Metabolic fate of 4-hydroxynonenal in hepatocytes: 1,4-dihydroxynonene is not the main product

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Abstract 4-Hydroxynonenal (HNE) is a major aldehydic product of lipid peroxidation known to exert several biological and cytotoxic effects. The metabolic fate of this aldehyde was investigated in hepatocytes as a cell type with a rapid HNE degradation. The experiments were carried out in rat hepatocytes at 37°C at initial HNE concentrations of 1 μM—that means in the range of physiological and pathophysiologically relevant HNE levels—, 5 μM or 100 μM, respectively. About 95% of 100 μM HNE was degraded within 3 min of incubation. At 1 μM HNE the physiological level of about 0.1 to 0.2 μM was restored already after 30 sec. As primary products of HNE in hepatocytes the glutathione-HNE- 1:1- adduct, the hydroxynonenoic acid and the corresponding alcohol of HNE, the 1,4-dihydroxynon-2-ene, were identified. In contrast to previous reports, the corresponding alcohol of the HNE, 1,4-dihydroxynon-2-ene, was not the main HNE metabolite by far. The sum of these three primary HNE products accounts for about two-thirds of the total HNE degradation after 3 min of incubation. Furthermore, the beta-oxidation of hydroxynonenoic acid including the formation of water was demonstrated. The quantitative share of HNE binding to proteins, contrary to its great functional importance, is low with about 3% of total HNE consumption after 3 min incubation. The glycine–cysteine–HNE, cysteine–HNE adducts, and the mercapturic acid from glutathione-HNE adduct are not formed. In total, almost 90% of HNE degradation could be balanced by the formation of different HNE metabolites. The fast metabolism underlines the role of HNE degrading pathways in hepatocytes as one important part of the antioxidative defense system in order to protect proteins from modification by aldehydic lipid peroxidation products.—**Siems, W. G., H. Zollner, T. Grune, and H. Esterbauer.** Metabolic fate of 4-hydroxynonenal in hepatocytes: 1,4-dihydroxynonene is not the main product. *J. Lipid Res.* 1997. **38:** 612–622.

Supplementary key words 4-hydroxynonenal • aldehyde metabolism • lipid peroxidation • rat hepatocytes

Lipid peroxidation is always combined with the formation of reactive aldehydes (1–8). 4-Hydroxynonenal (HNE) is a major aldehyde produced in vivo during the peroxidation of n–6 polyunsaturated fatty acids (18:2, 20:4) (8). This compound has a high biological activity and exhibits a number of cytotoxic, e.g., hepatotoxic, mutagenic and genotoxic effects (7, 8). It is considered that at least some of the damage observed in free radical pathology is mediated by HNE and other aldehydes which may act as "second toxic messengers" of the primary free radical event. The hypothetic sequence: free radicals—lipid peroxidation—aldehyde formation—damage was proposed by Esterbauer et al. (7, 8).

HNE concentration is significantly increased in plasma, various organs, and cell types including liver and liver cells under conditions of oxidative stress (7–10). In hepatocytes it was observed that exogenously added HNE was degraded rapidly, leading to a rapid decline of the initially added HNE amount. The first attempts to measure the degradation of exogenously added 4-hydroxynonenal in hepatocytes were described by Esterbauer, Zollner, and Lang (11). They demonstrated that the degradation rate of HNE in liver was much faster than in other tissues (11). It was postulated (11) that the rapid conversion of the cytotoxic HNE and other reactive aldehydes to alcohols, which are probably less toxic, could play a role in the defense system of the liver against toxic substances arising from free radical-induced lipid peroxidation (7, 11). Those investigations searched for different metabolic pathways of HNE. To date, studies of HNE-metabolizing enzyme activities in hepatoma cells (12, 13) and studies on identification

Abbreviations: HNE, 4-hydroxy-2,3-*trans*-nonenal (4-hydroxynonenal); DHN, 1,4-dihydroxynonene; HNA, 1,4-dihydroxynonenoic acid; GSH, reduced glutathione; GST, glutathione-S-transferase(s); BHT, 2,6-di-tert-butyl-p-cresol (butylated hydroxytoluene); TLC, thin-layer chromatography; HPLC, high performance liquid chromatography.

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of pathways of hnE metabolism in ascites cells (14), mucosal cells (15) and in mitochondria from rat kidney cortex (16) have not indicated that the corresponding alcohol of HNE, 1,4-dihydroxynon-2-ene (DHN), is the main product of HNE metabolism. Therefore we investigated the HNE pathways to test whether the DHN is a major product in HNE metabolism of hepatocytes.

MATERIAL AND METHODS

Preparation of hepatocytes and incubation of the cells

Hepatocytes were prepared from male Wistar H-strain rats (180–250 g body weight) using published methods (17, 18). The final cell concentration in the incubation medium was 10^6/ml. That corresponds to a cytocrit of less than 1% (1% cytocrit corresponds to 1.68 $\pm$ 0.11 $\times$ 10^6 cells/ml). Trypan blue uptake was used to estimate cell damage. By this method the viability of the hepatocytes used for incubation experiments was 92% immediately after the preparation of the cells. During the incubation, the cells were gassed with 95% oxygen–5% carbon dioxide (carbogen). The pH value during the whole incubation period was constant at 7.4. The incubations were carried out in the presence of the radical scavenger 2,6-di-tert-butyl-*p*-cresol (BHT). Exogenous HNE was added at time zero in the incubation to give final concentrations of 1, 5, or 100 µM. After different incubation periods, aliquots of the suspensions were taken. The reaction was stopped by addition of one volume of acetonitrile–acetic acid 96:4 (v/v) mixture to one volume of suspension or by addition of 0.1 ml of concentrated perchloric acid to 4 ml of the suspension. The acetonitrile–acetic acid extracts were used for the determinations of 4-hydroxynonenal and 4-hydroxynonenoic acid concentrations by means of HPLC separation and for TLC separations. Perchloric acid extracts were used for the measurement of the glutathione-HNE adduct concentrations including the TLC separation procedure of different HNE adducts and also for the determination of the HNE binding to proteins.

