Abstract  Sphingosine 1-phosphate (S1P), a bioactive lipid involved in various physiological processes such as cell proliferation and apoptosis, can be irreversibly cleaved by S1P lyase, yielding phosphoethanolamine and (2E)-hexadecenal (2EHD). The latter metabolite, an ω,ω-unsaturated fatty aldehyde, may be susceptible to nucleophilic attack by cellular biomolecules. Hence, we studied whether 2EHD forms reaction products with GSH and proteins in vitro. Using LC-MS/MS and stable isotopically labeled reference material, we identified a total of nine novel reaction products of 2EHD in a cell-free approach: two GSH conjugates and seven ω-amino acid adducts. Both GSH conjugates were also found in HepG2 cell lysates incubated with 2EHD. Likewise, we detected four out of seven amino acid adducts released from the model protein, BSA, and proteins extracted from HepG2 cells. On this occasion, the 2EHD Michael adduct with l-histidine proved to be the most prominent adduct. Most interestingly, inhibition of the enzymatically driven oxidative degradation of 2EHD resulted in increased levels of both GSH conjugates and protein adducts in HepG2 cell lysates. Hence, our data provide new insights into sphingolipid metabolism and will be useful to investigate certain disorders linked to an impaired fatty aldehyde metabolism in more detail.—Schumacher, F., C. Neuber, H. Finke, K. Nieschalke, J. Baesler, E. Gulbins, and B. Kleuser. The sphingosine 1-phosphate breakdown product, (2E)-hexadecenal, forms protein adducts and glutathione conjugates in vitro. J. Lipid Res. 2017. 58: 1648–1660.

Sphingosine 1-phosphate (S1P) is a bioactive lipid that functions as signaling molecule in various cellular processes. Besides the direct effects of S1P, including the regulation of apoptosis, proliferation, and calcium homeostasis (1, 2), it mediates numerous extracellular effects via five G-protein-coupled receptors (S1P1-5) (3). For example, binding of S1P to the S1P1 receptor is a key event regulating both the homing of immune cells to lymphoid organs and their release into blood and lymph fluid (4). In order to maintain the balance between proliferative and pro-apoptotic sphingolipid species (e.g., S1P and sphingosine, respectively), the so-called “sphingolipid rheostat,” S1P levels are strictly regulated (2). On the one hand, S1P is generated by phosphorylation of sphingosine catalyzed by two isoforms of sphingosine kinases (SphK1 and SphK2) (5). On the other hand, the degradation of S1P can either be reversible via phosphorylase-catalyzed dephosphorylation (6) or by irreversible cleavage into (2E)-hexadecenal (2EHD) and phosphoethanolamine catalyzed by S1P lyase (Fig. 1) (7). While the S1P metabolism has been intensively studied, the fate and physiological functions of the S1P breakdown product (2EHD) remains poorly understood.

Abbreviations: Arg, L-arginine; Cys, L-cysteine; DBAP, 2,4-dibromoacetophenone; FALDH, fatty aldehyde dehydrogenase; Fmoc-His-OH, N-(9-fluorenylethoxycarbonyl)-L-histidine; Glu, L-glutamic acid; Glu, L-glutamic acid; His, L-histidine; LPO, lipid peroxidation; Lys, L-lysine; MRM, multiple reaction monitoring; NAC, N-acetylcysteine; Ser, L-serine; S1P, sphingosine 1-phosphate; t1/2, half-life; Trp, L-tryptophan; 2EHD, (2E)-hexadecenal; 2EHD-GSH, Schiff base of (2E)-hexadecenal and GSH; 2EHD-Lys, Schiff base of (2E)-hexadecenal and L-lysine; 3-Arg-HD, 3-argininylhexadecanal; 3-Cys-HD, 3-cysteinylhexadecanal; 3-(Fmoc)His-HD, 3-(Fmoc)histidinylhexadecanal; 3-His-HD, 3-histidinylhexadecanal; 3-Lys-HD, 3-lysylhexadecanal; 3-NAC-HD, 3-(N-acetylcysteinyl)hexadecanal; 3-Ser-HD, 3-serinylhexadecanal; 3-SG-HD, 3-glutathionylhexadecanal; 3-Trp-HD, 3-tryptophanylhexadecanal; 4-HNE, 4-hydroxy-2-nonenal; 4-ONE, 4-oxo-2-nonenal.

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lyase-mediated breakdown products are not fully understood. However, there is a need to examine 2EHHD, in particular, more closely, because this long-chain α,β-unsaturated fatty aldehyde is a potentially cytotoxic substance (8), known to induce cytoskeletal reorganization and apoptosis in various cell types (9). In recent years, cellular mechanisms leading to degradation and, therefore, detoxification of 2EHHD have been revealed. Briefly, 2EHHD is oxidized to (2E)-hexadecenoic acid by the fatty aldehyde dehydrogenase (FALDH; also known as ALDH3A2) followed by an activation of the fatty acid by acyl-CoA synthetases and conversion to glycerophospholipids (10, 11). However, in a previous study utilizing lysates of human hepatoblastoma (HepG2) cells that were co-incubated with stable isotopically labeled 2EHHD-d₅, and triacsin C, an inhibitor of acyl-CoA synthetases, we only observed an approximate 80% conversion of the fatty aldehyde into its corresponding fatty acid (12). Consequently, we were confronted with the question of what happened to the remainder of the 2EHHD.

Considering the chemical structure of 2EHHD (Fig. 1), reactions with nucleophilic structures of biomolecules, such as nucleic acids, proteins, and GSH, appear probable. Due to its α,β-unsaturated carbonyl function, 2EHHD possesses two electrophilic centers within the molecule. The β-carbon (C-3 atom) is a so-called Michael acceptor that is considered a soft electrophile according to the “Hard and Soft Acids and Bases” concept (13). The formation of covalent bonds, preferably with nucleophiles of comparable softness (e.g., thiols), would produce Michael adducts. Furthermore, the electrophilic carbonyl carbon (C-1 atom) can react with primary amino groups to form imines, more specifically secondary aldimines, often referred to as Schiff bases. Because of the variety of nucleophilic structures within the cells, both Michael adducts and Schiff bases of 2EHHD are imaginable. In fact, a cyclic reaction product of 2EHHD with the DNA nucleoside, 2′-deoxyguanosine, resulting from nucleophilic attack at both electrophilic carbons (C-1 and C-3) has already been identified (14). However, to the best of our knowledge, nothing is known about the reactivity of 2EHHD toward the most abundant cellular nucleophiles, GSH and free or protein-bound amino acid side chains. While GSH conjugation is a desired process, which typically contributes to the detoxification of electrophiles, the addition of proteins usually entails alteration of structure and function, and degradation of the affected protein (15, 16). Therefore, both mentioned biotransformations of 2EHHD are of toxicological and pathophysiological relevance, indicating the need for mechanistic studies. However, there are no appropriate analytical methods published yet.

