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Identification of metabolic pathways of the lipid peroxidation product 4-hydroxynonenal by mitochondria isolated from rat kidney cortex

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Abstract The cytosolic lipid peroxidation product 4-hydroxynonenal (HNE) is rapidly metabolized in mitochondria isolated from rat kidney cortex. About 80% of HNE was degraded within 3 min of incubation. Main products of HNE which were identified in mitochondria were the hydroxynonenoic acid, the 1,4-dihydroxynonene and the glutathione-HNE-conjugate. Furthermore, formation of metabolites of the tricarboxylic acid cycle from HNE is suggested. The quantitative share of HNE binding to proteins was high with about 8% of total HNE consumption after 3 min of incubation. Therefore, rapid degradation of HNE by mitochondria might be involved in an intracellular antioxidative defense system.

Key words: Mitochondria; 4-Hydroxynonenal; Aldehyde metabolism; 4-Hydroxynonenoic acid; 1,4-Dihydroxynonene; HNE-glutathione

1. Introduction

4-Hydroxynonenal is a major product formed by lipid peroxidation from omega-6-polyunsaturated fatty acids as linoleic acid and arachidonic acid (for review see [1]). This aldehyde is cytotoxic at high concentrations (in the range of 100 μM), disturbs cell proliferation at low concentrations and exhibits genotoxic effects [2,3]. Furthermore in the submicromolar range 4-hydroxynonenal is chemotactic [4] and stimulates phospholipase C [5]. 4-Hydroxynonenal is rapidly metabolized in eucariotic cells [6–9]. We studied the metabolism of 4-hydroxynonenal in isolated mitochondria by tracerkinetic experiments to investigate the 4-hydroxynonenal detoxication and the formation of intermediates from this lipid peroxidation product.

2. Materials and methods

4-Hydroxynonenal, radioactively labeled 4-hydroxynonenal ([2-^{3}H]-HNE; 68.2 mCi/mmol) and standards for TLC were obtained from Prof. H. Esterbauer, Graz. HNE was prepared from the corresponding diacetal. ADP and succinate were obtained from Boehringer Mannheim, Germany. TLC plates and other chemicals and biochemicals of analytical grade were purchased from Merck, Darmstadt, Germany.

Male Wistar rats weighing 200–250 g were used. Mitochondria were prepared as described by Jung et al. [10]. The mitochondria were finally resuspended in a solution of 210 mM mannitol, 70 mM sucrose, 1 mM Tris-HCl and 1 mM EDTA (pH 7.4). Protein was determined using the Biuret method [10] after precipitation of protein with trichloroacetic acid. The incubation was carried out in a medium of 210 mM sucrose, 10 mM KCl, 0.5 mM KH_2PO_4, 0.5 mM EDTA, 60 mM Tris-HCl (pH 7.4), 10 mM succinate and 1.5 mM ADP. The protein concentration in the incubation medium was about 1.2 mg/ml. After preincubation of mitochondria at 25°C for 1 min the reaction was started by adding radiolabeled HNE to give a final concentration of 100 μM (specific radioactivity for incubation after mixing radioactive labeled HNE and unlabeled HNE was 1.92 mCi/mmol corresponding to 4272 dpm/nmol HNE). The total incubation time was 10 min and the incubation temperature was 25°C. After different incubation periods aliquots of the suspensions were taken. The HNE metabolizing reactions were stopped by the addition of 0.5 ml mitochondria suspension to an equal volume of acetonitrile/acetic acid mixture (96:4; v/v). After centrifugation two aliquots of the supernatant were eluted on TLC plates. The TLC-separation and identification of HNE metabolites was carried out as described previously by Grune et al. [11]. 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. For quantification of HNE utilization rates the radiolabeled compounds were scanned by an automatic TLC linear analyzer (Berthold, Wildbach, Germany). The acetonitrile/acetic acid extracts were used for the determination of 4-hydroxynonenal and 4-hydroxynonenoic acid by an HPLC-method, too. The disappearance of 1 μM HNE was measured only by the HPLC-method (determination of remaining HNE during the whole experiment). The conditions were: (150 × 4.6 mm) C_{18} column, acetonitrile/water (4:6; v/v), 1 ml/min, 223 nm detection wavelength. The protein precipitate was washed with physiological saline solution, then dissolved in 100 μl Protosol-Tissue and Gel Solubilizer (New England, Nuclear, Boston, USA) and added to 10 ml of toluene scintillation fluid (0.4% 2-(4′-butyl phenyl)-5-(4″-biphenyl)-1,3,4oxadiazol in toluene) and measured by means of a beta-counter (Berthold, Wildbad, Germany).