Preparation and storage of HNE

4-Hydroxynonenal was prepared from the diacetal (19, 20). It was stored as a solution in chloroform at −20°C. [2-^{3}H]HNE (68.2 mCi/mmol or 589.4 mCi/mmol, respectively) was prepared according to Rees and van Kuijk (21).

Preparation of nonlabeled and radioactive labeled 1,4-dihydroxynonene

Preparation of the 1,4-dihydroxynon-2-ene was carried out by reduction of HNE with sodium boronate (22). Reduction was controlled by HNE measurement (HPLC separation, UV detection at 223 nm). After 45 min of reaction the remaining sodium boronate was destroyed by addition of 1 N HCl (final pH value 2–3). Dihydroxynonene was extracted with dichloromethane and the solution was dried over sodium sulfate. The purity of the dihydroxynonene was checked by TLC separation (diethyl ether–hexane 70:30 [v/v]). As reagent for detection of TLC spots, 10% phosphomolybdic acid in ethanol was used. The purified dihydroxynonene was extracted from TLC plates with CH_2Cl_2 and stored in this solvent.

For the preparation of the radioactive labeled dihydroxynonene, 3 ml of [2-^{3}H] *trans*-4-hydroxy-2-nonenal (4.05 mCi/15 ml ethanol; specific radioactivity 68.2 mCi/mmol) was dried under nitrogen. Solubilization in 6 ml water was improved by sonification. Then 150 mg sodium boronate was added for the reduction of labeled HNE within a period of 90 min. The solution was acidified with 1 N HCl to pH 2–3 for the destruction of sodium boronate. The labeled dihydroxynonene was extracted with dichloromethane. TLC separation for the evaluation of the preparation procedure and for the purification of the dihydroxynonene was as described above. The purification, especially the separation from small amounts of remaining HNE, was completed by elution on Bond Elut C18 columns (Analytichem, International, Harbor City, CA). The pure dihydroxynonene was eluted with 35% methanol in water (v/v). Traces of remaining HNE were eluted at a higher methanol concentration. By this procedure, 97.1% of the preparation was radioactive labeled dihydroxynonene.

Determination of concentrations of HNE and 4-hydroxynonenoic acid

After HNE and hydroxynonenoic acid were extracted from suspension with an equal volume of acetonitrile–acetic acid 96:4 (v/v), they were separated by isocratic HPLC using 42% acetonitrile–water (v/v) as eluent. All solvents used for HPLC separation were of the highest analytical grade available and were obtained from Merck Darmstadt, Germany. Columns were Spherisorb S5 ODS2, 250 $\times$ 4.5 mm (Phase Separations Ltd., Queensbury, UK). The HPLC equipment consisted of standard equipment suitable for isocratic operation with a UV monitor (Kontron, Uvikon 730LC). Both HNE and dihydroxynonene were detected at a wavelength of 223 nm. Flow was adjusted to 1 ml/min for HPLC measurement of HNE. Both compounds were identified by coelution with pure standard substances. Furthermore, hydroxynonenoic acid was identified by spiking the HPLC peak from suspension extracts with hydroxynonenoic acid produced in the simple system of

HNE, NAD^+, and aldehyde dehydrogenase. Aldehyde dehydrogenase was supplied by Boehringer Mannheim GmbH, Germany. Quantification of the acid was based on the comparison of the hydroxynonenoic acid peak with formation of NADH in experiments in which HNE reacted with aldehyde dehydrogenase. In those experiments, the rate of formation of NADH, which was observed spectrophotometrically at 340 nm, was equal to the consumption rate of HNE (measured by HPLC at 223 nm) and to the rate of formation of hydroxynonenoic acid. From the NADH formation rate (equal to the HNE consumption rate) and the peak integral of the hydroxynonenoic acid produced, one could calculate the extinction coefficient of the acid or the amount of hydroxynonenoic acid representing a defined peak area in the HPLC separation procedure. The HNE quantification was done by means of authentic HNE samples of known concentration, dissolved in methanol–water 80:20 (v/v), and comparing the peak height of the standard chromatogram to sample chromatograms.

