Toxicology and Applied Pharmacology 279 (2014) 43–52

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

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

Ruijin Zheng [b], Ana-Cristina Dragomir [b], Vladimir Mishin [b], Jason R. Richardson [a], Diane E. Heck [c], Debra L. Laskin [b], Jeffrey D. Laskin [a,*]

[a] Environmental & Occupational Medicine, Rutgers University-Robert Wood Johnson Medical School, Piscataway, NJ, USA
[b] Pharmacology and Toxicology, Rutgers University-Ernest Mario School of Pharmacy, Piscataway, NJ, USA
[c] Environmental Science, School of Health Sciences and Practice, New York Medical College, Valhalla, NY, USA

ARTICLE INFO

Article history:
Received 2 February 2014
Revised 18 April 2014
Accepted 28 April 2014
Available online 14 May 2014

Keywords:
4-Hydroxynonenal
Alcohol dehydrogenase
Lipid peroxidation
Reactive oxygen species
Pulmonary toxicity
Neurotoxicity

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.

Introduction

It is well recognized that excessive production of reactive oxygen species (ROS) can lead to oxidative stress and tissue damage (Flora, 2007; Hollan, 1995; Sinclair et al., 1990). 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 (LoPachin et al., 2009; Poli et al., 2008b).

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 (Arunachalam et al., 2010; Rahman et al., 2002), 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 (Galligan et al., 2012; Hartley et al., 1999; Terneus et al., 2008). 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 (Amunom et al., 2011; Burczynski et al., 2001; Dick et al., 2001; Forman, 2010; Hartley et al., 1995; Srivastava et al., 2000). Although metabolism of 4-HNE has been studied extensively in the liver, much less is known about its metabolism in the 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 the 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

* Corresponding author at: Department of Environmental and Occupational Medicine, Rutgers-Robert Wood Johnson Medical School, 170 Hoes Lane, Piscataway, NJ, USA. Fax: +1 732 445 0119.
E-mail address: jlaskin@eohsi.rutgers.edu (J.D. Laskin).

http://dx.doi.org/10.1016/j.taap.2014.04.026
0041-008X/

the 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 PerkinElmer 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 Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (pH 7.3) containing 25 mM HEPES and 0.5 mM EGTA, followed by Leibovitz L-15 medium containing HEPES and 0.2 U/ml Liberase 3 Blendzyme. The liver was then excised and disaggregated, and the resulting cell suspension was 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 (2×10^6) 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% CO_2 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 (2×10^6 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 cm^3 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 1500 ×*g*, 4 °C for 20 min to remove nuclei and cellular debris, and the supernatants were used in metabolism assays as total homogenates. S9 fractions were prepared by centrifuging homogenized tissues at 9000 ×*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 1000 ×*g* at 4 °C for 10 min and clear supernatants were analyzed by HPLC as described by Hartley et al. (1995) using a Jasco HPLC system (Jasco Corporation, Tokyo, Japan) fitted with a Phenomenex 5 μm C18 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 was 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× 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 (Zheng et al., 2013). Briefly, 10 μg of protein was 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 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