Here, we present the in vitro identification of two GSH conjugates and seven l-amino acid adducts of 2EHHD using stable isotopically labeled reference material and LC-MS/MS. We developed multiple reaction monitoring (MRM) methods for the sensitive and unequivocal detection of all adducts and characterized them in terms of product stability. Moreover, we present sample preparation protocols that allow for semi-quantitative determination of 2EHHD GSH conjugates and protein adducts in lysates of HepG2 cells. Using these methods, we investigated the influence of an inhibited 2EHHD degradation on levels of the reaction products with GSH and whole cellular protein. Such an impaired fatty aldehyde degradation is found, for example, in patients with Sjögren-Larsson syndrome, a rare autosomal recessive neurocutaneous disease. Taken together, we revealed nine novel reaction products of the S1P breakdown product, 2EHHD, with cellular nucleophiles and, thus, provide new insights into sphingolipid metabolism.

MATERIALS AND METHODS

Chemicals

The 2EHHD and 2EHHD-d₅ were purchased from Avanti Polar Lipids (Alabaster, AL). GSH was from Merck KGaA (Darmstadt, Germany) and l-lysine (Lys) monohydrochloride as well as l-serine (Ser) from Fluka Biochemika (Buchs, Switzerland). l-Arginine (Arg), l-cysteine (Cys), l-histidine (His), Nα-[9-fluorenylmethoxycarbonyl]-l-histidine (Fmoc-His(OH)), l-tryptophan (Trp), N-acyctyl-l-cysteine (NAC), 2,4′-dibromoacetophenone (DBAP), and protease from Streptomyces griseus (pronase E) were from Sigma-Aldrich (Steinheim, Germany). All solvents and additives used were of LC-MS grade. The 1-butanol and formic acid were from VWR (Darmstadt, Germany) and acetonitrile, ethanol, methanol, dipotassium hydrogen orthophosphate, potassium dihydrogen phosphate, BSA, and Roti²-Quant (for Bradford assay) from Carl Roth (Karlsruhe, Germany). Water purified by a MilliPore apparatus (Millipore GmbH, Darmstadt, Germany) was used. FBS was obtained from Moregate Biotech (Munich, Germany). RPMI-1640 medium, penicillin/streptomycin, and trypsin were purchased from Biochrom AG (Berlin, Germany).

Synthesis of GSH conjugates of 2EHHD and 2EHHD-d₅

The 2EHHD conjugates were synthesized by incubating 100 mM 2EHHD (stock solution dissolved in acetonitrile) and 400 µM GSH (stock solution dissolved in 100 mM potassium phosphate, pH 7.4) in a mixture of 100 mM potassium phosphate (pH 7.4)/acetonitrile/ethanol 2:1:1 (v:v:v) at 37°C. The reaction was quenched after 4 h by adding 0.3% formic acid (v:v). Accordingly, GSH conjugates of 2EHHD-d₅ to be utilized as internal standard were synthesized by incubating 2EHHD-d₅ (stock solution dissolved in ethanol) with GSH. Both mixtures were applied for development of a LC-MS/MS method for GSH conjugates of 2EHHD.
and 2EHD-d₅. A working solution of the internal standard was obtained after 1:20 (v/v) dilution with 100 mM potassium phosphate (pH 7.4)/acetonitrile/ethanol 2:1:1 (v/v/v) containing 0.3% formic acid. All reaction solutions were used without further purification.

**Cell culture and treatment of cell lysates for analysis of GSH conjugates of 2EHD**

HepG2 cells between passages 2 and 12 were grown in 15 cm dishes in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Optimal growing conditions were chosen with 37°C in an atmosphere of 5% CO₂ and 95% air-humidity. Cells in the exponential phase were harvested by trypsinization and stored as cell pellets containing 2 × 10⁵ cells at −80°C until usage.

In order to investigate the time-dependent formation of GSH conjugates of 2EHD in vitro, cell pellets were resuspended in 456.5 µl 100 mM potassium phosphate (pH 7.4) and sonicated three times for 20 s on ice using an ultrasonic homogenizer (Bandelin, Berlin, Germany). Cell suspension (364 µl), equivalent to 1.6 × 10⁶ lysed cells, was preheated for 5 min at 37°C prior to addition of 4 mM GSH in 100 mM potassium phosphate (pH 7.4) and 8 µM 2EHD in acetonitrile, resulting in a final volume of 400 µl (2% acetonitrile, v/v). In experiments without GSH supplementation, an equivalent volume of 100 mM potassium phosphate (pH 7.4) was added instead. At defined time points of incubation at 37°C (0, 2.5, 5, 7.5, 10, 12.5, 15, 30, and 60 min) 40 µl of the cell lysates were immediately mixed with 55 µl ice-cold water containing 0.55% formic acid (v/v) and 10 µl of the internal standard working solution. Protein precipitation was carried out by adding 195 µl ice-cold acetonitrile. After 15 min at room temperature the samples were centrifuged at 10,500 g for 10 min and the supernatants were subjected to LC-MS/MS analysis. In parallel, an incubation of 8 µM 2EHD and 4 mM GSH in 100 mM potassium phosphate (pH 7.4) was conducted analogously to study the time-dependent formation of GSH conjugates in a cell-free system.

In a second set of samples, we were interested in whether the GSH conjugate levels could be modulated by an impaired oxidative degradation of 2EHD. Hence, we repeated the cell lysate experiment described above, but co-incubated 8 µM 2EHD and 4 mM GSH with 100 µM NAC (dissolved in 50 mM potassium phosphate, pH 7.4) were added to scavenge unreacted 2EHD-d₅ (molar ratio NAC:2EHD-d₅ = 10:1) and the incubation was resumed for 1 h under the same conditions. Then, 1,190 µl of 50 mM potassium phosphate (pH 7.4) were added to prepare a working solution with 1 mg/ml protein content. If this working solution is stored at −80°C, it is stable for several weeks.

**Cell culture and generation of cell lysates for protein adduct analysis**

Cultivation of HepG2 cells was as described for the GSH conjugate experiments. However, cells between passages 3 and 14 were grown in 10 or 15 cm dishes. Pellets containing 4 × 10⁶ cells were generated and stored at −80°C. On the day of the investigations, cell pellets were resuspended in 300 µl 50 mM potassium phosphate (pH 7.4) and homogenized using a Bead Ruptor 12 (Omni International, Kennesaw, GA). For that, 15 ceramic beads were added to each cell suspension that, consecutively, was homogenized three times (30 s each) at medium speed. In between homogenization steps, samples were placed on ice for 150 s. After centrifugation of the cell debris (1 min, 2,000 g) 270 µl of the cell lysate supernatants were transferred to new sample tubes. Ten microliters of the supernatants (5 µl in duplicates) were used for protein quantification via Bradford assay. The absorption was measured at λ = 595 nm with an Ultrospec™ 2100 pro UV/Visible spectrophotometer (GE Healthcare, Little Chalfont, UK).