Measurement of the concentration of the GSH adduct of HNE

The glutathione-HNE-adduct that is formed on the basis of 1 to 1 stoichiometry was measured as an isoindol derivative after the reaction of the adduct existing in neutralized perchloric acid extracts with *o*-phthaldialdehyde in the presence of mercaptoethanol. The reaction was started by addition of a sample aliquot to the *o*-phthaldialdehyde solution. The reaction mixture was transferred into a Hamilton syringe and the reaction was stopped by injection of the mixture into the HPLC system after 2 min. HPLC separation was carried out in an isocratic manner with 65% methanol–0.1 M sodium acetate buffer, pH 6.5 (v/v) in the presence of an ion-pair reagent (tetraethylammoniumhydroxide). Isoindol was detected by means of a fluorescence detector at wavelengths of 345 nm and 445 nm for excitation and emission, respectively. The formation of enantiomer products in the reaction of GSH-HNE adduct with *o*-phthaldialdehyde (23) led to the appearance of a double peak of the adduct isoindol. The reaction product of free reduced glutathione (GSH) with *o*-phthaldialdehyde eluted much earlier than the adduct isoindol within the isocratic HPLC separation method. HPLC equipment consisted of a Kontron LD pump, a Rheodyne injector, a Shimadzu C-R1B Chromatopac integrator, and a Shimadzu RF 530 fluorescence detector. The columns used were ODS 5 μm, 250 mm × 4.6 mm i.d. Precise measurement of the adduct concentration requires an extensive preparation of the neutralized perchloric acid extract of hepatocyte suspension with the aim of removing disturbing influences of amino acid derivatives after HPLC separation. For the purification of the extracts, Bond Elut C_{18} columns purchased from Analytichem International (Harbor City, CA) and a Baker-10 Extraction system (J.T. Baker Chemical Co., Philipsburg, NJ) were used. The columns were rinsed with methanol and equilibrated with water. After injection of the sample the column was rinsed with 0.1M sodium acetate buffer, pH 5, then rinsed with hexane. The sample was eluted with methanol, evaporated to dryness, and resolved in a defined volume of methanol.

Thin-layer chromatographic separations

After a defined incubation time, aliquots of suspensions were added to an equal volume of acetonitrile–acetic acid 96:4 (v/v). Supernatants of this extraction were used for TLC separations. Two different TLC separations were performed on aluminum sheets of silica gel 60 (without fluorescent indicator, layer thickness 0.2 mm, from Merck, Darmstadt, Germany). *i*) An elution with diethylether–hexane 70:30 (v/v) allowed the separation of hydroxynonenoic acid, 1,4-dihydroxynon-2-ene, HNE, and cyclic HNE-acetal spots. Reference spots were detected with phosphomolybdic acid in ethanol. The spots in parallel runs were scraped off for radioactivity measurements. At the conditions described, the hydrophilic glutathione-HNE adduct did not move from the injection point. By intramolecular cyclization, *cis*-HNE was transformed in the presence of palladium into the diethylacetal. The sample was spotted on a TLC plate, ethanolic sulfuric acid was added, the TLC elution was started, and the spot of the compound prepared was identified by means of phosphomolybdic acid reagent. *ii*) The second TLC separation was an elution with butanol–acetic acid–water 4:1:1 (v/v/v). This allowed the separation of the GSH-HNE adduct and other forms of HNE adducts such as cysteinyl-glycine-HNE and N-acetyl-cysteine-HNE from further HNE metabolites. Spots with amino functions were detected with ninhydrin (2,2-dihydroxyindane-1,3-dion) dissolved in ethanol (19).

High performance liquid chromatographic separations

HPLC separations were applied in tracer kinetic experiments with the aims of identification and quantitative determination of radioactive labeled water. Further peaks of radioactivity that were found in HPLC runs were not used for the investigation of HNE metabolism. Therefore, the gradient of the elution and the other conditions of HPLC separation are not described here. As equipment for the HPLC separations the System Gold from Beckman was applied. The columns were Ultrasphere ODS 5 μm, 250 mm × 4.6 mm i.d.

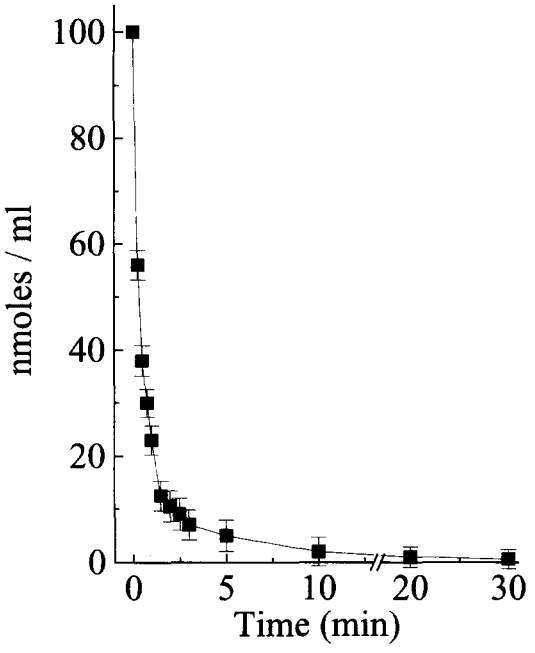

Fig. 1. Decrease of HNE concentration in hepatocyte suspensions. HNE concentration after addition to hepatocyte suspensions (10^6 cells/ml suspension) was 100 μM HNE. The measurement of HNE was carried out by means of HPLC separations with UV detection at 223 nm as described in Material and Methods. Values are given as mean ± SE. Number of independent experiments: n = 8.

RESULTS

Hepatocytes rapidly consume HNE

HNE was added to a suspension of freshly isolated hepatocytes (1×10^6 cells/ml) in a final concentration of 100 μM and the disappearance of the aldehyde was then followed by HPLC. **Figure 1** shows that 50% of the HNE was consumed within less than 30 sec. After 10 min of incubation less than 2% of the initially added HNE was present. Similar results were obtained in incubations with 5 or 1 μM HNE (time course not documented here, data after 3 min of incubation in Table 1). More than 80% of the aldehyde was consumed by the hepatocytes within about 15 sec.

