Identification of metabolic pathways of the lipid peroxidation product 4-hydroxynonenal in in situ perfused rat kidney

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Abstract The metabolism of the cytotoxic lipid peroxidation product 4-hydroxynonenal was studied in perfused rat kidney. We investigated the total capacity of the rat kidney to metabolize 4-hydroxynonenal (HNE) and quantified the metabolites in the venous effluents as well as in the excreted urine. A rapid utilization of HNE was demonstrated, due to its immediate reactions with cellular compounds and its metabolism. During the first 3 min more than 80% of the infused HNE was metabolized in the perfused kidney. Glutathione-HNE conjugate (GSH-HNE: 35%), the corresponding alcohol 1,4-dihydroxynonene (1,4-DHN: 12%), HNE-mercapturic acid conjugate (HNE-MA: 4%), 4-hydroxynonenoic acid (HNA: 7%), tricarboxylic acid (TCA-cycle metabolites), and water (32%) were identified as primary and secondary metabolic products. We postulated that the total capacity of rat kidney to metabolize 4-hydroxynonenal with about 160–190 nmol/g wet wt/min. (initial influent concentration was 100 nmol/ml HNE) and other aldehydic products of lipid peroxidation is in the same range as that in other organs, e.g., intestine with 22 nmol/g wet wt/min (initial 70 nmol/ml HNE) (Siems et al. 1995. *Life Sci.* **57:** 785–789) and heart with about 50 nmol/g wet wt/min (initial 10 nmol/ml HNE) (Grune et al. 1994. *Cell Biochem. Funct.* **12:** 143–147). Compared to other organs, liver and kidney seemed to be the most important organs for the elimination of the final products of metabolism. The importance of the kidney in the formation of HNE-mercapturic acid conjugate was demonstrated (Alary et al. 1995. *Chem. Res Toxicol.* **8:** 34–39). The selective excretion of this final metabolite of aldehyde metabolism may be of central importance in the detoxification of a number of lipid peroxidation products.—**Grune, T., W. G. Siems, and T. Petras.** Identification of metabolic pathways of the lipid peroxidation product 4-hydroxynonenal in in situ perfused rat kidney. *J. Lipid Res.* 1997. **38:** 1660–1665.

Supplementary key words glutathione • HNE-mercapturic acid • HNE metabolism

4-Hydroxy-2-nonenal (HNE) has been proposed to be one of the major diffusible toxic products of lipid peroxidation (1). At low subtoxic concentrations it may have a physiological role in the regulation of cell division (2). Experiments with human tubular kidney cells demonstrated that pathophysiological values are characterized by the dynamics of formation and degradation of the lipid-peroxidation product (3). Therefore the formation rates of HNE are much higher than the measured HNE concentrations. However, animals may have adequate defense systems to detoxify aldehydic products of lipid peroxidation, such as HNE (4). Experiments with excessive application of HNE (10–1000 mg/kg body wt.) can cause acute and extensive renal toxicity and lethal renal damage before severe hepatic damage occurs (5). Experiments with cell suspensions and lower HNE concentrations demonstrated a rapid degradation of the aldehyde in hepatocytes (6) and enterocytes (7), and a lower utilization rate in renal tubular cells (3). Esterbauer, Zollner, and Lang (8) postulated that the 4-hydroxynonenal-metabolizing activity in rat kidney is 10-fold lower than in rat liver. One of the significant qualitative specialities of renal cells, in comparison to parenchymal cells from other organs in the metabolic pathways of HNE, is the formation of mercapturic acid conjugate as a secondary product of the metabolism of HNE, which is effectively excreted via kidneys. The formation of HNE-mercapturic acid conjugate in perfused rat kidney was demonstrated previously (9). The main enzymes involved in the metabolism of HNE are glutathione transferases, aldehyde dehydrogenase, and alcohol dehydrogenase (10). We suggest that because of the

Abbreviations: HNE, 4-hydroxy-2,3-*trans*-nonenal (4-hydroxynonenal); 1,4-DHN, 1,4-dihydroxynonene; HNA, 4-hydroxynonenoic acid; HNE-GSH, 4-hydroxynonenal-glutathione conjugate; TCA, tricarboxylic acid; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography.