In additional fractionation experiments after 10 min of incubation of mitochondrial suspension in presence of radioactive labeled HNE the reaction was stopped by addition of an ice cold digitonin solution (Merck, Darmstadt; 1.2 mg/10 mg mitochondrial protein) and the suspension was brought rapidly to the temperature of 4°C. The proteins of the outer membrane, of the inner membrane, of the intermembrane space and of the matrix were isolated according to the method described in [12].

3. Results

First we measured the rate of catabolism of HNE within 10 min of incubation after treatment with 100 μM HNE. From the decrease of the HNE concentration in the first 30 s (56.1 nmol/mg protein) one can calculate the maximal HNE detoxification

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

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rate of 112.2 nmol/mg protein/min. The degradation of HNE approaches a steady state after 3 min. This is more clearly observed by investigating the HNE disappearance rate after adding 1 μM HNE (Fig. 1). Fig. 1 gives the degradation of 1 μM HNE. After 2 min of incubation, a steady state for the remaining HNE level has been adjusted to about 0.1 μM. HNE is metabolised by renal mitochondria to 4-hydroxynonenoic acid, 1,4-dihydroxynonene and the GSH-HNE-adduct (Table 1). All of the three metabolites increased very rapidly within the first minutes. The HNE-GSH-adduct and the 4-hydroxynonenoic acid reached the maximum value after about 2 min, and thereafter these metabolites slowly decreased. Within the incubation time the concentration of 4-hydroxynonenoic acid increased. The sum of the three primary metabolites DHN+HNA+GSH-HNE is 25.2% of the total initial HNE concentration after 30 s and 22.6% after 3 min of incubation. After 3 min about 10% of the radioactivity used was found to be in the HNE-GSH pool and 8% of the radioactivity was bound to proteins. Table 2 includes the percentage of radioactivity which was measured in the different mitochondrial fractions and the values of nmol HNE bound per mg protein in these fractions. The protein bound radioactive HNE (about 6% of total radioactivity after 10 min of incubation) is found predominantly bound to the proteins of the intermembrane space (5.25% of total radioactivity). Lesser amounts are found bound to the proteins of the inner membrane (0.28 %) and to the proteins of the matrix (0.18%). The nmol HNE per mg protein was found to be very high in the intermembrane space and almost similar for outer and inner membrane and matrix proteins.

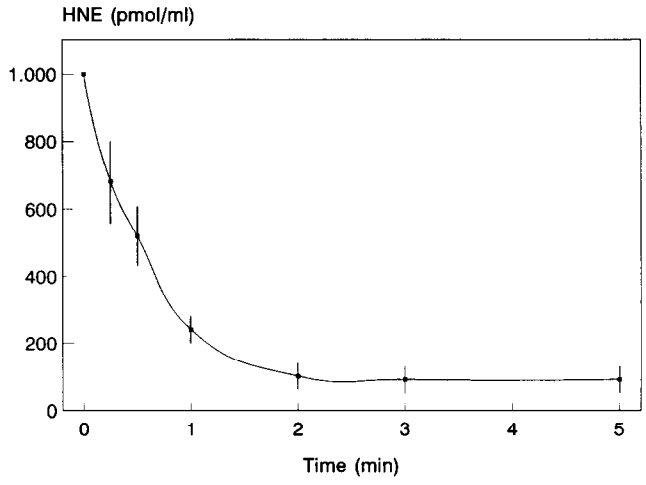

Fig. 1. HNE degradation in mitochondria from rat kidney cortex after addition of 1 μmol/l HNE. The HNE concentration was determined by the HPLC-method, as described in section 2. Values are given as mean ± S.D.

