Vol. 207, No. 2, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 15, 1995 Pages 477-484

METABOLISM OF 4-HYDROXY-2-NONENAL AND AGING

E. Chiarpotto, F. Biasi, A. Scavazza, S. Camandola, M.U. Dianzani and G. Poli*

Department of Experimental Medicine and Oncology, General Pathology Section, University of Torino, C. Raffaello 30, I-10125, Torino, Italy

CNR Centre of Immunogenetics and Experimental Oncology, Via Santena 19, I-10126, Torino, Italy

Received January 2, 1995

Hepatocytes isolated from male Wistar rats of 2-3 and 20-24 months of age were compared as regards concentration and metabolism of 4-hydroxy-2-nonenal, one of the major aldehyde products of n-6 fatty acid oxidative breakdown. A significant accumulation of fluorescent 4-hydroxy-2-nonenal-membrane lipid adducts was found in the cells from the old rats. The mechanism mainly responsible for such aldehyde accumulation was shown to be the impairment of its enzymatic metabolism. In fact, while endogenous, that is non-stimulated, aldehyde production was not impaired, the reductive pathway of 4-hydroxy-2-nonenal metabolism in hepatocytes from old rats was strongly depressed. The decrease of 4-hydroxy-2-nonenal consumption with age was confirmed in homogenates from liver and kidney, while there were no differences between young and old animals in heart, lung or brain. © 1995 Academic Press, Inc.

It has been suggested that free-radical-mediated reactions could contribute to the progressive accumulation of structural and functional changes which characterize the aging of tissues (1). The production of free radicals is a continuous biological process, which challenges a number of defensive enzymatic and non-enzymatic systems. Having overwhelmed the defensive barriers, free radicals can damage molecules that are important in cellular function. Indeed, virtually all cellular components appear to be sensitive to radical/oxidant damage: proteins (2,3), lipids (4,5), nucleic acids (6,7) and carbohydrates (8). Moreover, the peroxidative breakdown of membrane polyunsaturated fatty acids is an important means whereby tissue damage through the formation of lipid hydroperoxides (LOOH) is amplified. These LOOH easily decompose into multiple breakdown products, i.e. epoxides, alcohols and aldehydes (malondialdehyde: MDA and other carbonyl compounds of different classes), most of which display biological activity (9). In particular, bifunctional aldehydes act as cross-linking reagents, thus playing a role in the protein aggregation which contributes to the formation of the age

*Corresponding author. Fax : 39-11-6707753.

0006-291X/95 $5.00
Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

pigment lipofuscin (10). Among lipid peroxidation-derived aldehydes, 4-hydroxy-2-nonenal (HNE) is one of the most reactive (11,12). Indeed, its active binding to proteins and lipids causes the formation of oxidized derivatives that accumulate as lipofuscin in the tissues during senescence (13).

Since the extent of cellular damage due to aldehydes depends on the balance between the rates of their production and its elimination, we aimed to measure the production, but mainly to analyse the metabolism of HNE by hepatocytes from young and old rats.

MATERIALS AND METHODS

Animals. Male Wistar rats (Nossan, Correzzana, Italy) aged 2-3 months (young rats: YR) or 20-24 months (old rats: OR) were used. In a set of experiments concerning HNE metabolism, rats aged 12-14 months (middle-aged rats) were also employed. All animals were maintained on a semisynthetic rodent diet (n. 48 Piccioni, Gessate Milanese, Italy) and had free access to water. The treatment of the animals was approved by the Local Committee for Animal Experimentation.

Materials. Collagenase type IV, ethylene-glycol*bis*(ß-amino-ethylether)-N,N,N',N'-tetra-acetic acid (EGTA), N-2-hydroxy-ethyl-piperazine-N,N'-2-ethane sulphonic acid (HEPES), and reduced glutathione, were obtained from Sigma Aldrich, Milano, Italy; adenosine diphosphate (Na salt), reduced nicotinamide adenine dinucleotide phosphate, and glutathione reductase from Boehringer Mannheim Italia, Milano, Italy; 4-hydroxy-2-nonenal (HNE) was a kind gift from Prof. Esterbauer from the Biochemistry Institute of the University of Graz, Austria. All other chemicals were from BDH Chemicals Ltd., (Poole, Dorset, U.K.) and Merck (Darmstadt, Germany).