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different activities of these enzymes in rat kidney (11, 12), the amounts of the metabolites of HNE in the rat kidney differ from the amounts formed in rat hepatocytes (13). The aims of this study were to identify and quantify metabolites of HNE in kidney, which is one of the major organs of detoxification of exogenous as well as endogeneous substances.

MATERIALS AND METHODS

Materials

All chemicals were obtained from Merck, Darmstadt, Germany, including the TLC plates (silica gel 60, 0.2 mm thickness). 4-Hydroxynonenal (HNE) and radioactively labeled 4-hydroxynonenal ([2-^{3}H]HNE; 68.2 mCi/mmol) were obtained from Prof. Esterbauer, Graz, Austria. The detection of HNE was performed according to Esterbauer and Weger (14) and Lang, Celotto, and Esterbauer (15). For tracer-kinetic investigations we used a solution of mixed radioactive and non-radioactive HNE and diluted the aldehyde up to a concentration of 100 nmol/ml by addition of Krebs-Henseleit buffer.

Organ preparation and perfusion

Animals used were male Wistar rats, weighing 240 ± 50 g. They were anesthetized with diethyl ether. The abdominal cavity was opened and the left kidney, the abdominal aorta, and the left ureter were prepared for the experiments. Catheters were placed into the left renal artery to perfuse the kidney. The catheter in the left renal vein collected the perfused effluent; the third catheter in the left ureter was used for measuring the urine outflow of the kidney.

The kidney was first perfused with a Krebs-Henseleit buffer supplemented with 5 mM glucose, aerated with 95% O_2/5% CO_2 at 37°C for several minutes. Then the kidney was continuously perfused intra-arterially using a renal blood flow (RBF) of 3.5 ± 0.3 ml/min. Urine production was about 25 µl/min without pathological excretion of glucose into urine (Test-Diabur 5000, Boehringer Mannheim). As the second solution we used a Krebs-Henseleit buffer with a total concentration of 100 µM HNE and 5 mM glucose. In the case of tracer-kinetic experiments, we used radioactive labeled HNE with a specific radioactivity of 40 µCi/mmol. During the first 5 min of the experiment the effluent was collected every 30 sec from the left renal vein. The urinary volume was 19.7 ± 2.8 µl/min. The wet weight of the kidney was 1.68 ± 0.17 g.

Identification of HNE and its metabolites

An equal volume of ice-cold acetonitrile–acetic acid 96:4 (v/v) was immediately added to the perfusate to stop further reactions. After centrifugation, aliquots of the supernatant were eluted on TLC-plates. To identify the HNE metabolites we used the method of Grune et al. (7). The elution with hexane–diethylether 3:7 (v/v) allowed the separation of 4-hydroxynonenal, 4-hydroxynonenoic acid, and 1,4-dihydroxynonene. The elution with butanol–acetic acid–water 4:1:1 (v/v/v) allowed the determination of GSH-adducts of 4-hydroxynonenal and of TCA-cycle products. A single radioactive TLC spot coeluted with some known standards of TCA-cycle products (succinate, malate, citrate) and therefore it was suggested that at least some of the radioactivity in this spot was formed due to the oxidation of the 4-hydroxynonenoic acid via the β-oxidation and TCA-cycle. The separations on the TLC-plates were quantified by using an automatic TLC-linear analyzer (Berthold, Wildbach, Germany). In parallel, the acetonitrile–acetic acid extracts were used for the determination of 4-hydroxynonenal and 4-hydroxynonenoic acid by means of HPLC, basing on the molar absorption rate ε_{223nm} = 13750 cm^2/mol. The HPLC equipment consists of an M510 pump system (Waters, Milford, MA), a tunable absorbance detector (Waters 486, Milford, MA), a Rheodyne-injector (Rheodyne, Cotati, CA) and an LCI-100 integrator (Shimadzu, Tokyo, Japan). The column used was an Ultrasphere ODS 5 µm; 250 mm × 4.6 mm i.d. The eluent was acetonitrile–water 4.6 (v/v), 1 ml/min, and the detection wavelength was 223 nm.