4. Discussion

It could be demonstrated that the primary products (4-hydroxynonenoic acid, 1,4-dihydroxynonene and HNE-GSH) are formed in mitochondria of rat kidney cortex after addition of HNE to the mitochondria suspension in state 3 respiration. The formation of hydroxynonenoic acid was also measured by HPLC (data consistant with the radioactive method). As secondary products of HNE metabolism up to now GSH-DHN conjugate and water, which is generated through the beta-oxidation of 4-hydroxynonenoic acid could be identified in rat hepatocytes [7,13,14]. The water formation from the acid could be inhibited by 4-pentenoic acid as inhibitor of beta-oxidation of fatty acids, particularly an inhibitor of acyl CoA dehydrogenase [13]. Our data suggest that the primary formed 1,4-dihydroxynonene was metabolized by oxidation to 4-hydroxynonenoic acid, which can be subsequently degraded by betaoxidation and water formation. It can be assumed that the mitochondrial low-specific low K_m-aldehyde dehydrogenase (EC 1.2.1.3) oxidizes HNE to 4-hydroxynonenoic acid. 4-Hydroxynonenal was shown to be a substrate for oxidative metabolism by mitochondrial aldehyde dehydrogenase isoenzymes [15].

HNE reacts with sulfhydryl and amino groups of proteins. The share of the protein-HNE adducts formed was about 10% and decreased slowly to about 6% after 10 min. The results of fractionation experiments argue in support of the statement that the inner membrane acts like a barrier for the HNE penetration into the matrix. The HNE protein binding shows high values in the intermembrane space. Nevertheless, the protein binding in the inner membrane and in the matrix is high enough to suggest that there exists a penetration of HNE via the inner membrane into the matrix. The protein binding in mitochondria is expected to be of great functional importance for mitochondrial damage by HNE in situations of increased HNE formation. In Ehrlich mouse ascites cells the fraction of HNE binding to proteins was high in the proliferating phase (up to 8%) and low in the resting phase of tumor growth (about 2% [14]. In hepatocytes the protein-HNE adducts amounts to about 3% of the total HNE. That was significantly lower than in our experiments with mitochondria.

4-Hydroxynonenal reacts via Michael addition of a pH-dependent manner with glutathione producing a glutathione-4-hydroxynonenal conjugate molecule [16]. The depletion of GSH by 4-hydroxynonenal in a variety of experimental cellular

Table 1
Concentration of HNE intermediates in rat mitochondrial suspensions from kidney cortex compared with those in hepatocyte suspensions [6,13] and in rat enterocyte suspensions [11,13] after the addition of 100 μM HNE

Metabolite		Mitochondria		Hepatocytes	Enterocytes
Time	0.5 min	3 min	10 min	3 min	3 min
1,4-Dihydroxynonene	14.6 ± 2.6	9.3 ± 1.8	7.7 ± 0.9	8.1 ± 2.1	5.4 ± 0.6
HNE-GSH-adduct	8.4 ± 1.2	9.8 ± 1.2	7.4 ± 0.8	27.5 ± 2.5	11.0 ± 0.5
4-Hydroxynonenoic acid	2.2 ± 0.7	5.9 ± 0.7	6.2 ± 0.8	25.3 ± 5.6	4.2 ± 0.6
HNE-modified proteins	10.0 ± 1.5	8.4 ± 1.4	5.8 ± 0.9	3.0 ± 0.6	1.3 ± 0.2

Incubations with hepatocytes and enterocytes were carried out at pH 7.4, 37°C and with 10^6 cells/ml suspension, with mitochondria at pH 7.4, 25°C and with 1.2 mg protein/ml suspension. Values as nmol/ml suspension which is equal to % of the initial HNE concentration (mean ± S.D.).