Formation of primary HNE metabolites

A single dose of 100 μM HNE was added to a hepatocyte suspension and the amounts of the HNE-GSH conjugate, the 4-hydroxynonenoic acid, and the 1,4-dihydroxynonene present at different time intervals were determined over a 30-min incubation period (**Fig. 2A**). All three metabolites increased very rapidly within the first minute and reached the maximum value after about 2 min, and thereafter slowly decreased. At all time points the HNE-GSH conjugate was the main product followed by the carboxylic acid (HNA). Concentration of the alcohol (DHN) was always considerably lower. The relative proportion of the three metabolites was approximately 6:4:1. Formation of the HNE-GSH conjugate leads to a decrease of intracellular GSH concentration (**Fig. 3**). In the first 5 min of the experiments with 100 μM HNE, the GSH concentration was lower than half of the initial value. After that time the new steady-state level of GSH was a third of the initial concentration.

The kinetics of the formation of the three metabolites, HNE-GSH conjugate, HNE, and DHN, and their relative proportion showed a high reproducibility within different hepatocyte preparations. The kinetics of the formation of the metabolites was also measured in incubation systems with 5 and 1 μM HNE (curves not shown). **Table 1** reports the distribution of the three primary HNE metabolites after 3 min of incubation for initial HNE concentrations of 5 μM and 1 μM HNE. At that time point, 94–97% of the added HNE was consumed. The sum of the three primary metabolites, DHN + HNA + HNE-GSH, in all three cases after 3 min of incubation ranged between 60 and 65% of the added HNE (Fig. 2B). Taking into account the small fraction of the nonmetabolized HNE, it follows that about 33 to 36% of the HNE was converted into other metabolites. From the difference of time profiles of total HNE disappearance and sum of DHN + HNA + HNE-GSH (Fig. 2B) we examined the extent of this fraction of unknown metabolites. All kinetics experiments showed that the fraction of unknown metabolites was below 10% within the very first initial phase of about 30 sec of incubation. With increasing duration of the incubation this fraction steadily increased and reached a value of nearly 50% after 30 min (Fig. 2B). Such a finding strongly suggests that the HNE-GSH conjugate, the 4-hydroxynonenoic acid, and the 1,4-dihydroxynonene are primary metabolites that are further metabolized to other products. Additional experiments were therefore designed to identify these products.

Secondary products of HNE metabolism and quantitative share of HNE binding to proteins

It is known from many other studies that HNE can bind to thiol-groups of proteins and likely also to amino-groups. To determine the fraction of HNE covalently bound to proteins, hepatocytes were incubated with ^{3}H-labeled HNE for up to 10 min and the radioactivity associated with the hepatocyte proteins (TCA precipitate) was determined after different time intervals (**Table 2**). Binding of HNE reached a maximum after 3 min. The fraction of HNE bound to the proteins was 3% and 0.8% with 100 μM or 5 μM HNE, respectively.

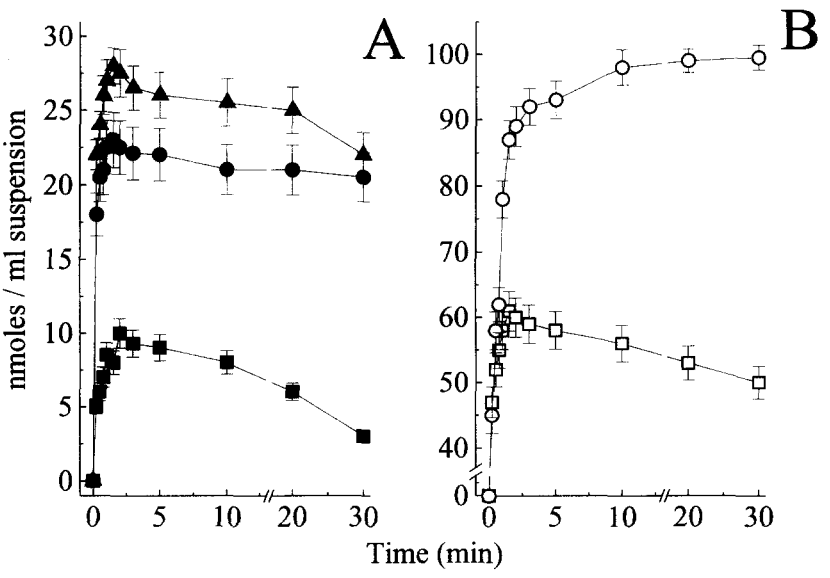

Fig. 2. Formation of primary HNE metabolites. A: Formation of HNE-glutathione (1:1) conjugate (▲), of 4-hydroxynonenoic acid (●), and of 1,4-dihydroxynon-2-ene (■) after the addition of HNE (100 μM) to hepatocyte suspensions. Conditions were as described in Fig. 1. Results are mean ± SE from 8 experiments. B: Disappearance of HNE (○) in hepatocyte suspensions in comparison to the sum of HNE-GSH conjugate, hydroxynonenoic acid, and dihydroxynonene formation rates (□) at initial HNE concentration of 100 μM. The values are given as mean ± SE in nmol/ml suspension. The incubation conditions were the same as given in Fig. 1. Number of independent experiments: 8. The difference between curves of HNE consumption and sum of the three main products of HNE represents the disappearance of HNE into other intermediates than HNE-GSH adduct, 4-hydroxynonenoic acid, or dihydroxynon-2-ene.

