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Metabolism of 4-Hydroxynonenal, a Cytotoxic Lipid Peroxidation Product, in Thymocytes as an Effective Secondary Antioxidative Defense Mechanism[1]

Werner G. Siems,* Alexej M. Pimenov,† Hermann Esterbauer,‡ and Tilman Grune†,[2]

**Herzog-Julius Hospital for Rheumatology and Orthopaedics, Kurhausstraße 13-17, D-38655 Bad Harzburg, Germany; †Clinics of Physical Therapy and Rehabilitation, Medical Faculty (Charité), Humboldt University, Schumannstraße 20/21, D-10098 Berlin, Germany; and ‡Institute of Biochemistry, Karl-Franzens University, Schubertstraße 1, A-8010 Graz, Austria*

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The metabolism of the aldehydic lipid peroxidation product, 4-hydroxynonenal (HNE), was studied in suspensions of mouse thymocytes. Thymocytes are characterized by low lipid peroxidation in comparison with other cell types notwithstanding their high content of arachidonic acid. In our study a very high capacity of HNE metabolism in thymocytes was observed: 27.7 nmol/mg w.w./min. That is about the same HNE degradation rate as determined in liver cells or small intestinal enterocytes, which are the cells with the by far highest capacity for the degradation of HNE and other aldehydic lipid peroxidation products in comparison with other cell types. The primary and secondary HNE metabolites in thymocytes were identified and quantified after the addition of 100 μM HNE to thymocyte suspensions: the glutathione-HNE conjugate, the hydroxynonenoic acid, the 1,4-dihydroxynonene, water, and the glutathione-dihydroxynonene conjugate. Furthermore, the HNE binding to proteins was measured. The very rapid HNE degradation in thymocytes besides the high amounts of lipophilic chain-breaking antioxidants is postulated to be an important secondary antioxidative mechanism and the main factor for the low accumulation of lipid peroxidation products in these cells.

Key words: aldehydes, antioxidants, antioxidative defense, 4-hydroxynonenal (HNE), lipid peroxidation, mouse thymocytes.

Lipid peroxidation is always combined with the formation of reactive aldehydes (*1-4*). 4-Hydroxynonenal (HNE) is a major aldehyde produced *in vivo* during the peroxidation of omega-6-polyunsaturated fatty acids (18:2, 20:4) (*1*). Increased generation of HNE was observed during inflammatory processes (*5, 6*), postischemic reoxygenation (*7-11*), and the development of various diseases, such as atherosclerosis (*1*), perinatal hypoxia (*12*), infectious diseases (*13*), age-related macula densa-degeneration (*14*), diabetes (*15*), irradiation damage (*16*), *etc.* HNE exhibits high biological activity, and it also has a number of cytotoxic, *e.g.* hepatotoxic, mutagenic, and genotoxic, effects (*1, 2*). 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 hypothetical sequence: free radicals-lipid peroxidation-aldehyde formation-damage was proposed (*1, 2*). Furthermore, signal functions of HNE in proliferation were postulated (*1*), and it is now apparent that under certain conditions, low-level exposure of cells to free radicals, oxidants, and lipid peroxidation products triggers apoptosis rather than necrosis (*17*). Moreover, oxidants may be essential biochemical intermediates in the progress of many forms of apoptosis, which could be also of interest for cells of the thymus, an organ exhibiting massive cell death (*17-19*).

It is generally accepted that the metabolism of HNE and other aldehydic products of lipid peroxidation regulates the levels of these compounds in biological tissues, and therefore modulates the biological and regulatory effects of HNE and other aldehydes. The metabolism of HNE is a secondary antioxidative mechanism that decreases the binding of HNE to cysteine, lysine, and histidine residues of proteins. These interactions of HNE were investigated with different

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[2] To whom correspondence should be addressed. Phone: +49-30-2093-7514, Fax: +49-30-2093-7204, E-mail:grune@rz.charite.huberln.de