Isolation and incubation of rat hepatocytes. Rat hepatocytes were isolated by the collagenase *in situ* perfusion method. Suspensions, diluted to appropriate cell concentrations with a balanced salt solution (14), were incubated at 37° C for up to one hour in 50 ml flasks. In the experiments in which hepatocytes were incubated at 0° C, the liquid inside the incubation bath, used to achieve and maintain the temperature while avoiding ice formation, was a mixture of 95% ethanol and water.

Preparation and incubation of organ homogenates. Liver, kidney, lung, heart and brain 1% homogenates (w/v) were prepared in ice-cold 0.1 M Tris-HCl/ 0.15 M KCl (2:1 v:v), pH 7.4, using a Polytron tissue homogenizer (Kinematica GMBH, Luzern, Switzerland).

Lipid peroxidation studies. The oxidative breakdown of lipids was measured in terms of malondialdehyde (MDA) (15), total non-polar carbonyls (16,17) and fluorescent lipid-aldehyde adducts (18).

4-Hydroxy-2-nonenal metabolism studies. Exogenous HNE metabolism was evaluated by incubating isolated hepatocyte suspensions (10^5 cells/ml) in the presence of HNE 0.1 mM at 37°C and 0°C. HNE consumption was monitored on cell suspension aliquots (0.5 ml) taken at different times by the hplc method of Esterbauer et al. (19). When used, 0.1 mM disulphyram (dimethylsulphoxide solution) and 10 mM pyrazole (final concentrations in the incubation medium) were directly added to the cell suspension. In homogenate experiments, the incubation was carried out at 37°C in the presence of 0.1 mM HNE and HNE consumption was monitored on homogenate aliquots (0.5 ml) taken at different times, as for hepatocyte suspensions.

Other biochemical analyses. Total glutathione-S-transferase activity was evaluated by the method of Warholm et al. (20) in cell suspension aliquots (0.5 ml) after sonication. Total protein thiols were determined by the method of Di Monte et al. (21). Total protein content was evaluated by the Lowry method as modified by Peterson (22). Total lipid content was determined using a diagnostic kit (Boehringer total lipids test-combination, Mannheim, Germany).

Statistical analyses. Student's *t* test was used to determine the statistical significance of the difference between experimental groups.

RESULTS

The basal, non-stimulated, concentration of lipid-peroxidation-derived aldehydes does not show any difference between hepatocytes from young and old rats, when only the

amount of free malondialdehyde is measured (YR: 11 ± 1; OR: 10 ± 4 nmol/10 ^{8} cells). However, if total free non-polar carbonyls other than malondialdehyde are measured, a significant increase can be detected in hepatocytes from old animals (YR: 350 ± 160; OR: 585 ± 160 nmol/10 ^{8} cells; $P<0.05$). The latter finding is strengthened by the observation of a marked difference in the basal content of 4-hydroxy-2-nonenal bound to membrane lipids detected as fluorescence adducts in hepatocytes from young and old rats (Fig. 1). As shown in Table I, the half-elimination of free HNE externally added to rat hepatocyte suspensions was achieved within 5 minutes by cells from young rats and in double that time by cells from old rats. It is noteworthy that this impaired HNE metabolism is detectable in the rat strain used only with advanced age. In fact the rate of HNE consumption in cells from middle-aged animals is still very similar to that of the young group. Two orders of mechanisms can contribute to HNE consumption: the aspecific binding to nucleophiles and the biotransformation operated by a complex and still partly unclear enzymatic pathway (12).

To investigate the involvement of the enzyme-independent aldehyde metabolism in the two experimental groups, HNE consumption was monitored in cell suspensions incubated at 0°C. The results, shown in Table II, indicate that HNE consumption is up to 20-25% independent of enzymatic activities, and it shows a similar behaviour in cells from young and old animals. Table II also reports the effect of two inhibitors of enzyme-mediated HNE metabolism, disulphyram and pyrazole. The disulphyram-induced block of the oxidative metabolism through aldehyde dehydrogenase slows down the aldehyde consumption in the old rat to that achievable