**Treatment of BSA and HepG2 cell lysates for analysis of 2EHD protein adducts**

Cell lysates, each equivalent to 3.5 × 10⁶ cells, and BSA solutions (both in 200 µl 50 mM potassium phosphate, pH 7.4), adjusted to the same protein content (~2 mg), were warmed at 37°C for 5 min. Then, 12.6 µl 5.4 mM 2EHD (in acetonitrile) were added, resulting in a final concentration of 250 µM of the fatty aldehyde, followed by incubation at 37°C for 1 h under gentle shaking (300 rpm). Afterwards, 10 µl 70 mM NAC (in 50 mM potassium phosphate, pH 7.4) were added (molar ratio NAC:2EHD ~10:1) and the incubation was resumed for 1 h under the same conditions. Amicon® Ultra-15 centrifugal filters (molecular mass cut-off: 50 kDa), purchased from Millipore, were filled with 5 ml 50 mM potassium phosphate (pH 7.4) followed by addition of 10 µl internal standard working solution (1 mg/ml BSA adducted with 2EHD-d₅), as well as the whole incubation mixture. Filters were centrifuged at room temperature for 5 min at 3,500 g. Afterwards, 4 ml 50 mM potassium phosphate (pH 7.4) were added and blended using a pipette tip prior to centrifugation for 10 min at 3,500 g. The last-mentioned washing step was repeated once and the centrifugation was carried out until the volume of the recovered protein solution was around 500 µl. The enzymatic proteolysis was prepared by dissolving 1 mg pronase E in 770 µl 50 mM potassium phosphate (pH 7.4) per cell lysate (or BSA solution) in a new sample tube. Then, samples were transferred from the centrifugal filter into the enzyme solution. Additionally, 400 µl 50 mM potassium phosphate (pH 7.4), used to rinse the filter unit,
were combined with the proteolysis solution. The protein degradation into amino acids was carried out overnight (∼16 h) at 37°C with gentle shaking (300 rpm). After brief centrifugation the protein hydrolysates were transferred into new sample tubes together with an equal volume (∼1.5 ml) of water-saturated 1-butanol. Extraction of amino acid adducts was achieved by extensive shaking at 1,500 rpm for 5 min. After centrifugation for 5 min at 3,500 g approximately 1 ml of the upper organic phase was transferred into a new sample tube and, subsequently, evaporated to dryness under reduced pressure using a Savant SpeedVac concentrator (Thermo Fisher Scientific, Dreieich, Germany). The dried residues were reconstituted in 50 μl methanol and thoroughly vortexed, followed by ultrasonication for 5 min and centrifugation for 1 min at 16,000 g. The supernatants were subjected to LC-MS/MS analysis.

**Investigation of the impact of an inhibited oxidative degradation of 2EHD on its protein adduct levels in HepG2 cell lysates**

Cell lysates (260 μl), each equivalent to 3.5 × 10⁶ cells, were warmed at 37°C for 5 min. Samples were treated with 5 μl 5.4 mM DBAP (in acetonitrile) or 5 μl acetonitrile (controls) followed by incubation for 3 min at 37°C under gentle shaking (300 rpm). Thereafter, all samples were treated with 5 μl 5.4 mM 2EHD (in acetonitrile) resulting in final concentrations of 100 μM for both the fatty aldehyde and the FALDH inhibitor. Incubation was carried out at 37°C for 1 h with gentle shaking (300 rpm). Afterwards, 10 μl 28 mM NAC (in 50 mM potassium phosphate, pH 7.4) were added to reach a molar NAC-to-2EHD ratio of approximately 10. Incubation was resumed for another hour under the same conditions. All further sample preparation steps necessary to obtain the ready-to-analyze sample solutions are analogous to those described in the previous section.

**LC-MS/MS analysis of GSH conjugates and amino acid adducts of 2EHD and 2EHD-d₅**

All analyses were conducted with an Agilent 1260 Infinity LC system coupled to an Agilent 6490 triple-quadruple-mass spectrometer (Agilent Technologies, Waldbronn, Germany) interfaced with an electrospray ion source operating in the positive ion mode (ESI+). However, settings of the chromatographic analyte separation and parameters of the ion source, as well as the MS/MS detector, differed between studies on GSH conjugation and protein adduction. In the following, the LC-MS/MS configurations used to analyze GSH conjugates of 2EHD and 2EHD-d₅, are referred to as “system 1” and those applied for detection of the corresponding amino acid adducts are referred to as “system 2.”

**System 1.** Analyte separation was carried out using an Agilent ZORBAX Eclipse XDB-C18 column (4.6 × 50 mm, 1.8 μm) equipped with an Agilent ZORBAX Extend-C18 guard column (2.1 × 12.5 mm, 5 μm). Water and methanol/acetonitrile 1:1 (v:v), both acidified with 0.1% formic acid, were used as eluents A and B. Samples of 2–10 μl were injected into a mobile phase consisting of 60% eluent B. Analytes were eluted from the column, which was tempered at 30°C, with a 2 min linear gradient to 80% eluent B and a subsequent isocratic stage for 3 min at 80% eluent B at a flow rate of 0.8 ml/min. The total run time for one analysis was 9 min, including re-equilibration of the HPLC system.

**System 2.** Analytes were separated by means of an Agilent Poroshell 120 EC-C18 column (3.0 × 150 mm, 2.7 μm) connected to a pre-column of the same material (4.6 × 5 mm, 2.7 μm). Water and acetonitrile, both containing 1% formic acid, were used as eluents A and B. The initial mobile phase composition and the sample injection volume were 60% eluent A and 5–15 μl, respectively. Analyte elution from the column (tempered at 30°C) was achieved with a 15 min linear gradient to 100% eluent B and a subsequent isocratic stage for 2 min at 100% eluent B at a flow rate of 0.5 ml/min. The total run time for one analysis, including re-equilibration of the HPLC, was 21 min.

The following ion source parameters were determined after repeated injection of a 2EHD GSH conjugate synthesis mixture (system 1) or a mixture of 2EHD Michael adducts with His and Lys (system 2) using the Source Optimizer tool of the Agilent MassHunter Workstation software (version B.06.00): drying gas temperature = 250°C (system 1) or 280°C (system 2), drying gas flow = 15 l/min (system 1) or 11 l/min (system 2) of nitrogen, sheath gas temperature = 380°C (system 1) or 350°C (system 2), sheath gas flow = 11 l/min (system 1) or 12 l/min (system 2) of nitrogen, nebulizer pressure = 40 psi (system 1) or 25 psi (system 2), capillary voltage = 4,500 V (system 1) or 1,000 V (system 2), nozzle voltage = 500 V (systems 1 and 2). The optimized ion funnel parameters were: high pressure RF voltage = 150 V (system 1) or 190 V (system 2) and low pressure RF voltage = 60 V (system 1) or 160 V (system 2). Quantification of GSH conjugates and amino acid adducts of 2EHD and 2EHD-d₅ was carried out using the MRM approach. The optimized collision energies for each MRM transition, which were determined using the Optimizer tool of the MassHunter software, as well as the applied dwell times, are given in **Table 1** (system 1) and **Table 2** (system 2).

**RESULTS**

**General statement**

The adducts described below were assigned to certain chemical structures using MS/MS data and comparative experiments utilizing stable isotopically labeled or protecting group-reactants. A clear assignment to possible isomeric structures requires NMR spectroscopic investigations, which have not been carried out in this study and need to be addressed in future experiments.

