petersen DR. Metabolic interactions of 4-hydroxynonenal, acetaldehyde and glutathione in isolated liver mitochondria. *Adv Exp Med Biol.* 1993; 328:27–36. [PubMed: 8493904]
- Hartley DP, Ruth JA, Petersen DR. The hepatocellular metabolism of 4-hydroxynonenal by alcohol dehydrogenase, aldehyde dehydrogenase, and glutathione S-transferase. *Arch Biochem Biophys.* 1995; 316:197–205. [PubMed: 7840616]
- Hollan S. Free radicals in health and disease. *Haematologia (Budap).* 1995; 26:177–189. [PubMed: 7590512]
- Honzatko A, Brichac J, Murphy TC, Reberg A, Kubatova A, Smoliakova IP, Picklo MJ Sr. Enantioselective metabolism of trans-4-hydroxy-2-nonenal by brain mitochondria. *Free Radic Biol Med.* 2005; 39:913–924. [PubMed: 16140211]
- Houglum K, Filip M, Witztum JL, Chojkier M. Malondialdehyde and 4-hydroxynonenal protein adducts in plasma and liver of rats with iron overload. *J Clin Invest.* 1990; 86:1991–1998. [PubMed: 2123889]
- Kirichenko A, Li L, Morandi MT, Holian A. 4-hydroxy-2-nonenal-protein adducts and apoptosis in murine lung cells after acute ozone exposure. *Toxicol Appl Pharmacol.* 1996; 141:416–424. [PubMed: 8975766]
- Laurent A, Perdu-Durand E, Alary J, Debrauwer L, Cravedi JP. Metabolism of 4-hydroxynonenal, a cytotoxic product of lipid peroxidation, in rat precision-cut liver slices. *Toxicol Lett.* 2000; 114:203–214. [PubMed: 10713486]
- Leonarduzzi G, Parola M, Muzio G, Garramone A, Maggiora M, Robino G, Poli G, Dianzani MU, Canuto RA. Hepatocellular metabolism of 4-hydroxy-2,3-nonenal is impaired in conditions of chronic cholestasis. *Biochem Biophys Res Commun.* 1995; 214:669–675. [PubMed: 7677780]
- LoPachin RM, Gavin T, Petersen DR, Barber DS. Molecular mechanisms of 4-hydroxy-2-nonenal and acrolein toxicity: nucleophilic targets and adduct formation. *Chem Res Toxicol.* 2009; 22:1499–1508. [PubMed: 19610654]
- Luckey SW, Petersen DR. Metabolism of 4-hydroxynonenal by rat Kupffer cells. *Arch Biochem Biophys.* 2001; 389:77–83. [PubMed: 11370675]
- Meyer MJ, Mosely DE, Amarnath V, Picklo MJ Sr. Metabolism of 4-hydroxy-trans-2-nonenal by central nervous system mitochondria is dependent on age and NAD⁺ availability. *Chem Res Toxicol.* 2004; 17:1272–1279. [PubMed: 15377162]
- Mitchell DY, Petersen DR. The oxidation of alpha-beta unsaturated aldehydic products of lipid peroxidation by rat liver aldehyde dehydrogenases. *Toxicol Appl Pharmacol.* 1987; 87:403–410. [PubMed: 3564015]
- Mitchell DY, Petersen DR. Inhibition of rat hepatic mitochondrial aldehyde dehydrogenase-mediated acetaldehyde oxidation by trans-4-hydroxy-2-nonenal. *Hepatology.* 1991; 13:728–734. [PubMed: 2010168]
- Moreb JS, Baker HV, Chang LJ, Amaya M, Lopez MC, Ostmark B, Chou W. ALDH isozymes downregulation affects cell growth, cell motility and gene expression in lung cancer cells. *Mol Cancer.* 2008; 7:87. [PubMed: 19025616]
- Murphy T, Amarnath V, Picklo MJ. Oxidation of 4-hydroxynonenal in rat brain slices. *Chem Biol Interact.* 2003; 143-144:101–105. [PubMed: 12604194]
- Paradis V, Kollinger M, Fabre M, Holstege A, Poynard T, Bedossa P. In situ detection of lipid peroxidation by-products in chronic liver diseases. *Hepatology.* 1997; 26:135–142. [PubMed: 9214462]
- Perluigi M, Sultana R, Cenini G, Di Domenico F, Memo M, Pierce WM, Coccia R, Butterfield DA. Redox proteomics identification of 4-hydroxynonenal-modified brain proteins in Alzheimer's

disease: Role of lipid peroxidation in Alzheimer's disease pathogenesis. *Proteomics Clin Appl*. 2009; 3:682–693. [PubMed: 20333275]