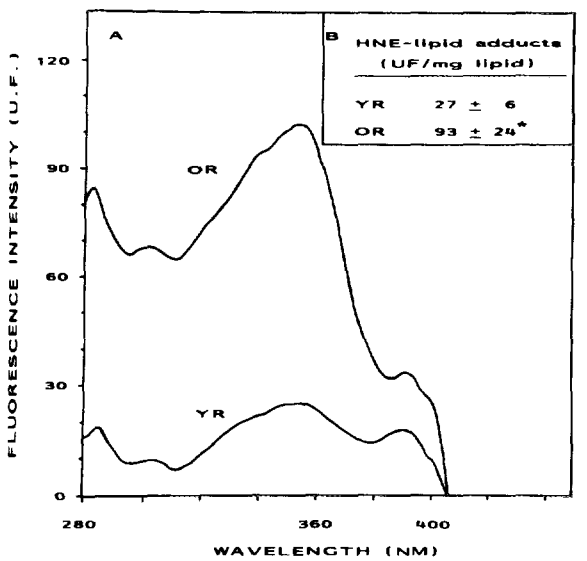

Fig. 1. **Basal content of 4-hydroxy-2-nonenal-membrane lipid adducts in hepatocytes from young and old rats.**
A: Comparison of two typical excitation spectra at 460 nm emission wavelength of total lipids of hepatocytes from young and old rats. B: Means ± S.D. * Old rat (OR) significantly different from young rat (YR): $P<0.05$.

TABLE I

Consumption of exogenous 4-hydroxy-2-nonenal (HNE) in hepatocytes from young, middle aged and old rats

Time (min)	HNE (% consumption)		
	Young rats (2-3 months)	Middle aged rats (12-14 months)	Old rats (20-24 months)
5	50 ± 6	45 ± 4	36 ± 1 [a]
10	70 ± 2	65 ± 4	51 ± 9 [a]
15	78 ± 3	74 ± 2	62 ± 4 [a]
30	84 ± 1	82 ± 3	75 ± 2 [a]
45	89 ± 2	86 ± 2	82 ± 1 [a]
60	96 ± 2	90 ± 4	88 ± 3 [a]

Hepatocyte suspensions (10^5 cells/ml) were incubated at 37°C in the presence of 0.1 mM HNE. Values are means ± S.D. of four experiments in duplicate and are referred to time zero HNE consumption (0%).
[a]Significantly different from young rats ($P<0.05$).

in the same cells at 0°C, while in the young rat the consumption rate is still double that at 0°C. The additional inhibition of the reductive metabolism through alcohol dehydrogenase induced by simultaneous cell treatment with pyrazole does not further affect HNE metabolism in old rats, but leads to a significant further decrease of aldehyde metabolism in hepatocytes from young animals. In this experimental group, however, the enzymatic metabolism of HNE appears to be also achieved, even if to a lesser extent, by pathways other than the oxidative and reductive ones. For this reason, the specific activity of glutathione transferase using HNE as substrate was evaluated in hepatocytes from young rats (201 ± 40 nmol/mg protein/min) and old rats (223 ± 37 nmol/mg protein/min) but no statistical difference was observed between the two groups. Following the demonstration of a significantly lower rate of HNE metabolism in the hepatocytes from old as against young rats, the metabolism of this toxic aldehyde by various other organs of the same animals was checked, i.e. in liver, kidney, lung, heart and brain homogenates (Table III). The half life of HNE in the liver homogenates appears shorter than that detected in intact hepatocyte suspensions, while in all other cases it is much longer. Among the organs examined, a significant difference in the rate of HNE metabolism between young and old animals was only seen in the kidney. Once again, HNE consumption in the aged tissue proceeded more slowly.

DISCUSSION

Variations of endogenous membrane lipid peroxidation, clearly related to the gender and strain of the animals used, as well as to the dietary regimen, can be better estimated when the production of several aldehyde compounds other than malondialdehyde is considered. In fact, it has been demonstrated that in isolated rat

TABLE II

Consumption of exogenous 4-hydroxy-2-nonenal (HNE) in hepatocytes, obtained from young (2-3 months) and old (20-24 months) rats, and incubated at 0°C, or in the presence of either disulphyram or pyrazole at 37°C

	HNE (% consumption)		
	Time (min)		
	5	15	30
Incubation at 0°C			
Young rats	14 ± 9	18 ± 7	20 ± 7
Old rats	13 ± 4	23 ± 5	27 ± 7
Incubation at 37°C plus 0.1 mM disulphyram			
Young rats	26 ± 3 [b]	43 ± 3 [b]	53 ± 1 [b]
Old rats	14 ± 7 [a]	21 ± 12 [a]	31 ± 14 [a]
Incubation at 37°C plus 0.1 mM disulphyram and 10 mM pyrazole			
Young rats	12 ± 6 [c]	27 ± 8 [c]	35 ± 9 [b,c]
Old rats	14 ± 4	20 ± 4	27 ± 4