**Formation of Michael adducts and Schiff bases of 2EHD with GSH in vitro**

The C-5 carbon at the double bond of the α,β-unsaturated carbonyl compound, 2EHD (Fig. 1), represents a soft electrophilic center. Therefore, it was obvious to study the reactivity of 2EHD toward soft cellular nucleophiles, such as thiols. Hence, we incubated 2EHD with GSH and analyzed the reaction mixture for accordan product by LC-MS/MS. Indeed, we detected a product represented by the protonated

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|---|---|
| **TABLE 1.** MRM parameters for the detection of GSH conjugates of 2EHD and 2EHD-d₅, | **Analyte** | **MRM Transition (m/z)** | **Collision Energy (eV)** |
| 3-SG-HD | 546.3 > 399.2 | 16 |
| 2EHD-GSH (imine) | 528.3 > 350.4 | 16 |
| 3-SG-HD-d₅ | 551.4 > 404.3 | 16 |
| 2EHD-d₅-GSH (imine) | 533.3 > 355.4 | 16 |

Values were determined using the Optimizer tool of the Agilent MassHunter Workstation software. Bold indicates mass transitions used for quantification. Additional mass transitions were recorded for structure identification of the analytes. The dwell time for all mass transitions was 100 ms.
molecular ion, [M+H]+ m/z 546.3, that can be attributed to a Michael adduct [3-glutathionyl-hexadecanal (3-SG-HD)]. Additionally, we were able to detect a precursor ion with m/z 528.3 that, due to the mass loss of 18 Da, is a product of a condensation instead of an addition reaction. In this case, we proposed the Schiff base (imine) formation of the primary amino group of the γ-glutamyl moiety of GSH with the carbonyl carbon of the fatty aldehyde (2HD-GSH).

Next, we performed product ion scans of both precursor ions, m/z 546.3 and 528.3, in order to obtain structural information on the conjugates detected. The resulting product ion mass spectra are given in Fig. 2. The putative Michael adduct shows a main product ion with m/z 399.2 that can be explained by the fragment [M+H-Glu-H2O]+. Likewise, the most intense fragment of the assumed Schiff base product (m/z 350.4) may be attributable to the cleavage of the glycine (Gly)-Cys moiety of GSH. Subsequently, we developed MRM methods for the detection of both adducts of 2HD with the remaining amino acids investigated as well as for 3-NAC-HD can be found in the supplemental material.

Stability of 3-SG-HD in HepG2 cell lysates compared with a cell-free system

As the Michael adduct, 3-SG-HD (the major GSH conjugate of 2HD), still contains an electrophilic carbonyl function in the molecule, it is still susceptible to nucleophilic attack. Irrespective of the nature of the nucleophile, this would mean the loss of the analyte using our targeted LC-MS/MS approach. To test this hypothesis, we studied the time-course of the 3-SG-HD formation in lysates of HepG2 cells and a cell-free system using identical buffered conditions. It should be mentioned that no 3-SG-HD was detectable in cell lysates without incubation with 2HD. But, as depicted in Fig. 3, the GSH conjugates of interest were immediately formed in HepG2 cell lysates after treatment with 2HD. The highest concentration was detectable even after 2.5 min. An equally prompt formation was found after additional GSH supplementation; however, maximum amounts of 3-SG-HD were approximately 30-fold higher. In both cases, the rapid formation of 3-SG-HD was followed by an equally rapid decrease. As early as 30 min after the incubation, no more GSH conjugates could be detected. On the contrary, 3-SG-HD synthesized under buffered cell-free conditions was stable throughout the experiment (until 60 min). Here, the formation of 3-SG-HD reached its plateau at 10 min and resulted in slightly higher maximum adduct concentrations (~150%) compared with those in HepG2 cell lysates (with GSH supplementation).

Formation of Michael adducts and Schiff bases of 2HD with free l-amino acids in vitro

Next, we aimed to develop a protein adduct analysis for 2HD by means of LC-MS/MS detection of amino acid adducts. First of all, candidates for adduct formation needed to be elucidated. As known from the GSH conjugation study, 2HD can react with thiols and primary amino groups. Thus, we included Cys, Lys, and Arg in our study. We selected three further amino acids with a nucleophilic attack. Irrespective of the nature of the nucleophile, this would mean the loss of the analyte using our targeted LC-MS/MS approach. To test this hypothesis, we studied the time-course of the 3-SG-HD formation in lysates of HepG2 cells and a cell-free system using identical buffered conditions. It should be mentioned that no 3-SG-HD was detectable in cell lysates without incubation with 2HD. But, as depicted in Fig. 3, the GSH conjugates of interest were immediately formed in HepG2 cell lysates after treatment with 2HD. The highest concentration was detectable even after 2.5 min. An equally prompt formation was found after additional GSH supplementation; however, maximum amounts of 3-SG-HD were approximately 30-fold higher. In both cases, the rapid formation of 3-SG-HD was followed by an equally rapid decrease. As early as 30 min after the incubation, no more GSH conjugates could be detected. On the contrary, 3-SG-HD synthesized under buffered cell-free conditions was stable throughout the experiment (until 60 min). Here, the formation of 3-SG-HD reached its plateau at 10 min and resulted in slightly higher maximum adduct concentrations (~150%) compared with those in HepG2 cell lysates (with GSH supplementation).
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344.3, 360.3, 385.4, 394.3, 413.4 and 443.3, that may stand for the Michael adducts of Ser, Cys, Lys, His, Arg, and Trp, respectively. Additionally, we detected a possible Schiff base product (m/z 367.3) after reaction of Lys with 2EHD. Subsequently, we subjected all found precursor ions to product ion scans and identified suitable fragments for development of MRM methods. As can be taken from Table 2, we optimized four mass transitions per amino acid adduct. Analogously, we proceeded with incubations of those six amino acids and 2EHD-d5. Comparing the optimized MRM parameters for adducts of 2EHD (Table 2) and 2EHD-d5 (Table 2, supplemental Table S1), it becomes clear that the most intense fragment ions, which were used for the quantifier mass transitions, significantly differed in their structures depending on the respective amino acid. While those fragments of adducts with Trp, Arg, Cys, and Ser contained the fatty aldehyde moiety, smaller and aldehyde-free fragments were most intense for His and both Lys adducts. Figure 4 presents the quantifier MRM chromatograms of the Michael adducts of Lys, Arg, His, Ser, Trp, and Cys [in the following referred to as 3-lysyl-hexadecanal (3-Lys-HD), 3-arginyl-hexadecanal (3-Arg-HD), 3-histidinyl-hexadecanal (3-His-HD), 3-serinyl-hexadecanal (3-Ser-HD), 3-tryptophanyl-hexadecanal (3-Trp-HD), and 3-cysteinyl-hexadecanal (3-Cys-HD), respectively]. Furthermore, an accordant chromatogram for 2EHD-Lys, the Schiff base product of 2EHD and Lys, is illustrated. Amino acid adducts eluted in a retention time window of 5–14 min, going from 3-Lys-HD (5.2 min) to 3-Cys-HD (14.0 min). The latter adduct occurred as a broad double peak (10.1 and 14.0 min), while the remaining adducts showed sharp peaks with negligible isomeric signals. Because both peaks were detected in the same intensity ratio in all four recorded MRM
transitions of 3-Cys-HD (supplemental Fig. S1), they can both be assigned to the presumed Michael adduct. For this reason, the accordant signal was integrated over both peaks, as shown in Fig. 4G. The cause of this signal splitting remains unknown. However, we were able to determine a strong influence of the amount of formic acid added to the LC eluents on the peak shape and intensity of 3-Cys-HD, suggesting a pH-dependent peak splitting. The addition of 1% formic acid to the solvents resulted in approximately 4-fold higher peak areas as compared with 0.1% formic acid, albeit the signal splitting became stronger (supplemental Fig. S2). Based on the applied settings of the ion source and MS/MS detector, the intensities of the amino acid adducts were in the following order: 3-Cys-HD > 3-His-HD >> 2EHD-Lys = 3-Trp-HD > 3-Lys-HD = 3-Arg-HD >> 3-Ser-HD. We also studied the stability of the 2EHD amino acid adducts after incubation under aqueous buffered conditions in a period of 48 h (Fig. 4). Maximum amounts of adducts were already formed after 30 min of incubation, followed by a substantial decrease. After 48 h, less than 5% of the respective maximum product yields were recovered. Interestingly, 3-His-HD is a clear exception. For this particular Michael adduct, the highest concentrations were determined after 8 h of incubation, followed by a plateau phase until 30 h. Two days after starting the reaction, 41% of the maximum product amount was still detected. This time-course experiment, conducted to identify the most stable amino acid adduct of 2EHD for further studies, was carried out once. However, repeated measurements of selected adducts and time points proved the reproducibility (data not shown). Furthermore, we developed a MRM method for detection of 2EHD adducts with NAC, as we utilized this Cys derivative as a scavenging agent for unreacted 2EHD in our protein adduct analysis protocol. The corresponding Michael adduct with the [M+H]+ of m/z 402.3 eluted from the analytical separation column at 13.7 min (data not shown). The optimized MRM parameters for the detection of 3-(N-acetylcysteinyl)-hexadecanal (3-NAC-HD) are given in supplemental Table S1.