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

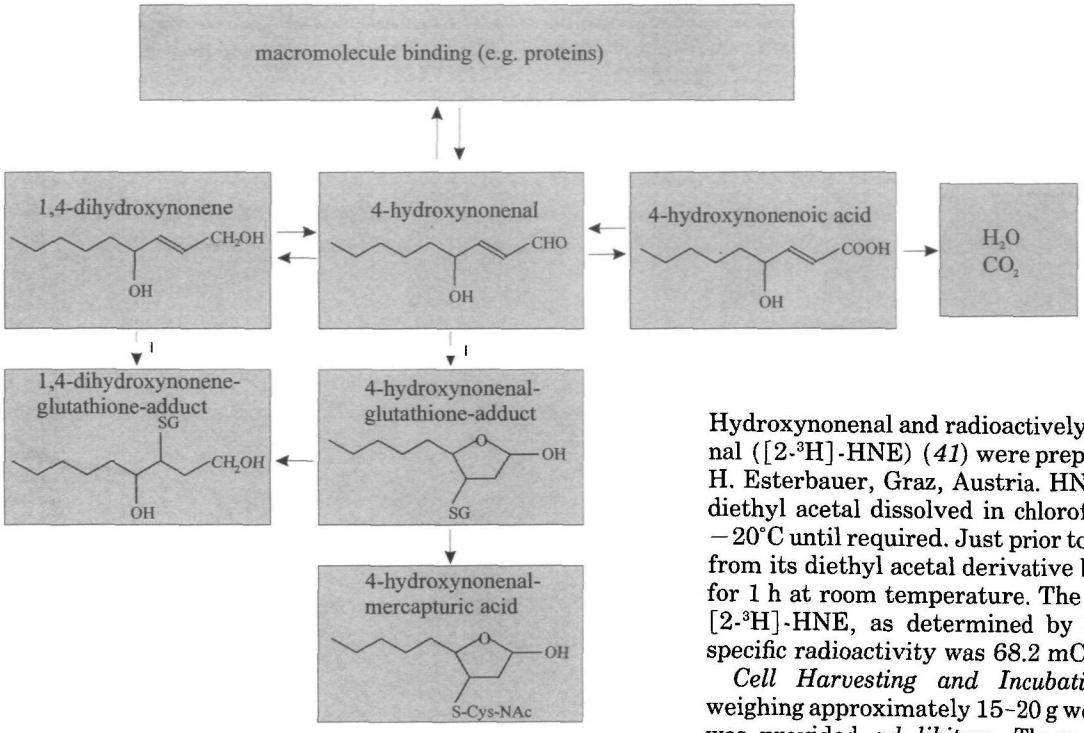

Fig. 1. Main pathways of HNE metabolism.

proteins (*20-26*). The metabolism of HNE was analyzed in various cell types, tissues, and organs (*27-32*). Figure 1 shows the established scheme of metabolic pathways. The highest capacity to degrade HNE was found in hepatocytes and enterocytes of the small intestine (*9, 33*). The rate of HNE degradation in the liver and small intestinal cells is by far higher than that in all other cell types investigated, such as kidney cells (*34*), tumor cells (*28*), skeletal muscle, and heart (*35, 36*). Nothing is known about HNE degradation capacity in thymocytes.

Previously, low susceptibility of the thymus and thymocytes to lipid peroxidation was postulated on the basis of the finding of low levels of lipid peroxidation products in these cells, notwithstanding the high content of arachidonic acid in comparison with in other cell types (*37*). The low level of lipid peroxidation products in thymocytes is in accordance with the high resistance of tumor cells to inducers of free radical oxidative stress, resulting in low steady-state concentrations of endogenous lipid peroxidation products in tumor cells (*4, 38-40*). The low accumulation of lipid peroxidation products in thymocytes was explained by their high amounts of lipophilic chain-breaking antioxidants (*37*). In this study the capacity of HNE degradation in mouse thymocytes was measured as a potential factor for the prevention of high levels of aldehydic lipid peroxidation products in thymocytes. Furthermore, the different pathways of HNE metabolism were measured.

MATERIALS AND METHODS

Materials—The standards for TLC were prepared as described previously (*27*). All other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO). TLC plates were obtained from Merck. 2-(4′-tertbutylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazol was obtained from Beckman Instruments (Fullerton, CA). 4-Hydroxynonenal and radioactively labeled 4-hydroxynonenal ([2-^{3}H]-HNE) (*41*) were prepared in the laboratory of H. Esterbauer, Graz, Austria. HNE was supplied as HNE diethyl acetal dissolved in chloroform, and was stored at −20°C until required. Just prior to use, HNE was prepared from its diethyl acetal derivative by 1 mM HCl hydrolysis for 1 h at room temperature. The radiochemical purity of [2-^{3}H]-HNE, as determined by HPLC, was 96.5%. Its specific radioactivity was 68.2 mCi/mmol.

Cell Harvesting and Incubation—Female ICR mice weighing approximately 15-20 g were used. Food and water was provided *ad libitum*. Thymocytes were isolated as described (*42*), and diluted to a final density of 28×10^6 cells/ml with Eagle-Tris medium, pH 7.4. The cell suspension was preincubated at 37°C for 5 min. After the preincubation, 100 μM HNE was added. The maximal incubation time was 30 min.