Determination of glutathione

The analysis of GSH was described by Beutler, Duron, and Kelly (16). The concentrations of GSH were measured after reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) photometrically at 412 nm after 30 min.

RESULTS

Experiments for the measurement of 4-hydroxynonenal and its metabolites were carried out with continuously perfused rat kidneys by infusion of an 100 µM HNE solution into the left renal artery. During the first 10 min we measured the HNE utilization in perfused rat kidney as the difference between arterial influent concentration (100 µM) and venous effluent on the basis of known flow rates of perfusate and urine (**Fig. 1**), We observed that during the first 2 min about 90 nmol/g wet wt per min and after 10 min about 80 nmol/g wet wt per min of the infused HNE were metabolized in the kidney. Most (99.7%) of the non-metabolized HNE was detected in the venous effluent; 0.3% was detected in the urine. After 5 min of continuous perfusion, the consumption rate of HNE reached a steady state of nearly 160 nmol/g wet wt per min.

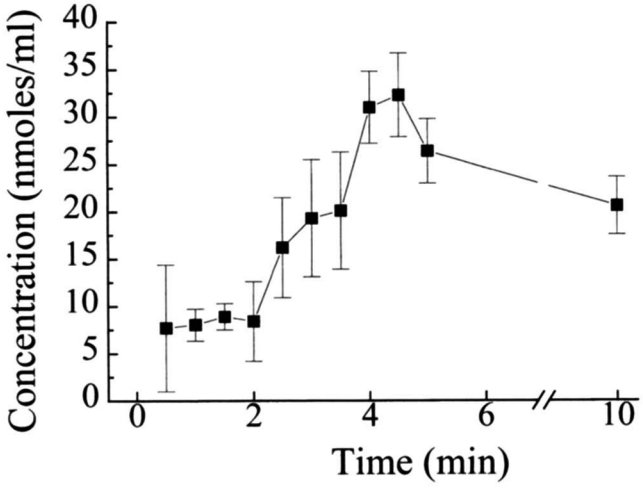

Fig. 1. HNE concentration in venous effluent of perfused rat kidney. HNE concentration of arterial influent was 100 μM. Mean wet weight of rat kidney was 1.68 ± 0.17 g. Perfusion flow 2.1 ± 0.3 ml/g wet wt per min. The influent consists of Krebs-Henseleit buffer; pH 7.4, temp. 37°C (Mean ± SD; n = 9).

HNE is metabolized to 4-hydroxynonenoic acid (HNA), 1.4-dihydroxynonene (1.4-DHN), GSH-HNE conjugate, mercapturic acid conjugate of HNE (MA), and products of the tricarboxylic acid cycle (TCA-cycle). **Figure 2** shows the measured total concentrations of these metabolites in the venous effluent and the urine after 5 min of perfusion. At this time the total concentration of HNE in the effluent was 26 nmol/ml (the total added HNE concentration was 100 nmol/ml). All metabolites of HNE were also found in the urine. 4-Hydroxynonenoic acid, the HNE-mercapturic acid conjugate, and 1.4-dihydroxynonene had a higher concentration in urine than in the venous effluent. The highest concentration was found for 1,4-DHN, about 53

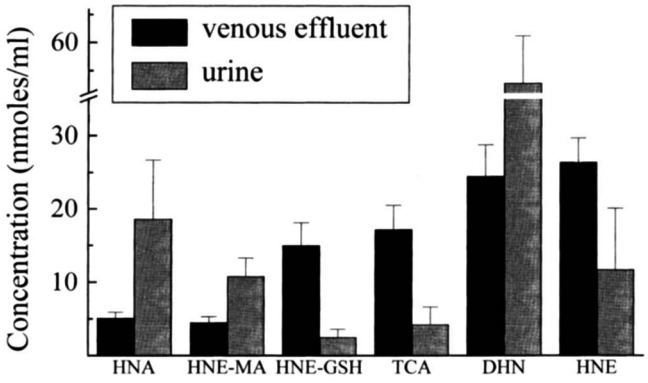

Fig. 2. Concentrations of the products of HNE metabolism and of HNE in urine and venous effluent. HNE-MA, HNE-mercapturic acid; TCA, products of tricarboxylic acid cycle. Measured concentrations after 5 min of perfusion of rat kidney. Same conditions as described in Fig. 1. (Mean ± SD; n = 5).