- Petersen DR, Doorn JA. Reactions of 4-hydroxynonenal with proteins and cellular targets. *Free Radic Biol Med*. 2004; 37:937–945. [PubMed: 15336309]
- Poli G, Biasi F, Leonarduzzi G. 4-Hydroxynonenal-protein adducts: A reliable biomarker of lipid oxidation in liver diseases. *Mol Aspects Med*. 2008a; 29:67–71. [PubMed: 18158180]
- Poli G, Schaur RJ, Siems WG, Leonarduzzi G. 4-hydroxynonenal: a membrane lipid oxidation product of medicinal interest. *Med Res Rev*. 2008b; 28:569–631. [PubMed: 18058921]
- Rahman I, van Schadewijk AA, Crowther AJ, Hiemstra PS, Stolk J, MacNee W, De Boer WI. 4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2002; 166:490–495. [PubMed: 12186826]
- Roede JR, Stewart BJ, Petersen DR, McQueen CA. Hepatotoxicity of Reactive Aldehydes. *Comprehensive Toxicology*. 2010:581–594.
- Sidell KR, Montine KS, Picklo MJ Sr, Olsen SJ, Amarnath V, Montine TJ. Mercapturate metabolism of 4-hydroxy-2-nonenal in rat and human cerebrum. *J Neuropathol Exp Neurol*. 2003; 62:146–153. [PubMed: 12578224]
- Siems W, Grune T. Intracellular metabolism of 4-hydroxynonenal. *Mol Aspects Med*. 2003; 24:167–175. [PubMed: 12892994]
- Siems WG, Zollner H, Grune T, Esterbauer H. Metabolic fate of 4-hydroxynonenal in hepatocytes: 1,4-dihydroxynonene is not the main product. *J Lipid Res*. 1997; 38:612–622. [PubMed: 9101442]
- Sinclair AJ, Barnett AH, Lunec J. Free radicals and antioxidant systems in health and disease. *Br J Hosp Med*. 1990; 43:334–344. [PubMed: 2194616]
- Singhal SS, Zimniak P, Awasthi S, Piper JT, He NG, Teng JJ, Petersen DR, Awasthi YC. Several closely related glutathione S-transferase isozymes catalyzing conjugation of 4-hydroxynonenal are differentially expressed in human tissues. *Arch Biochem Biophys*. 1994; 311:242–250. [PubMed: 8203886]
- Smathers RL, Galligan JJ, Stewart BJ, Petersen DR. Overview of lipid peroxidation products and hepatic protein modification in alcoholic liver disease. *Chem Biol Interact*. 2011; 192:107–112. [PubMed: 21354120]
- Spitz DR, Sullivan SJ, Malcolm RR, Roberts RJ. Glutathione dependent metabolism and detoxification of 4-hydroxy-2-nonenal. *Free Radic Biol Med*. 1991; 11:415–423. [PubMed: 1797627]
- Srivastava S, Dixit BL, Cai J, Sharma S, Hurst HE, Bhatnagar A, Srivastava SK. Metabolism of lipid peroxidation product, 4-hydroxynonenal (HNE) in rat erythrocytes: role of aldose reductase. *Free Radic Biol Med*. 2000; 29:642–651. [PubMed: 11033416]
- Terneus MV, Brown JM, Carpenter AB, Valentovic MA. Comparison of S-adenosyl-L-methionine (SAmE) and N-acetylcysteine (NAC) protective effects on hepatic damage when administered after acetaminophen overdose. *Toxicology*. 2008; 244:25–34. [PubMed: 18068290]
- Tjalkens RB, Cook LW, Petersen DR. Formation and export of the glutathione conjugate of 4-hydroxy-2, 3-E-nonenal (4-HNE) in hepatoma cells. *Arch Biochem Biophys*. 1999; 361:113–119. [PubMed: 9882435]
- Ullrich O, Grune T, Henke W, Esterbauer H, Siems WG. Identification of metabolic pathways of the lipid peroxidation product 4-hydroxynonenal by mitochondria isolated from rat kidney cortex. *FEBS Lett*. 1994; 352:84–86. [PubMed: 7925950]
- Vila A, Tallman KA, Jacobs AT, Liebler DC, Porter NA, Marnett LJ. Identification of protein targets of 4-hydroxynonenal using click chemistry for ex vivo biotinylation of azido and alkynyl derivatives. *Chem Res Toxicol*. 2008; 21:432–444. [PubMed: 18232660]
- Zarkovic K. 4-hydroxynonenal and neurodegenerative diseases. *Mol Aspects Med*. 2003; 24:293–303. [PubMed: 12893007]
- Zheng R, Po I, Mishin V, Black AT, Heck DE, Laskin DL, Sinko PJ, Gerecke DR, Gordon MK, Laskin JD. The generation of 4-hydroxynonenal, an electrophilic lipid peroxidation end product, in rabbit cornea organ cultures treated with UVB light and nitrogen mustard. *Toxicol Appl Pharmacol*. 2013; 272:345–355. [PubMed: 23845594]