Characterization of the Thymocytes—The thymocyte "stem suspension" with 2.8×10^9 cells/ml and a cytocrit value of 63% was diluted 1:100 (v/v) with the Eagle-Tris buffer, pH 7.4. The mean cell volume was 225 μm^3, the mean thymocyte diameter was 7.5 μm. The viability of the thymocytes at the end of the preincubation was 92.1%, as determined by the trypan-blue exclusion test.

The cytocrit value is the volume content of the cells in the suspension as to the whole volume of the suspension. The cytocrit value is a similar value to the haematocrit value characterizing the volume content of red blood cells in the whole blood volume. How was the cytocrit value measured? Also—as the hematocrit—it was measured by means of centrifugation and reading of the cell column as a percentage of the suspension column. In the case of a very low cytocrit in a diluted cell suspension the cytocrit measurement is only correct if one measures the cytocrit of the stem solution, which is then divided by the dilution factor.

Analysis of HNE and HNE Degradation Products—At different times during the incubation aliquots 0.5 ml of the suspension were taken, and the protein was precipitated with an equal volume of acetonitrile:acetic acid (96:4, v/v). After centrifugation, two portions of the supernatant were eluted on TLC plates for the determination of HNE metabolites with hexane:diethylether (3:7, v/v) as the eluent, and for the determination of glutathione conjugates with butanol:acetic acid:water (4:1:1, v/v/v).

TLC allows, in the first case (about 1 h elution time), the separation of 4-hydroxynonenal, 4-hydroxynonenoic acid, and 1,4-dihydroxynonene, or in the second case (about 4 h separation), the determination of GSH-adducts of 4-hydroxynonenal and 1,4-dihydroxynonene. For quantification,

the TLC plates with labeled compounds were scanned by an automatic TLC linear analyzer (Berthold, Wildbach). HNE and 4-hydroxynonenoic acid were analyzed in parallel by the high performance liquid chromatography procedure (*27*).

The precipitate was washed with a physiological saline solution and then dissolved in 100 μl of Protosol-Tissue and Gel Solubilizer (New England Nuclear, Boston). Then it was added to 10 ml of a toluene scintillator [0.4% 2-(4′-tertiary butylphenyl)-5-(4″-biphenyl)-1,3,4-oxodiazol in toluene] and measured with a beta-scintillation counter. The difference between the radioactive count for the solution after acetonitrile precipitation and that on TLC analysis was attributed to water. Radioactively labeled water was quantitated by means of HPLC separation, too. The HPLC equipment and separation conditions were the same as in Ref. *27*.

RESULTS

HNE Degradation—In Fig. 2, the HNE degradation in suspensions of thymocytes that occurred within 30 min incubation is shown. 16 % of the HNE, corresponding to 16 nmol/28×10^6 cells, had already been degraded after the first 5 s of incubation at 37°C. From that value the capacity of HNE degradation in mouse thymocytes can be calculated: 27.7 nmol/mg w.w./min. After 1 min two-thirds of the HNE had already been degraded, and after 5 min almost 90%.

Products of HNE Metabolism and Their Time-Dependent Formation—In Fig. 3, the oxidative pathways of HNE metabolism are summarized. The overall oxidation of HNE amounted to one-fifth at 1 min, 40% at 5 min, 50% at 20 min, and about 60% of the total HNE degradation at 30 min incubation. At all time points, the HNE consumption *via* oxidative pathways exceeds the HNE consumption *via* reductive pathways, and also the rate of GSH conjugate formation.

Figure 4 shows the time-dependent formation of 1,4-dihydroxynonene (DHN) and GSH-DHN conjugate, *i.e.* the reductive metabolism of HNE in thymocytes. After 10 min incubation a slight decrease in the dihydroxynonene concentration in suspensions was observed. The reductive metabolism during the whole experiments amounted to about 15% of the total HNE degradation rate.