**Formation of a Michael adduct of N-terminally protected His with 2EHD**

In order to verify the His side chain as linkage site in the formation of the most stable 2EHD amino acid adduct detected, we incubated N-terminally protected Fmoc-His-OH with 2EHD and analyzed the reaction mixture in the product ion scan mode. As can be seen in Fig. 5, we were able to detect a precursor ion, [M+H]+ with m/z 616.4, that may represent the Michael adduct 3-(Fmoc)histidinyl-hexadecanal [3-(Fmoc)His-HD] and that further confirms the adduct formation via the imidazole moiety of His. This supports the assumption that the adduct formation in the presumed unprotected Michael adduct, 3-His-HD, is also carried out via either the π or the τ nitrogen of His, because the precursor ion and most intense fragment ion of 3-His-HD, m/z 394 and m/z 110, respectively, were found as product ions in the mass spectrum of 3-(Fmoc)His-HD (Fig. 5).

**The 3-His-HD-d5 as internal standard for 2EHD protein adduct analysis**

Studies with free l-amino acids suggested that His adducts of 2EHD (3-His-HD) are promising protein adducts in terms of intensity of formation and stability. Consequently, we decided to include stable isotopically labeled
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3-His-HD-d5, as an internal standard in our sample preparation set-up and MRM analysis. Figure 6 confirms the structural consistency of 3-His-HD and 3-His-HD-d5 on the basis of their product ion mass spectra and identical fragmentation patterns seen therein. In order to correct the analytical outcome for sample losses during size exclusion filtration for variations in enzymatic protein hydrolysis, as well as adduct losses during liquid-liquid extraction (see the Materials and Methods), protein (BSA)-bound 3-His-HD-d5 was applied as internal standard. Because the exact proportion of the adducted His residues in this standard is not known, this approach only permits a semi-quantitative determination of 3-His-HD in proteins.

Detection of 2EHD protein adducts in BSA and HepG2 cell lysates

After establishment of a protein adduct analysis based on MRM detection of 2EHD amino acid adducts, BSA and whole protein of HepG2 cell lysates, incubated with 2EHD, were analyzed for accordant protein adducts. For the determination of protein adducts in treated cell lysates, in particular, we have integrated several steps into our protocol to prevent protein adducts from being confused with peptide or amino acid adducts because all three species would be similarly detected using the developed method. This would lead to an overestimation of adduct levels. First, we quenched the reaction of 2EHD with BSA or cellular nucleophiles by addition of 10 molar equivalents of NAC prior to sample preparation. The efficiency of this step can be monitored by determining 3-NAC-HD (using the recommended protocol without size exclusion filtration). Second, we performed a centrifugal filtration of the incubation mixture to assure that only molecules with a molecular mass of at least 30 kDa were subjected to enzymatic proteolysis. Both steps prevent low molecular mass adducts of 2EHD, formed during cell lysate treatment or during protein digestion by carryover of unreacted 2EHD, from falsifying the analysis result. Finally, the usage of a protein-based internal standard allows the correction of analyte losses from the very beginning of the sample preparation process and a relative quantification of protein adducts. A comparative protein adduct analysis of treated BSA and HepG2 cell lysates is shown in Fig. 7.

For both types of samples, a total of four protein adducts of 2EHD was detectable: 3-His-HD, 2EHD-Lys, 3-Trp-HD, and 3-Cys-HD. The amino acid adducts detected had similar retention times, as in the experiments with free amino acids (compare Figs. 4, 7). For both sample types, 3-His-HD showed, by far, the highest signal intensity, followed by 2EHD-Lys and similar amounts of 3-Trp-HD and 3-Cys-HD. Differences between BSA and cell lysates were also observed. Thus, at comparable amounts of 3-His-HD, approximately 4-, 2-, and 5-fold higher values for 2EHD-Lys, 3-Trp-HD, and 3-Cys-HD in cell lysates could be determined. Consequently, the later eluting and less intense signal of 3-Cys-HD (at 14.3 min) could only be detected in cell lysates. In HepG2 cell lysates that served as solvent controls (without addition of 2EHD), no accordant protein adducts were detectable.
Modulation of GSH conjugate and protein adduct levels of 2EHD in HepG2 cell lysates by inhibition of the oxidative 2EHD degradation

The initial motivation for the development of the methods presented here was the investigation of the influence of 2EHD on the pathogenesis of certain metabolic disorders, which are characterized by an impaired fatty aldehyde metabolism, for example, by reaction with cellular macromolecules leading to their depletion or dysfunction. On this background, we applied our novel LC-MS/MS methods for determination of 2EHD GSH conjugates and protein adducts to HepG2 cell lysates that were co-incubated with 2EHD and DBAP, a known inhibitor of the FALDH (17) responsible for the oxidative degradation of 2EHD to (2E)-hexadecenoic acid. As depicted in Fig. 8, we observed increased amounts of 3-SG-HD, the major GSH conjugate of 2EHD, in cell lysates treated with DBAP compared with corresponding solvent controls throughout the whole time-course experiment (lasting 60 min). The maximum conjugate yield, after DBAP treatment, amounted to 138% of that in controls. Moreover, 3-SG-HD was still detectable in DBAP-treated cell lysates 60 min after incubation; whereas, in control samples, no 3-SG-HD was found after only 30 min. Similar results were obtained for 2EHD protein adducts (Fig. 9). The analysis of 3-His-HD, identified as the predominant protein adduct of 2EHD, revealed a 19% increase in the corresponding modification of HepG2 cell whole protein after DBAP treatment versus untreated controls that was of borderline significance (P = 0.063).