This corresponds to 3 and 0.04 nmol HNE/10^6 cells. There was also protein-bound radioactivity in trace kinetic experiments started with radioactive labeled dihydroxynonene (Table 2).

Considering the steady decrease of 4-hydroxynonenoic acid concentration with prolonged incubation, we suspect that it might be further metabolized to CO_2 and H_2O. If so, radioactive water should be formed. This point was checked by an HPLC separation that allowed the separation of radioactive substances present in the acetonitrile extract of hepatocytes treated with tritium-labeled HNE. Such an acetonitrile extract contained about 95% of the added radioactivity; the remaining radioactivity was likely protein-bound and therefore precipitated with acetonitrile. The almost complete recovery of the radioactivity indicated that no metabolites were lost during incubation or extraction procedures, and it follows that all HNE metabolites can be injected into the HPLC columns and should appear in the column effluent with proper conditions of elution. Eight radioactive peaks clearly separated from each other were found in HPLC elutions. Based on the elution behavior of standards, we identified the positions of HNE, HNE-GSH conjugate, dihydroxynonene, and hydroxynonenoic acid. The first radioactive peak was eluted at the same position as a tritium-labeled water standard. This first peak was not detected in lyophilized samples, which indicates that the compound is vol-

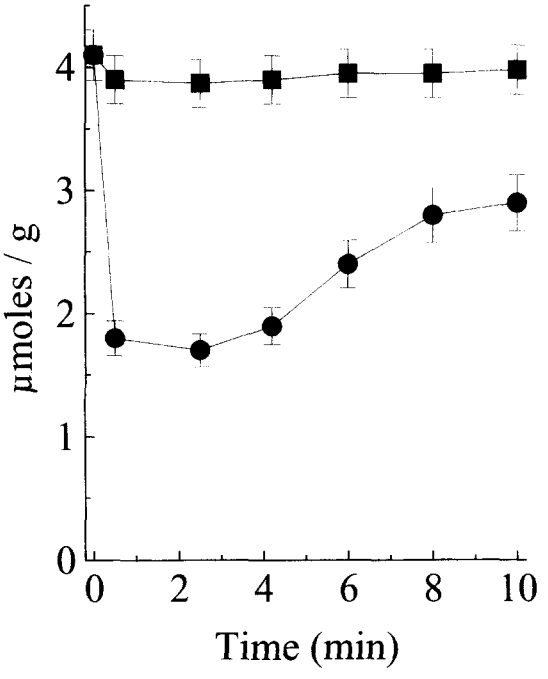

Fig. 3. Concentration of reduced glutathione in rat hepatocytes after addition of 4-hydroxynonenal; (■) control; (●) 100 μM HNE; temperature 37°C; pH 7.4. Values are given as μmol/g wet weight. The molar initial GSH level of the cells was about 4 mM. Number of experiments: 8.

TABLE 1. Consumption of 4-hydroxynonenal in rat hepatocytes (10^6 cells/ml suspension) at 37°C and pH 7.4 after 3 min of incubation

	Initial Concentration of 4-HNE				
	100 μM	5 μM		1 μM	
Variable	% = nmol/ml susp.	%	nmol/ml susp.	%	nmol/ml susp.
4-HNE	6.31 ± 1.69	2.5 ± 0.2	0.13 ± 0.01	5.7 ± 1.1	0.06 ± 0.10
Consumption of 4-HNE (HNE)	93.69 ± 1.69	97.5 ± 0.1	4.87 ± 0.01	94.3 ± 1.1	0.94 ± 0.01
1,4-Dihydroxynon-2-ene (DHN)	8.11 ± 2.12	6.4 ± 2.2	0.32 ± 0.11	11.0 ± 4.3	0.11 ± 0.04
Glutathione-HNE adduct (GSH-HNE)	27.50 ± 2.47	26.3 ± 2.2	1.32 ± 0.11	25.6 ± 2.4	0.27 ± 0.02
Hydroxynonenoic acid (HNA)	25.28 ± 5.61	28.5 ± 2.0	1.43 ± 0.10	23.7 ± 5.3	0.24 ± 0.05
Sum of DHN, HNE-GSH, and HNA	60.92 ± 4.18	61.4 ± 2.0	3.07 ± 0.10	60.1 ± 3.2	0.60 ± 0.03
HNE-consumption − sum of DHN, HNE-GSH, and HNA	32.81 ± 4.36	36.1 ± 3.1	1.80 ± 0.15	34.2 ± 5.1	0.34 ± 0.05
Number of experiments (n)	8	4	4	5	5

The measurements of the metabolites were carried out as described in Material and Methods. The values are given as mean ± SE.