One pathway of HNE degradation is the formation of glutathione adducts of HNE or HNE products *via* glutathione transferases. The formation of these conjugates is demonstrated in Fig. 5. Additionally, the formation of conjugates of HNE with proteins is shown in this figure. The contribution of glutathione conjugate formation to the total HNE metabolism is 20 to 25% after 30 min incubation. The

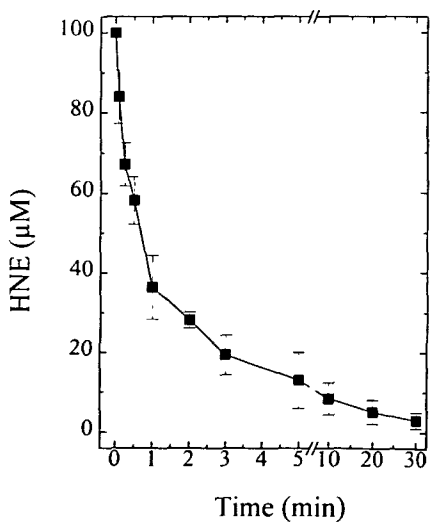

Fig. 2. **HNE degradation in mouse thymocytes.** The initial HNE concentration was 100 μM. The cell content was 28×10^6 cells/ml. For further experimental conditions see "MATERIALS AND METHODS." Values are given as nmol/ml suspension, which is identical to the percentage of the initial HNE concentration (mean of 6 independent experiments with SD of less than 8%).

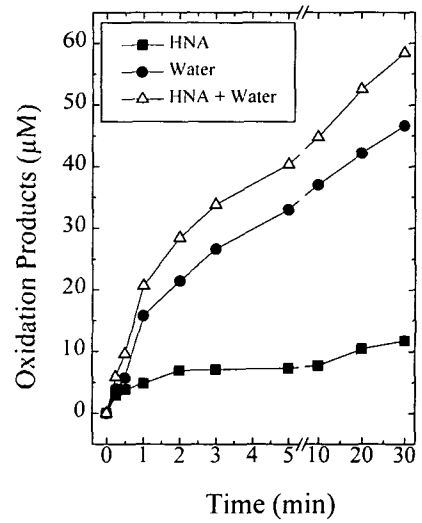

Fig. 3. **Oxidative metabolism of exogenously added HNE in mouse thymocytes.** Values are given as nmol/ml suspension (mean, SD less than 15%, $n=6$). For the experimental conditions see the legend to Fig. 2 and "MATERIALS AND METHODS."

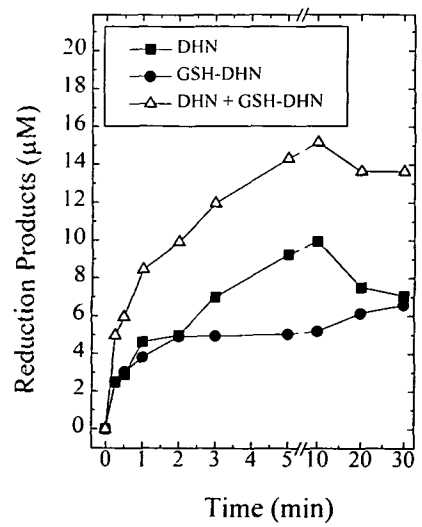

Fig. 4. **Reductive metabolism of HNE in mouse thymocytes.** Values are nmol/ml suspension (mean, SD less than 15%, $n=6$). For experimental conditions see the legend to Fig. 2 and "MATERIALS AND METHODS."

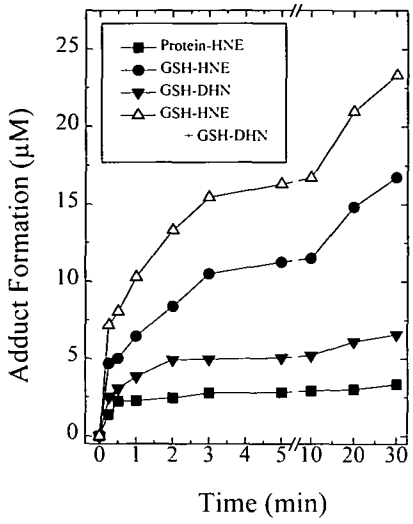

Fig. 5. **Glutathione or protein-adduct formation after the addition of radiolabeled HNE to thymocytes.** Values are given as nmol HNE bound in the adducts per ml suspension. This is in the case of GSH-HNE and GSH-DHN identical to the concentration of the formed metabolite. For the experimental conditions see the legend to Fig. 2 and "MATERIALS AND METHODS." Values are given as the mean of 6 independent experiments with SD of less than 13%.

