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

Oliver Ullrich*a, Tilman Grunea, Wolfgang Henkeb, Herrmann Esterbauer*, Werner G. Siemsd*

*aClinics of Physical Therapy and Rehabilitation and bResearch Division of the Urological Clinic, Medical Faculty (Charite), Humboldt-University Berlin, Schumannstr. 20–21, D-10098 Berlin, Germany
bInstitute of Biochemistry, Karl-Franzens-University, Schubertstr. 1, A-8010 Graz, Austria
cHerz-Julus-Hospital for Rheumatology and Orthopaedics, Kurhausstr. 13–17, D-38655 Bad Harzburg, Germany

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 hydroxynoenoic acid, the 1,4-dihydroxynone 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-Dihydroxynonenone; HNE-glutathione

1. Introduction

4-Hydroxynonenal, a major product formed by lipid peroxidation from omega-6-polyunsaturated fatty acids as linolic 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 eucaryotic cells [6–9]. We studied the metabolism of 4-hydroxynonenal in isolated mitochondria by tracekinetic 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 (2-3H)-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, Tris-HCl and 1 mM EDTA (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 precubication 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 μCi/mmol corresponding to 472 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/aetic 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-hydroxy-4-hydroxynonenonoic acid and 1,4-dihydroxynonenone. 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, Wildbad, Germany). The acetonitrile/aetic acid extracts were used for the determination of 1-hydroxynonenal and 4-hydroxynonenonoic 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 x 4.6 mm) C18 column, acetonitrile/aetic acid (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'-hhiphenyl)-1,4oxadiazol 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...
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 metabolized by renal mitochondria to 4-hydroxynonenoic acid, 1,4-dihydroxynone 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-hydroxy-}

...added 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 metabolized by renal mitochondria to 4-hydroxynonenoic acid, 1,4-dihydroxynone 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 DNH+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.

4. Discussion

It could be demonstrated that the primary products (4-hydroxynonenoic acid, 1,4-dihydroxynone 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 consistent 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-dihydroxynone was metabolized by oxidation to 4-hydroxynonenoic acid, which can be subsequently degraded by beta-oxidation 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 sulphydryl 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 2  
Distribution of protein bound radioactivity within mitochondrial compartments after 10 min of treatment of isolated kidney cortex mitochondria with 100 μM HNE

<table>
<thead>
<tr>
<th>Compartment</th>
<th>% of total radioactivity</th>
<th>% of protein bound radioactivity</th>
<th>nmol HNE/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer membrane</td>
<td>0.02 ± 0.002</td>
<td>0.35</td>
<td>0.53</td>
</tr>
<tr>
<td>Intermembrane space</td>
<td>5.25 ± 0.55</td>
<td>91.67</td>
<td>69.11</td>
</tr>
<tr>
<td>Inner membrane</td>
<td>0.28 ± 0.04</td>
<td>4.90</td>
<td>0.27</td>
</tr>
<tr>
<td>Mitochondrial matrix</td>
<td>0.18 ± 0.02</td>
<td>3.08</td>
<td>0.39</td>
</tr>
</tbody>
</table>

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-hydroxynonenenoic acid. In mitochondrial suspension the enzymatic conversion of 4-hydroxynonenenal 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 hydroxynonenenoic 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