DISCUSSION

The intention of this study was to provide new insights into the metabolic fate of 2EHD, an irreversible cleavage product of the bioactive lipid, S1P. Due to its α,β-unsaturated carbonyl function, it is a reactive compound that can potentially interact with cellular macromolecules and, thus, may exert adverse effects (e.g., cytotoxicity). In recent years, metabolic pathways that lead to a detoxification of 2EHD have been identified. These include oxidation of 2EHD to (2E)-hexadecenoic acid by the FALDH followed by an activation of the fatty acid by acyl-CoA synthetases and conversion to glycerophospholipids (10, 11). However, in an earlier study, we were able to show that, at least in lysates of HepG2 cells, the conversion of 2EHD to the activated fatty acid does not proceed quantitatively (12). Hence, we aimed to identify cellular targets of 2EHD in order to provide a possible contribution to the elucidation of the pathogenesis of certain metabolic diseases that are linked to an impaired fatty aldehyde metabolism, such as the Sjögren-Larsson syndrome. For 4-hydroxy-2-nonenal (4-HNE), an α,β-unsaturated aldehyde endogenously formed during lipid peroxidation (LPO), adduct formation with GSH (18), proteins (19), and DNA nucleobases (20) has been observed. As DNA adduct formation of 2EHD in vitro has already been shown (14), we focused on identification of accordant reaction products with GSH and proteins within this study.

Using state-of-the-art mass spectrometric techniques, we were able to detect nine novel reaction products of 2EHD
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with nucleophilic biomolecules, first of all, in a cell-free in vitro approach. These reaction products are two GSH conjugates, one Michael adduct and one Schiff base, and seven amino acid adducts, Michael adducts with Lys, Arg, His, Ser, Trp, and Cys, as well as a Schiff base product with Lys. Although we have not verified the structures of these reaction products by NMR spectroscopy, we provide strong evidence for their identity with our MS/MS experimental data. For the main GSH conjugate detected, 3-SG-HD, the product ion scan revealed an intense fragment with \( m/z \) 399.2, explainable by \([M+H-Glu-H_2O]^+\) (Fig. 2). This fragment ion was also observed for the isobaric GSH conjugate of 2-chlorohexadecanal, an \( \alpha \)-chloro fatty aldehyde structure closely related to 2EHD, that is produced by hypochlorous acid targeting plasmalogens during neutrophil activation (21). We observed a second minor GSH conjugate of 2EHD that, due to the mass loss of 18 Da, most likely is a condensation product of both educts. The obtained product ion spectrum confirms the assumption of an imine (Schiff base) formation of the \( \alpha \) amino group of the \( \gamma \)-glutamyl unit of GSH with the carbonyl carbon of 2EHD because at least two fragments, \( m/z \) 453.0 (putative cleavage of Gly) and \( m/z \) 350.4 (putative cessation of Gly-Cys), exclude another possible linkage site (Fig. 2). Incubations with 2EHD-d5 and GSH further confirmed the proposed structures. For all 2EHD amino acid adducts, with the exception of 3-Cys-HD that appeared as broad double peak, we obtained one major product, as can be seen in the MRM chromatograms (Fig. 4). Comparing the retention times of 3-Ser-HD (7.24 min) with 3-Cys-HD (first peak: 10.10 min), the large difference appears surprising because both assumed structures differ only in the atom presumably involved in the Michael addition, oxygen or sulfur. However, while 3-Ser-HD eluted as a relatively sharp signal, 3-Cys-HD eluted over a wide retention time range, starting at about 7 min. Considering this, the difference in retention time seems to be less pronounced. Furthermore, it is also known from already published protein adduct studies that Ser and Cys modified by the same adduct differ quite significantly in their retention time (22), even though a direct comparison is difficult because of varying chromatographic conditions. There are several clear indications that the monitored compounds are adducts with the amino acid side chains, rather than with the \( \epsilon \) amino group. First, at physiological pH (as in our experiments), the dissociation equilibrium of the \( \epsilon \) amino group of all amino acids is on the side of the protonated form (\( -\text{NH}_3^+ \)), which degrades its nucleophilicity. It must be mentioned that this is also true for the \( \epsilon \)-amino group of Lys. Second, clear hints can be taken from the MS/MS data of the adducts. For instance,
both Cys and NAC form an adduct with a mass gain of 238 Da, which corresponds to the molecular mass of 2EHD. As the Cα amino group in NAC is protected by acetylation, this indicates Michael adduction via the thiol group. Likewise, a collision-induced retro-Michael addition (m/z 360.3 > 122.1 for 3-Cys-HD and m/z 402.3 > 164.1 for 3-NAC-HD) was observed for both adducts. Moreover, N-terminally protected Fmoc-His-OH forms a Michael adduct with 2EHD that can be fragmented, among others, into the ions, m/z 394 and m/z 110, representing the [M+H]+ and the most intense product ion of 3-His-HD. Further strong evidence for the adduct formation with amino acid side chains is provided by the fact that corresponding 2EHD adducts were also found in proteins (four out of seven candidates in 2EHD-treated BSA) in which the Cα amino groups are part of peptide bonds. The N-terminal amino acid serves as an exception, which, however, is aspartic acid in the case of BSA (23). Regarding the stability of amino acid adducts with α,β-unsaturated aldehydes, it has frequently been reported that the formation of corresponding Michael adducts and Schiff bases is reversible and the products obtained are more or less short-lived (24). For example, 4-HNE Michael adducts with the ε-amino group of Lys are formed reversibly and can be isolated only after reductive aldehyde-to-alcohol conversion using sodium borohydride (NaBH4) (25, 26). Therefore, we included stability investigations for 2EHD amino acid adducts and GSH conjugates in our study. We allowed the six amino acids, Arg, Cys, His, Lys, Ser, and Trp, to react with 2EHD in a mixture of phosphate buffer (pH 7.4) and ethanol 1:1 (v/v) and monitored the product yields over a period of 48 h. As reported for other α,β-unsaturated aldehydes, it turned out that six out of seven adducts were not stable during this experiment. The half-life (t1/2) for all amino acid adducts, except 3-His-HD, that were estimated by second-order polynomial regression using the time-courses given in Fig. 4 were in the range of 11–19 h. Interestingly, the Michael adduct of His and 2EHD had a t1/2 of about 45 h and, therefore, possessed substantially higher stability. Due to its imidazole moiety, His plays multiple roles in molecular interactions, particularly in proteins, with hydrogen bonding being an example for such an interaction (27). Possibly, such an intramolecular hydrogen bond between the basic imidazole nitrogen and the carbonyl function of 2EHD, present in the enol form, contributes to the stabilization of the adduct. However, this is speculative and further studies should be envisaged to clarify this issue. Regarding the stability of GSH conjugates of 2EHD, we compared the time-course of product formation in aqueous buffered solution with that in HepG2 cell lysates. Here, we observed a clear difference between cell-derived and cell-free systems (Fig. 3). Whereas the Michael adduct, 3-SG-HD, was stable for at least 1 h under aqueous buffered conditions, a substantial product degradation starting a
few minutes after incubation was determined in cell lysates. On the one hand, it is conceivable that an enzymatically driven degradation of 2EHD GSH conjugates to mercapturic acids takes place in cell lysates. Another possibility that could be considered was discovered for 4-oxo-2-nonenal (4-ONE) several years ago. This LPO product, once conjugated by GSH via Michael addition, was shown to effectively prevent Schiff base formation of 2E. Although this is a plausible mechanism, not least due to the -lactoglobulin (28). Aiming NaBH4 (C. Neuber, F. Schumacher, and B. Kleuser; unpublished observations). Consequently, mass spectrometric studies on protein adduction with α,β-unsaturated aldehydes that follow protocols omitting treatments for adduct stabilization, as is the case for the present study, will reveal only those adducts that are stable throughout the sample preparation procedure. The protocol presented herein takes at least 24 h from the initial protein solution to the ready-to-analyze sample. Based on this information, it is not surprising that 3-His-HD, proven to be the second most abundant amino acid adduct with comparatively high stability, is by far the most intense protein adduct of 2EHD detected in treated BSA and cell lysates (Fig. 7). Furthermore, the ionization parameters selected for MS/MS detection of the amino acid adducts have an influence on their respective detection sensitivities. The ion source parameters used herein were optimized for 2EHD Michael adducts of His and Lys. It can, therefore, not be ruled out that the high relative intensities of 3-His-HD and 2EHD-Lys (with probably similar MS response as 3-Lys-HD) in comparison to the remaining adducts detected in 2EHD-treated protein are at least partially caused by this. On the basis of the amino acid sequence of BSA, the protein adduct data were not necessarily to be expected. Among the nucleophilic amino acids investigated, this model protein contains 50× Lys, 35× Cys (Cys-34 as only free thiol), 28× Ser, 23× Arg, 17× His, and 2× Trp (23). Once again, this underlines the suitability of 3-His-HD as the best marker substance for the protein adduction of 2EHD because approximately 50 times more adducts were found than for Lys residues that are 3.5 times more abundant in the primary structure of BSA. However, not only the mere number of corresponding amino acids in the protein is crucial for the adduction, but also their spatial accessibility. Comparisons with the HepG2 total protein (≥30 kDa fraction) of unknown composition confirmed 3-His-HD as major adduct. The higher yields of adducts with Lys, Trp, and Cys were probably due to the deviating composition of the total cell lysate protein compared with pure BSA. Future investigations, currently planned in our working group, should include studies on the stability of 2EHD protein adducts and their potential to form cross-links with other proteins or further biomolecules, as demonstrated for various LPO products, such as acrolein (29, 30), 4-ONE (31), and 4-HNE (32). The S1P lyase responsible for irreversible cleavage of S1P and subsequent formation of 2EHD is expressed in many tissues, including liver (33). In the present study, we used human hepatoblastoma-derived HepG2 cells for metabolic studies. Adapting a LC-MS/MS-based assay published by Berdyshev et al. (34), we determined a S1P lyase activity in HepG2 cell lysates that amounted to 0.360 ± 0.02 pmol 2EHD/min/μg protein (data not shown; unpublished observations, C. Neuber and B. Kleuser). Nevertheless, we could not detect basal levels of 2EHD adducts with GSH or proteins in HepG2 cell lysates in the present study. This was only possible after 2EHD supplementation. However, we were able to increase the amounts of 2EHD GSH conjugates and protein adducts formed in HepG2 cell lysates by inhibiting the oxidative degradation of 2EHD (Figs. 8, 9). This was achieved by co-incubation with DBAP in concentrations for which effective inhibition of the FALDH was shown (17). The FALDH inhibition was tested in a separate set of experiments by simultaneous analysis of 2EHD-d5 and (2E)-hexadecenoic acid-d5 in HepG2 cell lysates co-incubated with 2EHD-d5 and DBAP according to our method, previously described (12). Thus, a significant reduction in the fatty aldehyde-to-acid conversion could be observed in the presence of DBAP (supplemental Fig. S3). These findings clearly underline the value of the methodology presented here for in-depth studies of metabolic disorders characterized by disrupted fatty aldehyde degradation. Probably, the most well-known example of such a disorder is the Sjögren-Larsson syndrome. For this autosomal recessive neurocutaneous disease, more than 70 mutations, especially missense mutations (38%), in the FALDH gene are described that lead to reduced activity of the encoded enzyme of up to 90% (35). Affected patients suffer from ichthyosis, spastic paraplegia, and mental retardation. While the above-mentioned gene defect is recognized as the cause of the syndrome, the molecular pathogenesis leading to the corresponding symptoms is not fully understood. In general, GSH conjugation is a desirable detoxification mechanism. However, an excessive occurrence of electrophiles, such as 2EHD, can lead to GSH depletion and thus to a cellular redox imbalance. Furthermore, protein adducts can lead to the destabilization or even loss of function of the affected protein (15, 16). Both are pathogenic processes. Therefore, the methodology presented here offers the possibility to investigate the disease patterns of the Sjögren-Larsson syndrome and further disorders in more detail.