Highlights

Lipid peroxidation generates 4-hydroxynonenal, a highly reactive aldehyde. Rodent liver, but not lung or brain, is efficient in degrading 4-hydroxynonenal. 4-hydroxynonenal persists in tissues with low metabolism, causing tissue damage.

Figure 1. Effects of pyridine nucleotides on 4-HNE degradation in homogenates from mouse tissues

Total homogenates from mouse liver, lung and brain were incubated with 100 μ M 4-HNE in the absence or presence of 1 mM of NAD⁺, NADP⁺, NADH, or NADPH. After 30 min, samples were extracted and analyzed for 4-HNE content using HPLC. Controls (C) were from samples extracted at 0 time. Bars are the mean $\pm$ SE (n = 3). ^a Significantly ($p < 0.05$) different from control; ^b Significantly ($p < 0.05$) different from no cofactors.

Figure 2. Effects of pyridine nucleotides on 4-HNE degradation in S9 fractions from mouse and rat tissues

S9 fractions from mouse (upper panels) and rat (lower panels) liver, lung or brain were treated with 100 μ M 4-HNE in the absence or presence of 1 mM NAD⁺, NADP⁺, NADH, or NADPH. After 30 min, samples were extracted and analyzed for 4-HNE content using HPLC. Controls (C) were from samples extracted at 0 time. Bars are the mean $\pm$ SE (n = 3). ^a Significantly ($p < 0.05$) different from control; ^b Significantly ($p < 0.05$) different from no cofactors.

Figure 3. Metabolism of 4-HNE by mouse tissues

4-HNE (100 μ M) was added to reaction mixes containing S9 fractions of mouse liver, lung or brain. At the indicated times (0, 15 and 30 min), residual 4-HNE and metabolites in the reaction mixes were extracted and analyzed by HPLC. Upper panels, Kinetics of 4-HNE degradation in S9 fractions in the absence or presence of 1 mM NADH or NADPH. Heat inactivated enzyme was used as control. Lower panels, HPLC analysis of 4-HNE metabolism in S9 fractions in presence of 1 mM NADH.

Figure 4. Metabolism of 4-HNE by rat tissues

4-HNE (100 μ M) was added to reaction mixes containing S9 fractions of rat liver, lung or brain. At the indicated times (0, 15 and 30 min), residual 4-HNE and metabolites in the reaction mixes were extracted and analyzed by HPLC. Upper panels, Kinetics of 4-HNE degradation in S9 fractions in the absence or presence of NADH or NADPH. Heat inactivated enzyme was used as control. Lower panels, HPLC analysis of 4-HNE metabolism in S9 fractions in presence of 1 mM NADH.

Figure 5. NADH-dependent 4-HNE metabolism in mouse liver

S9 fractions, prepared from mouse liver, were treated with 100 μM 4-HNE in the presence of increasing concentrations of NADH. Panel A, HPLC analysis of 4-HNE metabolism. Metabolites were analyzed after a 30 min incubation. Panel B, Kinetics of 4-HNE degradation after increasing periods of time. Data are presented as the percentage of 4-HNE recovered relative to the amount added at the start of the assay. Panels C and D, Kinetics of formation of metabolites a and b.