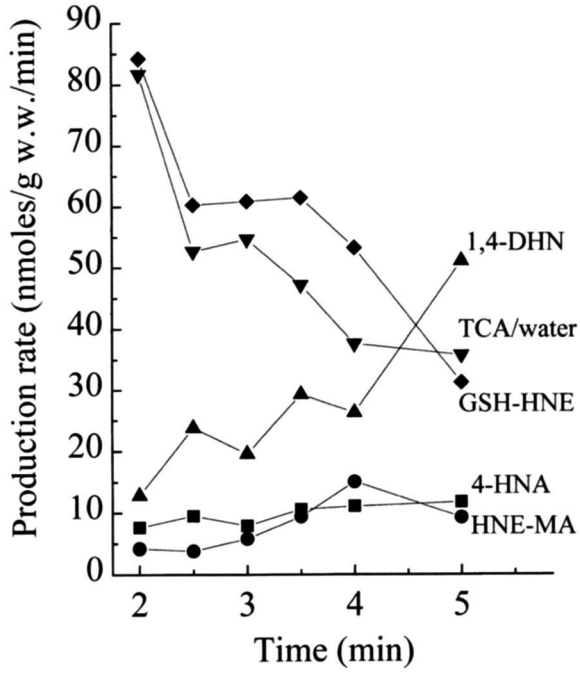

Fig. 3. Production rates of the metabolites of HNE. 4-Hydroxynonenoic acid (■) and mercapturic acid (●), 1,4 dihydroxynonene (▲), GSH-HNE (◆) products of TCA-cycle (▼). Conditions as described in Fig. 1. (Mean ± SD; n = 5).

nmol/ml in urine and 24 nmol/ml in venous effluent. The GSH-HNE conjugate and the products of the TCA-cycle never reached concentrations above 5 nmol/ml in urine. In the venous effluent their concentrations were about 15–17 nmol/ml; this was 4- to 6-fold higher than in urine.

Figure 2 shows the concentration gradients of the metabolites and their distribution (as percent) found in urine and venous effluent. The highest concentration gradient of the kidney was found for HNA. The gradient for the mercapturic acid was comparably high. The gradients of the products of TCA-cycle and the GSH-HNE adduct were below 1. After 5 min of perfusion, about 5% of the produced metabolites were detected in urine, about 95% was returned to the circulation. About 0.1% of the total formed GSH-HNE and the products of the TCA-cycle were found in urine.

Figure 3 shows the total formation rates of the metabolites of HNE from the second minute until the fifth minute of perfusion (measured in the venous effluent and the urine). After 2 min the major production rates were found for the formation of the GSH-HNE conjugate (about 85 nmol/g wet wt per min) and the formation of the products of the TCA-cycle (about 80 nmol/g wet wt per min). Five min after perfusion the formation of 1,4-DHN reached the highest formation level of about 50 nmol/g wet wt per min., while GSH-HNE and

the products of the TCA-cycle showed a rapid decrease to a level of about 30 nmol/g wet wt per min. Only HNE-mercapturic acid and 4-hydroxynonenoic acid showed a small increase from about 5 nmol/g wet wt per min up to about 8–10 nmoles/g wet wt per min. The concentration of reduced glutathione (GSH) in the kidney is important for the rate of formation of the HNE-GSH–conjugate 1:1 and its metabolic product HNE-mercapturic acid. Figure 3 shows an increase of the production rate of HNE-mercapturic acid during the first minutes. After 4 min, the production rate stabilized at about 8 nmol/g wet wt per min. GSH-HNE formation showed an decrease from 84 nmol/g wet wt per min (in the second minute) to 31 nmol/g wet wt per min (in the fifth minute). The initial value of the GSH level in the rat kidney was about 1.26 ± 0.23 μmol/g wet wt. After 5 min, GSH concentration was about 0.96 ± 0.10 μmol/g wet wt. Therefore, 24% of the initial GSH was used for further reactions, such as formation of the GSH-HNE adduct and HNE-mercapturic acid. Less than 2% of the infused radioactivity was found in the kidney itself.