In summary, we have detected a total of nine novel reaction products of the S1P degradation product, 2EHD, with cellular nucleophiles by means of LC-MS/MS in a cell-free approach: two GSH conjugates and seven amino acid adducts. Both GSH conjugates were also found in HepG2 cell lysates incubated with 2EHD. Likewise, we were able to detect four out of seven amino acid adducts released from the model protein, BSA, and HepG2 whole protein, with 3-His-HD being the most prominent adduct. Most interestingly, inhibition of the enzyme responsible for oxidative degradation of 2EHD (FALDH) resulted in increased levels of both
GSH conjugates and protein adducts in HepG2 cell lysates. Hence, our data and developed mass spectrometric approaches will be useful to investigate physiological conditions or certain disorders that are linked to an impaired fatty aldehyde metabolism in more detail.

REFERENCES

1. Mattie, M., G. Brooker, and S. Spiegel. 1994. Sphingosine-1-phosphate, a putative second messenger, mobilizes calcium from internal stores via an inositol trisphosphate-independent pathway. J. Biol. Chem. 269: 3181–3188.

2. Spiegel, S., and S. Milstien. 2003. Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat. Rev. Mol. Cell Biol. 4: 397–407.

3. Rosen, H., R. C. Stevens, M. Hanson, E. Roberts, and M. B. A. Oldstone. 2013. Sphingosine-1-phosphate and its receptors: structure, signaling, and influence. Annu. Rev. Biochem. 82: 637–662.

4. Rivera, J., R. L. Proia, and A. Olivera. 2008. The alliance of sphingo-

ine-1-phosphate and its receptors in immunity. Nat. Rev. Immunol. 8: 753–763.

5. Hatt, N. C., C. A. Oskeritzian, S. W. Paugh, S. Milstien, and S. Spiegel. 2006. Sphingosine kinases, sphingosine-1-phosphate, apoptosis and diseases. Biochim. Biophys. Acta. 1758: 2016–2026.

6. Le Stunff, H., C. Peterson, H. Liu, S. Milstien, and S. Spiegel. 2002. Sphingosine-1-phosphate and lipid phosphohydrolases. Biochim. Biophys. Acta. 1582: 8–17.

7. Serra, M., and J. D. Saba. 2010. Sphingosine-1-phosphate lyase, a key regulator of sphingosine-1-phosphate signaling and function. Annu. Rev. Biochem. 63: 349–362.

8. James, P. F., and R. A. Zoeller. 1997. Isolation of animal cell mutants defective in long-chain fatty aldehyde dehydrogenase. Sensitivity to fatty aldehydes and Schiff's base modification of phospholipids: implications for Sjögren-Larsson syndrome. J. Biol. Chem. 272: 29532–29539.