Table 2
Distribution of protein bound radioactivity within mitochondrial compartiments after 10 min of treatment of isolated kidney cortex mitochondria with 100 μM HNE

	% of total radioactivity	% of protein bound radioactivity	nmol HNE/mg protein
Outer membrane	0.02 ± 0.002	0.35	0.53
Intermembrane space	5.25 ± 0.55	91.67	69.11
Inner membrane	0.28 ± 0.04	4.90	0.27
Mitochondrial matrix	0.18 ± 0.02	3.08	0.39

Fragmentation of mitochondria was carried out according to Schnaitman [12]. Values represent the mean ± S.D. ($n = 4$).

systems has been reported by many authors. Table 1 shows the concentration of HNE intermediates in hepatocyte [13], enterocyte [11] and mitochondrial suspensions. From a quantitative point of view, the main products of HNE in hepatocyte suspension were the HNE-GSH conjugate and the 4-hydroxynonenoic acid. In mitochondrial suspension the enzymatic conversion of 4-hydroxynonenal with GSH to the GSH-HNE adduct seems to be of minor importance. Until now no mitochondrial-specific form of glutathione-*S*-transferase was described. Obviously the relatively low formation rate of HNE-GSH conjugate is due only to the chemical reaction between HNE and GSH even at very high intramitochondrial GSH levels. The low concentration of hydroxynonenoic acid suggests a high metabolic rate of this acid and low steady state concentration.

The HNE catabolism is an important component of the antioxidative defense system of cells because of the cytotoxic effects of the secondary product of lipid peroxidation. Our data suggest that mitochondrial HNE metabolism as part of the whole intracellular HNE degradation also is very important for the antioxidative protection of cells.

References

[1] Esterbauer, H., Schauer, R.J. and Zollner, H. (1991) Free Rad. Biol. Med. 11, 81–128.
[2] Poot, M., Verkerk, A., Koster, J.F., Esterbauer, H. and Jongkind, J.F. (1988) Mech. Ageing Dev. 43, 1–9.
[3] Eckl, P. and Esterbauer, H. (1989) Adv. Biosci. 76, 141–157.
[4] Curzio, M. (1988) Free Rad. Res. Commun. 5, 55–66.
[5] Esterbauer, H., Zollner, H. and Schauer, R.J. (1988) ISI Atlas Sci. Biochem. 1, 311–317.
[6] Siems, W.G., Zollner, H. and Esterbauer, H. (1990) Free Rad. Biol. Med. 9, 110.
[7] Esterbauer, H., Zollner, H. and Lang, J. (1985) Biochem. J. 228, 363–373.
[8] Ferro, M., Marinari, U.M., Poli, G., Dianzani, M.U., Fauler, G., Zollner, H. and Esterbauer, H. (1988) Cell Biochem. Func. 6, 245–250.
[9] Poot, M., Verkerk, A., Koster, J.F., Esterbauer, H. and Jongkind, J.F. (1987) Eur. J. Biochem. 162, 287–291.
[10] Jung, K. and Pergande, M. (1988) Biomed. Biochim. Acta 47, 455–460.
[11] Grune, T., Siems, W., Kowalewski, J., Zollner, H. and Esterbauer, H. (1991) Biochem. Int. 25, 963–971.
[12] Schnaitman, C. and Greenawalt, J.W. (1968) J. Cell Biol. 38, 158–175.
[13]
[14] Siems, W.G., Grune, T., Zollner, H. and Esterbauer, H. (1993) in: Free Radicals: From Basic Science to Medicine (Poli, G., Albano, E. and Dianzani, M.U., Eds.) pp. 89–101, Birkhaeuser, Basel, Switzerland.
[15] Siems, W.G., Grune, T., Beierl, B., Zollner, H. and Esterbauer, H. (1992) in: Free Radicals and Aging (Emerit, I. and Chance, B., Eds.) pp. 124–135, Birkhaeuser, Basel, Switzerland.
[16] Mitchell, D.Y. and Petersen, D.R. (1987) Toxicol. Appl. Pharmacol. 87, 403–410.
[17] Esterbauer, H., Zollner, H. and Scholz, N. (1975) N. Naturforsch. 30, 466–473.
[18] Hartley, D.P. and Petersen, D.R. (1993) Adv. Exp. Med. Biol. 32, 27–36.