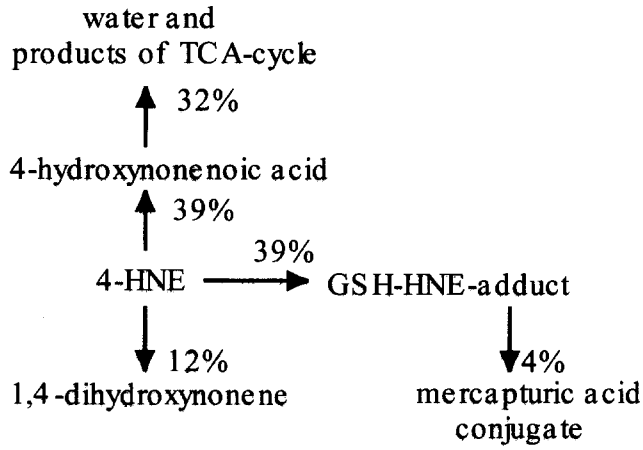

Fig. 4. Main pathways of HNE metabolism in perfused rat kidney after 3 min of perfusion. Formation of metabolites is shown as percentage of the total consumed HNE. Glutathione-HNE conjugate (GSH-HNE: 35%), the corresponding alcohol 1,4-dihydroxynonene (1,4-DHN: 12%), HNE-mercapturic acid conjugate (HNE-MA: 4%), 4-hydroxynonenoic acid (HNA: 7%), tricarboxylic acid (TCA-cycle metabolites), and water (32%). About 10% of the total measured radioactivity were unidentified metabolites of HNE. Conditions as described in Fig. 1.

DISCUSSION

HNE and its metabolism

The processes by which cells and organs detoxify the secondary product of lipid peroxidation 4-hydroxynonenal (HNE) are of considerable interest. Therefore, we investigated HNE breakdown and formation of its metabolites in perfused rat kidney. Figure 1 shows that under normoxic conditions rat kidney is able to degrade the aldehyde 4-hydroxynonenal. During the first 2 min about 10% of the total added HNE was found in the venous effluent. We can calculate that rat kidney is able to utilize about 160–190 nmol/g wet wt per min. This is in the same order as the consumption rates measured in perfused rat hearts with about 50 nmol/g wet wt per min and a HNE influent concentration of 10μM (17) or in perfused small intestine with 22 nmol/g wet wt per min with initial 70 nmol/g wet wt (18). After 10 min the HNE concentration in the effluent reached a steady state concentration of about 20 nmol/ml.

It was demonstrated that the primary products (4-hydroxynonenoic acid, 1,4-dihydroxynonene, and GSH-HNE) are formed in the perfused rat kidney. GSH-DHN conjugate, water, and CO_2 were identified as secondary products of HNE metabolism in rat hepatocytes, enterocytes, and tumor cells (6, 13, 19). Water and CO_2 are generated by the β-oxidation and the TCA-cycle of 4-hydroxynonenoic acid. The water formation from the acid could be inhibited by 4-pentenoic acid as inhibitor of β-oxidation of fatty acids, particularly an inhibitor of acyl-CoA-dehydrogenase (13). The HNE-metabolism pathways are in accordance with those found by Siems, Zollner, and Esterbauer (20) and Grune et al. (7) in other cell types. **Figure 4** shows the main pathways of HNE metabolism in perfused rat kidney. It also describes the produced metabolites as a percent of the total consumed HNE after 3 min of perfusion. About 10% of the total measured radioactivity was found in unidentified metabolites of HNE. It is possible, that a part of this was bound to proteins and another part was finally degraded to water and CO_2. Due to the minimal amount of radioactivity in the kidney after 5 min (less then 2% of the infused radioactivity) one can calculate that in the kidney only a small amount of HNE binds to proteins and accumulates. Therefore, the defense of the kidney to protect the cellular proteins is very effective and in same range as shown for other cells and organs (7, 20).