9. Kumar, A., H-S. Byun, R. Bittman, and J. D. Saba. 2011. The sphingolipid degradation product trans-2-hexadecenal induces cytoskeletal reorganization and apoptosis in a JNK-dependent manner. Cell. Signal. 23: 1114–1112.

10. Nakahara, K., A. Ohkuni, T. Kitamura, K. Abe, T. Nagamura, Y. Ohno, R. A. Zoeller, and A. Kihara. 2012. The Sjögren-Larsson syndrome gene encodes a hexadecenal dehydrogenase of the sphingosine-1-phosphate degradative pathway. Mol. Cell. 46: 461–471.

11. Ohkuni, A., Y. Ohno, and A. Kihara. 2013. Identification of acyl-CoA synthetases involved in the mammalian sphingosine-1-phosphate metabolic pathway. Biochem. Biophys. Res. Commun. 442: 195–201.

12. Neuber, C., F. Schumacher, E. Gulbins, and B. Kleuser. 2014. Method to simultaneously determine the sphingosine-1-phosphate breakdown product (2E)-hexadecenal and its fatty acid derivatives using isotopodilution HPLC-electrospray ionization-quadrupole time-of-flight mass spectrometry. Anal. Chem. 86: 9065–9073.

13. Lopachin, R. M., T. Gavin, A. Decaprio, and D. S. Barber. 2012. Application of the hard and soft, acids and bases (HASB) theory to toxicant-target interactions. Chem. Res. Toxicol. 25: 259–251.

14. Upadhyaya, P., A. Kumar, H-S. Byun, R. Bittman, J. D. Saba, and S. S. Hecht. 2012. The sphingolipid degradation product trans-2-hexadecenal forms adducts with DNA. Biochem. Biophys. Res. Commun. 424: 18–21.

15. Lopachin, R. M., and A. P. Decaprio. 2005. Protein adduct formation as a molecular mechanism in neurotoxicity. Toxicol. Sci. 86: 214–225.

16. Castro, J. P. T. Jung, T. Grune, and W. Siems. 4-Hydroxynonenal (HNE) modified proteins in metabolic diseases. Free Radic. Biol. Med. Epub ahead of print. November 1, 2016; doi:10.1016/j.freeradbiomed. 2016.10.1497.

17. Kelson, T. L., J. R. Secor McVoy, and W. B. Rizzo. 1997. Human liver fatty aldehyde dehydrogenase: microsomal localization, purification, and biochemical characterization. Biochim. Biophys. Acta. 1355: 99–110.

18. Boon, P. J. M., H. S. Marinho, R. Oosting, and G. J. Mulder. 1999. Glutathione conjugation of 4-hydroxy-trans-2,5-nonenal in the rat in vivo, the isolated perfused liver and erythrocytes. Toxicol. Appl. Pharmacol. 159: 214–223.

19. Petersen, D. R., and J. A. Doorn. 2004. Reactions of 4-hydroxynonenal with proteins and cellular targets. Free Radic. Biol. Med. 37: 937–945.

20. Duerr, M. A., R. Aurora, and D. A. Ford. 2015. Identification of glu-
thione adducts of α-cholefatty aldehydes produced in activated neutrophils. J. Lipid Res. 56: 1014–1024.

21. Barknowitz, G., W. Engst, S. Schmid, M. Bernau, B. H. Monien, M. Kramer, S. Florian, and H. Glatt. 2014. Identification and quantification of protein adducts formed by metabolites of 1-methoxy-3-indolymlmethyl glucosinolate in vitro and in mouse models. Chem. Res. Toxicol. 27: 188–199.

22. Hirayama, K., S. Akashi, M. Furuya, and K. Fukuhara. 1990. Rapid confirmation and revision of the primary structure of bovine serum albumin by ESIMS and frit-FAB LC/MS. Biochem. Biophys. Res. Commun. 178: 639–646.

23. Sayre, L. M., D. Lin, Q. Yuan, X. Zhu, and X. Tang. 2006. Protein adducts generated from products of lipid oxidation: focus on HNE and ONE. Drug Metab. Res. 38: 651–675.

24. Nadkarni, D. V., and L. M. Sayre. 1995. Structural definition of early lysine and histidine addiction chemistry of 4-hydroxynonenal. Chem. Res. Toxicol. 8: 284–291.

25. Liu, D., H-G. Lee, Q. Liu, G. Perry, M. A. Smith, and L. M. Sayre. 2005. 4-Oxo-2-nonenal is both more neurotoxic and more protein reactive than 4-hydroxy-2-nonenal. Chem. Res. Toxicol. 18: 1219–1231.

26. Liao, S-M., Q-S. Du, J-Z. Meng, Z-W. Pang, and R-B. Huang. 2013. The multiple roles of histidine in protein interactions. Chem. Cent. J. 7: 44.

27. Zhu, X., M. M. Gallagol, J. J. Mieyal, V. E. Anderson, and L. M. Sayre. 2009. Covalent cross-linking of glutathione and carnosine to proteins by 4-oxo-2-nonenal. Chem. Res. Toxicol. 22: 1050–1059.

28. Furuhata, A., M. Nakamura, T. Osawa, and K. Uchida. 2002. Thiolation of protein-bound carcinogenic aldehyde: an electrophilic acrolein-histidine adduct that covalently binds to thiois. J. Biol. Chem. 277: 27919–27926.

29. Burcham, P. C., and S. M. Pyke. 2006. Hydralazine inhibits rapid acrolein-induced protein oligomerization: role of aldehyde scavenging and adduct trapping in cross-link blocking and cytoprotection. Mol. Pharmacol. 69: 1050–1065.

30. Stewart, B. J., J. R. Roede, J. A. Doorn, and D. R. Petersen. 2009. Lipid aldehyde-mediated cross-linking of apolipoprotein B-100 inhibits secretion from HepG2 cells. Biochim. Biophys. Acta. 1791: 772–780.

31. Kurtz, A. J., and R. S. Lloyd. 2003. L,N²-deoxyoxyno-20suggenates adducts of acrolein, crotonaldehyde, and trans-4-hydroxynonenal cross-link to peptides via Schiff base linkage. J. Biol. Chem. 278: 5970–5976.

32. Van Veldhoven, P. P., S. Gipsers, G. P. Mannakaerts, J. R. Vermesch, and V. Brys. 2000. Human sphingosine-1-phosphate lyase: cDNA cloning, functional expression studies and mapping to chromosome 10q221. Biochim. Biophys. Acta. 1487: 128–134.

33. Berdyshvev, E. V., J. Goya, I. Gershkova, G. D. Prestwich, H-S. Byun, R. Bittman, and V. Natarajan. 2011. Characterization of sphingo-

ine-1-phosphate lyase activity by electrospray ionization–liquid chromatography/tandem mass spectrometry quantitation of (2E)-hexadecenal. Anal. Biochem. 408: 12–18.

34. Rizzo, W. B., and G. Carney. 2005. Sjögren-larsson syndrome: diversity of mutations and polymorphisms in the fatty aldehyde dehydro-

genase gene (ALDH3A2). Hum. Mutat. 26: 1–10.