TABLE 2. Binding of 4-hydroxynonenal to proteins in rat hepatocytes

Additions	Incubation Time	Binding of Radioactivity to Proteins	Number of Experiments
	min	*% of total*	
4-Hydroxynonenal, 100 μM	3	3.02 ± 0.56	10
4-Hydroxynonenal, 100 μM	10	3.14 ± 0.68	10
4-Hydroxynonenal, 5 μM	10	0.81 ± 0.06	5
4-Hydroxynonenal, 1 μM	10	0.62 ± 0.08	3
1,4-Dihydroxynonene, 100 μM	3	0.88 ± 0.02	4

Values were measured in experiments with radioactive labeled 4-HNE or 1,4-dihydroxynon-2-ene from the perchloric acid extract after 3 and 10 minutes of incubation. Values as mean ± S.E.

atile, confirming the suggestion that it is water produced by the metabolism of HNE in hepatocytes. The rate of formation of radioactive water was slow, and the time curve showed not a transient maximum but a steady increase (**Fig. 4A**). In hepatocytes treated for 10 min with 100 μM HNE, 14% of the radioactivity appeared in the water peak. The most plausible explanation for the formation of radioactive water is the beta-oxidation of 4-hydroxynonenoic acid. If it is so, inhibition of beta-oxidation would lead to an accumulation of the 4-hydroxynonenoic acid, and to an inhibition of the formation of radioactive water. Indeed, incubation of hepatocytes with HNE (100 μM) in the presence of 4-pentenoic acid, an inhibitor of beta-oxidation, significantly changed the distribution of metabolites although it had no effect on the total HNE consumption rate. In the absence and presence of the inhibitor, 95% of HNE was metabolized in 3 min. In the presence of the inhibitor, there was a significant higher accumulation of the 4-hydroxynonenoic acid in comparison with control experiments without inhibitor. Furthermore, in the presence of the 4-pentenoic acid, only traces of radioactive water were formed (Fig. 4B). These findings clearly show that 4-hydroxynonenoic acid is an intermediary metabolite, which is further utilized most likely by beta-oxidation. If this further utilization is blocked, 4-hydroxynonenoic acid becomes the major HNE metabolite. During the first 60 sec of incubation, the inhibitor had almost no effect on the rate of the conversion of HNE to 4-hydroxynonenoic acid. Thereafter, however, the time profile was markedly different. In the presence of the inhibitor, the 4-hydroxynonenoic acid steadily increased, reaching 45 nmol/ml after 3 min, whereas in the absence of the inhibitor the concentration of the carboxylic acid slowly decreased again. These experiments indicate that 4-hydroxynonenoic acid is not an end product of HNE metabolism. It is further metabolized by beta-oxidation. The concentration of the cyclic HNE-acetal measured during the time course of the incubation experiments is shown in **Fig. 5.** This compound amounted to about 5% of initial HNE concentration after three min of incubation and about 10% after 10 min.

DISCUSSION

Comparison of degradation rate of HNE in different cell types

The degradation/disappearance of HNE and also of 4-hydroxypentenal has been studied in vitro with hepatocytes (11, 24), hepatoma cells (25, 26), isolated perfused rat hearts (27), enterocytes of the rat small intestine (15, 28), Ehrlich ascites tumor cells (14, 29), and kidney (30, 31). Furthermore, experiments with subcellular fractions of rat liver, cytosol, microcosms, and mitochondria as well as with purified enzymes of the HNE-degrading pathways (aldehyde dehydrogenases, alcohol dehydrogenase, and glutathione transferase) were carried out. One can find the highest HNE degradation

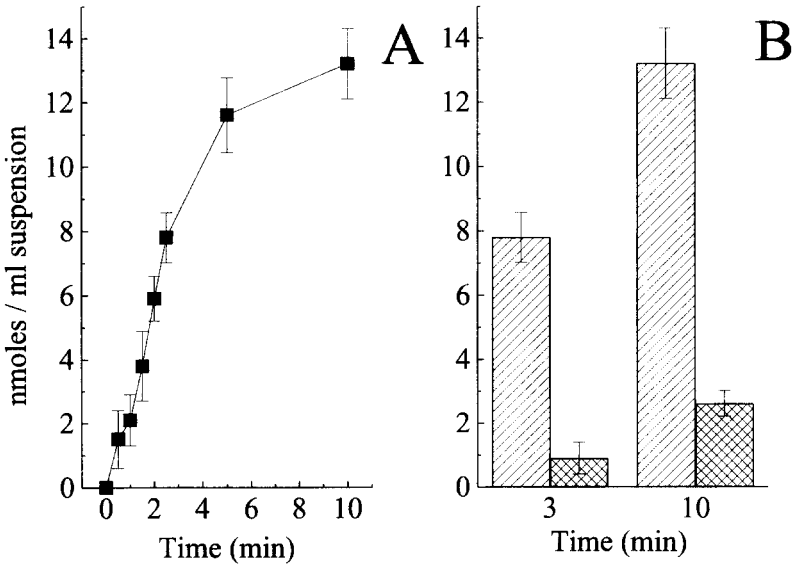

Fig. 4. Formation of water from HNE in isolated rat hepatocytes. A: HNE was added at 100 μM initial concentration to hepatocyte suspensions. Incubation conditions as in Fig. 1. The water formation was measured on the basis of radioactivity detection in HPLC separations of acetonitrile/acetic acid extracts of hepatocyte suspensions at different incubation periods. B: Water formation from HNE (initial concentration 100 μM) in control experiments (□) and in presence of 4-pentenoic acid (▨); 5 experiments.