GSH and GSH conjugated products in kidney

Via Michael addition, 4-hydroxynonenal reacts with glutathione producing a glutathione-4-hydroxynonenal conjugate molecule (8, 21, 22). From a quantitative point of view, the main product of the metabolized HNE in the perfused rat kidney after 3 min is the HNE-GSH conjugate (35%) and 4-hydroxynonenoic acid and its metabolites (39%, see Fig. 4). Experiments with hepatocyte suspensions (13, 23) demonstrated that after

3 min HNE-GSH and HNA were the main products of HNE as well.

The formation of the GSH-HNE adduct is a glutathione-transferase-catalyzed reaction. The glutathione transferases accelerate the spontaneous chemical adduct formation by a factor of 300–600 more than the spontaneous chemical reaction (24). Glutathione-S-transferase activity in the kidney is much lower than in the liver (11).

We examined the formation rates of the GSH-HNE adduct and its secondary product HNE-mercapturic acid conjugate (Fig. 4). The initial GSH value in rat kidney was about 1260 nmol/g wet wt. After 5 min the total production rate of both metabolites was maximally 360 nmol/g wet wt per 5 min (Fig. 3). Thus the maximal GSH utilization is estimated to be 28% for these metabolites. The total GSH pool of rat kidney is not exhausted. Schnellmann and Mandel (25) and Schnellman, Gilchrist, and Mandel (26) showed that the mitochondrial GSH pool in rabbit renal proximal tubules represents 15–72% of the total cellular GSH. Ullrich et al. (27) demonstrated evidence for the fact that HNE is not able to penetrate the inner mitochondrial membrane. Thus, the enzymatic conversion of HNE with GSH to the GSH-HNE adduct in isolated kidney cortex mitochondria seems to be of minor importance, even in the presence of high intramitochondrial GSH levels.

HNE-mercapturic acid seems to be a stable product of HNE metabolism. Experiments with injection of tritium-labeled 4-hydroxyhexenal (HHE) (28) and tritium-labeled 4-hydroxynonenal (HNE) (29) into the vein of rats have shown that a part of the radioactivity was excreted in the urine as mercapturic acid conjugate. From these experiments and the study presented here it can be concluded that the excretion of mercapturic acid conjugates as well as the excretion of 1,4-DHN is the main method of HNE-product disposal from the organism.

Formation of 1,4-dihydroxynonene

The reductive metabolism of HNE is characterized by the formation of 1,4-DHN and the oxidative metabolism is characterized by the formation of HNA, products of TCA-cycle, and water. During the first minutes the metabolism is characterized as a more oxidative process, after 5 min of perfusion the production rate of 1,4-dihydroxynonene increased up to a level of about 50 nmol/g wet wt per min, whereas the formation rates of the products of TCA-cycle and water formation decreased rapidly. Thus the metabolism of HNE after 5 min of perfusion is characterized by more reductive processes.

Aldehyde reductases are able to metabolize aldehydes to their corresponding alcohols (30). Previously it was demonstrated thataldose reductases, present in human kidney (31), play an important role in the reductive metabolism of endogenous aldehydes such as 4-hydroxynonenal (32). Both enzymes exhibit higher affinity for NADPH than NADP. Sawada et al. (12) showed that the activity of the aldehyde reductase in rat kidney is 2-fold higher than in rat liver. Thus the increase of the alcohol formation during perfusion is probably due to the high activity of the enzymes, primed for reductive metabolism.

We found that the metabolism of HNE in rat kidney is, in general, similar to the metabolism in other organs. The ability of the perfused kidney to metabolize HNE is on a level comparable to that in other organs such as heart and small intestine. The mean qualitative difference is the ability of the rat kidney to form the HNE-mercapturic acid-conjugate, a stable product of the HNE metabolism that is effectively excreted. Another important excretion product is 1,4-DHN. From a quantitative point of view, the formation of 1,4-dihydroxynonene is in a higher range in kidney than in other organs or cells. Specific carriers in the tubular cell can accelerate the active transport of the secondary products of HNE metabolism differently, which may be responsible for the different gradients in the venous effluent and urine.

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