Figure 6. Effects of enzyme inhibitors on 4-HNE metabolism

4-HNE degradation by S9 fractions from mouse and rat liver, lung or brain was analyzed in the absence and presence of 200 μ M 4-methylpyrazole or 100 μ M disulfiram. NADH (1 mM) was used to simulate 4-HNE metabolism. Left panels: Effects of inhibitors on 4-HNE degradation in mouse S9 fractions. Right panels: Effects of inhibitors on 4-HNE degradation in rat tissue fractions. Data are presented as the percentage of 4-HNE recovered relative to the amount added at the start of the assay.

Figure 7. Effects of enzyme inhibitors on 4-HNE metabolism in S9 fractions from mouse and rat liver

4-HNE degradation was quantified in S9 fractions from livers of mice (top panel) and rats (bottom panel) in the absence and presence of 200 μ M 4-methylpyrazole (MP) or 100 μ M disulfiram (DSF). The final concentration of pyridine nucleotides (NAD⁺, NADP⁺, NADH or NADPH) in the assay mixes was 1 mM. Assays were run for 30 min and then analyzed for 4-HNE content. Controls (C) were from samples extracted at 0 time. Bars represent the mean $\pm$ SE (n = 3). ^a Significantly ($p < 0.05$) different from control; ^b Significantly ($p < 0.05$) different from no inhibitor.

Figure 8. Effects of disulfiram and 4-methylpyrazole on 4-HNE metabolism in S9 fractions from mouse liver

S9 liver fractions were incubated with increasing concentrations of disulfiram (DSF) or 4-methylpyrazole (MP) in the presence of 1 mM NADH. Residual 4-HNE was analyzed after 30 min by HPLC as described in the Materials and Methods.

Figure 9. Effects of NADH and enzyme inhibitors on 4-HNE-protein adduct formation in mouse liver, lung and brain

S9 tissue fractions were incubated with 100 μ M 4-HNE in the absence or presence of 1 mM NADH, 200 μ M 4-methylpyrazole (MP) or 100 μ M disulfiram (DSF). At the indicated times, samples were analyzed for 4-HNE protein adducts using SDS-polyacrylamide gel electrophoresis and western blotting using a monoclonal 4-HNE antibody.

Figure 10. Effects of glutathione on 4-HNE-protein adduct formation in S9 fractions from mouse liver, lung and brain

S9 tissue fractions were incubated with 100 μ M 4-HNE in the absence or presence of 0.5 mM glutathione. At the indicated times, samples were analyzed for 4-HNE-protein adducts using SDS-polyacrylamide gel electrophoresis and western blotting using a monoclonal 4-HNE antibody.

Figure 11. Metabolism of 4-HNE in isolated mouse hepatocytes

Panels A and B. Cells were incubated with 100 μ M 4-HNE. After 0, 15, 30 and 60 min, reactions were stopped and the cells analyzed for 4-HNE and its metabolites, and protein adducts. Panel A, HPLC tracings of 4-HNE degradation and the appearance of metabolites in hepatocytes. Metabolites (c, d and e) are shown by arrows. Panel B, kinetics of uptake and disappearance of 4-HNE in the cells. Panel C, Cells were treated with 30 or 100 μ M 4-HNE and analyzed for 4-HNE-protein adducts after 0, 5, 15 and 30 min in western blotting using a monoclonal antibody to 4-HNE. Arrow heads show abundant 4-HNE-protein adducts.

Table 1

Distribution of glutathione S-transferase activity in mouse and rat tissues

Species	Tissue	Enzyme activity ^I (nmole/min/mg protein)
Mouse	liver	74.7 ± 10.6
	lung	50.0 ± 5.4 ^a
	brain	31.1 ± 2.0 ^a
Rat	liver	100.3 ± 7.7
	lung	141.5 ± 4.7 ^a
	brain	44.7 ± 5.4 ^a

^I Glutathione S-transferase reactions were run as indicated in the Materials and Methods and contained 100 μM 4-HNE, 500 μM glutathione and 100-200 μ of S9 tissue protein in 1 ml reaction mixes. 4-HNE remaining in the assays was assessed by HPLC. Background degradation of 4-HNE by glutathione conjugation in the absence of tissue fractions was subtracted from measurements of enzyme activity. Each value represents the mean ± SE (n = 3).

^aSignificantly different ($p < 0.05$) from liver.