rate in hepatocytes and enterocytes. Esterbauer et al. (11) already suggested that, in rats, liver has the highest capacity to metabolize HNE. In this study, we could measure that, within three min of incubation, 93.7% of HNE added with an initial concentration of 100 μM was utilized by 10^6 hepatocytes/ml suspension at 37°C and pH 7.4. The rates of HNE consumption in hepatocyte suspension were measured as 93.7 nmol/10^6 cells at 100 μM HNE initially, 4.9 nmol/10^6 cells at 5 μM HNE initially, and 0.9 nmol/10^6 cells at 1 μM HNE initially within three min of incubation (see Table 1). This result can be interpreted as a linear correlation between initial HNE concentration in cell suspension and HNE consumption rate as already found for kidney cells (30). The linear interrelationship that was measured in our experiments is therefore true also for physiological and pathophysiologically relevant conditions under which HNE levels in the range of 0.1 to 5 μM were measured in blood plasma or different tissues. Hartley, Ruth, and Petersen (24) found a decrease of HNE consuming rates with increasing HNE concentrations in the range of 50 to 250 μM, possibly due to a partial inhibition of metabolic enzymes. HNE utilization rates in different eucaryotic cell types have been compared (15, 16, 29). At 37°C and an initial HNE concentration of 100 μM, 28.4 ± 1.8 nmol HNE/mg wet weight per min in hepatocytes, 25.2 ± 2.7 nmol HNE/mg wet weight per min in enterocytes, and 8.7 ± 1.5 nmol HNE/mg wet weight per min in Ehrlich ascites tumor cells were degraded. The capacity of the Ehrlich ascites tumor cells to metabolize HNE (14, 29) was in the range of such organs as kidney or heart, but higher than in the skeletal muscle or the spleen (11). Thus, our results are consistent with those of Esterbauer et al. (8, 11) that liver cells have the highest capacity to detoxify HNE.

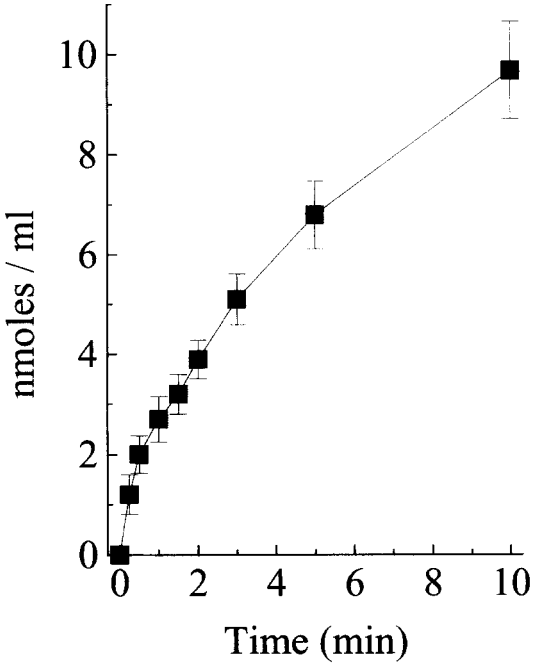

Fig. 5. Formation of cyclic HNE-acetal in hepatocyte suspension after the exogeneous addition of HNE (100 μM). Conditions as in Fig. 1. The measurement was carried out by determination of radioactivity which was scraped off from the TLC spot of this substance (see Material and Methods). Values as mean from 8 independent experiments.

Metabolic pathways of HNE in the liver

The capacity of cells to degrade HNE depends on the capacity of the HNE-metabolizing enzymes in those cell types. Current knowledge indicates that the main enzymes involved in the metabolism are glutathione transferases, aldehyde dehydrogenases, and alcohol dehydrogenases. The main primary metabolites of HNE are,

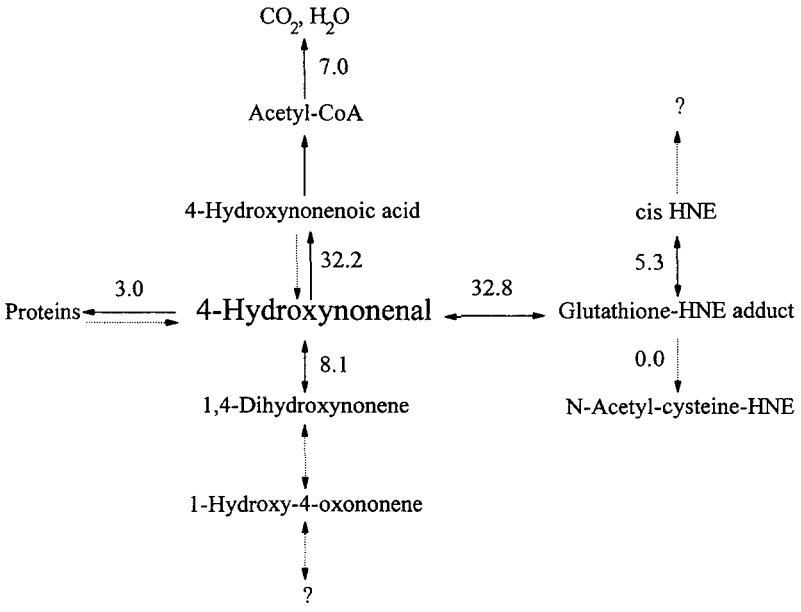

Fig. 6. Schematic representation of HNE metabolism in rat hepatocytes including the flux rates of the different pathways. These values are given as nmol/ml suspension/3 min. The incubation conditions were as in Fig. 1. The initial HNE concentration was 100 μM. The number of experiments was 8. Solid arrows represent pathways whose existence was demonstrated in this report or earlier. Dotted arrows represent pathways that hypothetically exist in the liver cells or can exist only in other tissues because of the exclusion of their existence in hepatocytes, as indicated by our results (e.g., no transformation into N-acetyl-cysteine-HNE). Nothing is known about the possibility of the back reaction from hydroxynonenoic acid to HNE.