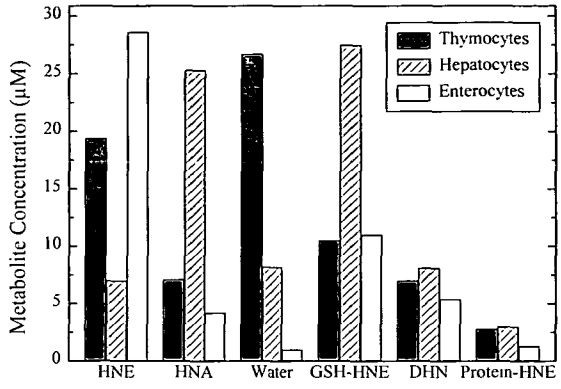

Fig. 6. **Comparison of the HNE metabolite patterns of hepatocytes, small intestinal enterocytes, and thymocytes.** The incubation time following the addition of radioactively labeled HNE for all three cell types was 3 min, and the pH was 7.4. Incubation was carried out as described under "MATERIALS AND METHODS." The values for hepatocytes and enterocytes were taken from (*9, 27, 33*).

formation of protein adducts was lower than 4% of HNE degradation during the whole experiment.

In Fig. 6, the formation of the three primary HNE metabolites (DHN, HNA, and GSH-HNE), and additionally that of water from HNA and that of HNE-protein conjugates, is compared for the three cell types showing the highest capacity for HNE degradation: hepatocytes, thymocytes, and small intestinal enterocytes. The rate of formation of DHN is quite similar in all three cell types. The GSH-HNE conjugate formation predominates in hepatocytes. Whereas hepatocytes exhibit high accumulation of HNA, this accumulation in enterocytes and thymocytes is prevented by rapid conversion of the acid and the formation of water as one of the final products. The protein adduct formation in all three cell types is quantitatively low: between 1.3 and 3.0% of the total HNE consumption rate.

DISCUSSION

Low Levels of Lipid Peroxidation Products in Tissues with Increased Proliferation and Massive Cell Death?—It is known from studies by different groups that most tumor cells are characterized by low steady-state concentrations of endogenous lipid peroxidation products (*4, 38-40*), *i.e.* it is generally accepted that there is an inverse correlation between the proliferation rate and the levels of lipid peroxidation products. The molecular basis of this correlation is not clearly understood. There are data on the fluctuation of the lipid peroxidation rate itself, and on the contents of lipophilic and hydrophilic antioxidants (*43*). Interestingly, one also finds very low steady-state levels of lipid peroxidation products in cells of the thymus (*37*), an organ in which cell death is massive. Thymus-derived T-lymphocytes constitute the cell-mediated effector arm of the immune system. It has been estimated that up to 90% of precursor cells never emerge at the periphery (*17, 18*). In studies on apoptosis in thymic selection, triggering of the T-cell antigen receptor, chemical agents and oxidants were favored as signals leading to cell activation (*44*). Antigen receptor triggering leads to an increase in the cytoplasmic Ca^{2+} concentration and protein kinase C inactivation (*44-49*). These changes are known to be induced also by low HNE concentrations (*1, 2*). Intracellular oxidants have the potential to trigger apoptosis in several ways (*17, 19*). Antioxidants have been reported to provide protection against many different types of apoptosis (*50*). Using rat thymocytes, it was found that free radical spin traps can also inhibit multiple forms of apoptosis (*51*). The effects of different antioxidants are manifested as the prevention of cell shrinkage and the reduction in the internucleosomal fragmentation of chromatin, the two most characteristic indicators of apoptosis in these cells (*17*). When thymocytes undergoing glucocorticoid-induced apoptosis were separated by Percoll density centrifugation, an increased level of oxidation was detectable in both pre-apoptotic and apoptotic cells (*51, 52*). All these findings add support to the hypothesis that intracellular oxidants play an important role in apoptosis, although neither the identity, site of generation or molecular targets of the putative oxidant species have been identified. If there is any specific impact of oxidants and antioxidants on apoptosis, studies on the influence of HNE and other cytotoxic aldehydic products of lipid peroxidation, and therefore, also on regulation of HNE levels by metabolic pathways of HNE, are interesting.

High Capacity of Thymocytes to Degrade HNE—It was found that thymocytes possess very high capacity for HNE consumption in comparison with other cell types: 27.7 nmol/mg w.w./min. Only hepatocytes exhibit a somewhat higher HNE degradation rate: 28.4 nmol/mg w.w./min. The value for thymocytes is unexpected high taking into account that hepatocytes have the by far most active pathways of metabolism of HNE and other aldehydic lipid peroxidation products (*1, 53, 54*). Therefore, the level of HNE in thymocytes should be very low due to the high metabolic rate. It might be suggested that the low conentrations of aldehydic lipid peroxidation products in thymocytes besides the high amounts of lipophilic antioxidants in

these cells are due to the extremely high capacity of the pathways of metabolism of aldehydes such as HNE.

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