therefore, the HNE-GSH adduct, the corresponding carboxylic acid of HNE (HNA), and the corresponding alcohol (DHN) (see Fig. 2A). In contrast to the previous report on DHN as by far the main metabolite of HNE in hepatocytes (11), the quantitative share of the formation of other HNE metabolites was found to be very high (24). Especially, the formation of HNE-GSH and HNA is much higher than that of DHN. A suggestion on the dominant role of DHN formation (11) was concluded from homogenate experiments in an oxygen-free, nitrogen/carbon dioxide (19:1) atmosphere and in the presence of high amounts of NADH. With the development of sensitive methods for the determination of several HNE metabolites in recent years, the almost complete balancing of HNE metabolites became possible, with the results demonstrated here. HNE-metabolizing aldehyde dehydrogenase isoenzymes are present in the hepatic cytosol, mitochondria, and probably also in microsomes (24, 32). The HNE-metabolizing NADH-dependent alcohol dehydrogenase is localized mainly in the hepatic cytosol (32). The glutathione transferases are ubiquitous enzymes, being particularly rich in hepatocytes and probably other liver cells. Even though a number of rat GST isozymes show catalytic activity toward HNE, rat liver glutathione transferase 8-8 has been demonstrated to be most effective in catalyzing the conjugation of this compound to GSH, and it has been suggested that this isozyme may be involved in the detoxification of HNE and related endogenous electrophiles (8, 32–35). A human acidic glutathione-S-transferase, hGST 5.8, was isolated from heart, pancreas, and brain (36). It was shown that this enzyme has about 20-fold higher specific activities for HNE than for 1-chloro-2,4-dinitrobenzene and expressed glutathione peroxidase activity toward phospholipid hydroperoxides. Berhane et al. (37) characterized the detoxification of base propenals and other unsaturated aldehyde products of free radical reactions and lipid peroxidation by human glutathione transferases (GST). They found that in general, GST A1-1 and GST M1-1, in contrast to GST P1-1, were more active with 4-hydroxyalkenals than with base propenals (37). HNE readily reacts with GSH also in a nonenzymatic reaction. The GSH transferase-catalyzed reaction can, however, proceed about 300 to 600 times faster (8, 32).

In this study the intracellular flux rates of HNE metabolism were measured. The approximate product distribution seen after 3 min of incubation of hepatocytes in presence of HNE was 33% HNE-GSH adduct, 32% 4-hydroxynonenoic acid, and only about 10% 1,4-dihydroxynon-2-ene, which is in agreement with other investigators (24). Three percent of exogenously added HNE was bound to proteins. The scheme presented in **Fig. 6** includes further metabolic steps that were partially measured in our experiments. Water formation from HNA amounted to about 7% after 3 min. The continuous water formation from HNE corresponds to the observation that after longer incubation, the concentration of HNA slowly decreased. The decreasing level of DHN can be explained by the equilibrium between HNE and DHN, only to a minor extent, with formation of further products formed from DHN (see postulated compound in Fig. 6). HNE-GSH conjugate formation leads to decreased cellular level of GSH (Fig. 3). GSH

concentration decreased to about 40% of initial value during incubation of hepatocytes in the presence of 100 μM HNE. This decrease was followed by a slow restoration of GSH level during incubation. Therefore, we were able to identify 90% of the HNE metabolites including primary and secondary metabolites, thus determining the metabolic fate of another 30% of metabolized HNE in addition to that cited in the study of Hartley, Ruth, and Petersen (24).

It is known that the HNE-GSH conjugate can be transported from one organ to another. The HNE-GSH conjugate release from cardiomyocytes into the heart perfusate was described by Ishikawa, Esterbauer, and Sies (27). In the whole animal the HNE-GSH conjugate is transported from the liver to the kidney and there further metabolized to a mercapturic acid (32). The formation of HNE mercapturic acid by kidney cells and by the isolated perfused rat kidney has been demonstrated (31, 32, 38). Degradation products of HNE-GSH adduct such as glycine-cysteine-HNE, cysteine-HNE, and the corresponding mercapturic acid in hepatocyte suspensions could not be detected after addition of HNE to the hepatocytes as shown by our experiments.

HNE metabolic pathways as secondary antioxidative defense system prevent aldehydic protein modification

The disappearance of HNE is connected on the one hand with reactions that contribute to the cellular damage and on the other hand with detoxification processes. In our opinion there is no doubt that the protein binding of HNE is of great importance concerning the pathophysiological consequences of free radical-initiated reactions.

The physiological and pathological meaning of protein binding of HNE was studied for specific proteins, e.g., for HNE binding to LDL (39), glyceraldehyde-3-phosphate dehydrogenase (40), glucose-6-phosphate dehydrogenase (41), and Na^+-K^+-ATPase (42). The chemical mechanisms of alkenale and hydroxyalkenale binding to proteins have been studied intensively by the group of Uchida and Stadtman and their colleagues (40, 41, 43–48). The findings of those authors may have important implications for the accumulation of proteins modified by aldehydes in vivo (47), a process that is believed to be involved in age- and disease-related impairment of cellular function (49, 50). Therefore, the primary importance of HNE-degrading pathways in hepatocytes as one important part of the antioxidative defense system, which functions also at physiological and pathophysiological HNE levels, seems to be the protection of proteins from modification by aldehydic lipid peroxidation products.

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