Hepatocyte suspensions (10^5 cells/ml) were incubated at 0°C or at 37°C in the presence of 0.1 mM HNE, 0.1 mM disulphyram and 10 mM pyrazole. Values are means ± S.D. of four experiments in duplicate and are referred to time zero HNE consumption (0%).
[a]Significantly different from young rats ($P<0.05$).
[b]Significantly different from the corresponding hepatocytes from young rats incubated at 0°C ($P<0.05$).
[c]Significantly different from the corresponding hepatocytes from young rats in the presence of disulphyram alone ($P<0.05$).

hepatocytes undergoing lipid peroxidation, MDA accounts for 9-19% of the detectable carbonyls, 30-48% consisting of various non-polar aldehydes, namely alkanals, 2-alkenals, 4-hydroxy-2-alkenals, and 44-53 % of polar carbonyls (16). In the hepatocytes from old rats, the increase of total non-polar carbonyls other than MDA confirms some accumulation of these compounds with age. Moreover, it is now possible to detect not only aldehydes free in the cell environment, but also some of them bound to target molecules. As regards in particular HNE, the main hydroxyalkenal deriving from the oxidative breakdown of n-6 polyunsaturated fatty acids, and far more toxic than MDA, the marked increase of its fluorescent adducts to hepatocyte lipids reported in old rats (Fig. 1) conclusively proves its involvement in lipofuscin formation and accumulation. Even if a transiently stimulated synthesis of HNE may be hypothesized, the constant impairment of its metabolism with advanced age (Table I) appears to be the mechanism mainly responsible for accumulation. The different rate of HNE

TABLE III

Consumption of exogenous 4-hydroxy-2-nonenal (HNE) from liver, kidney, lung, heart and brain homogenates of young (2-3 months) and old (20-24 months) rats

Organ	HNE (% consumption)		
	Time (min)		
	5	15	30
Liver			
Young rats	80 ± 1	92 ± 3	95 ± 2
Old rats	50 ± 3 [a]	64 ± 9 [a]	75 ± 9 [a]
Kidney			
Young rats	23 ± 4	40 ± 6	59 ± 5
Old rats	18 ± 3 [a]	24 ± 7 [a]	40 ± 9 [a]
Lung			
Young rats	13 ± 11	19 ± 11	31 ± 12
Old rats	12 ± 11	22 ± 6	32 ± 8
Heart			
Young rats	11 ± 10	27 ± 2	46 ± 7
Old rats	15 ± 14	31 ± 13	39 ± 14
Brain			
Young rats	11 ± 3	22 ± 6	32 ± 8
Old rats	20 ± 11	24 ± 12	42 ± 14

1% Homogenates (w/v) were incubated at 37°C in the presence of 0.1 mM HNE. Values are means ± S.D. of four experiments in duplicate and are referred to time zero HNE consumption (0%).
For experimental details, see Materials and Methods.
[a]Significantly different from young rats ($P<0.05$).

metabolism by hepatocytes from young and old rats is not due to variations in the reaction of carbonyl function with protein and non-protein thiols, as suggested by the similar protein thiol levels in the cells from the two groups (YR: 114 ± 15 and OR: 111 ± 6 nmol/mg protein) and shown by the similar trend of aldehyde consumption at 0°C (Table II). The use of certain enzyme inhibitors made it clear that the most important changes in the hepatic disappearance of HNE involve enzymatic pathways, especially those leading to reduction of the hydroxyalkenal. In fact, by subtracting the percentage of aldehyde consumed at 5, 15, 30 min in the presence of the aldehyde dehydrogenase inhibitor disulphyram (Table II) from that achieved at the same incubation times without inhibitors (Table I), it appears that the aldehyde dehydrogenase pathway has the same efficiency in young and old animals. The present results are in agreement with those of Mitchell and Petersen (23), who demonstrated that the oxidative metabolism of HNE is

responsible for nearly half its elimination. It is noteworthy that, in the aged group, aldehyde dehydrogenase activity seems to be the only one actually able to metabolize the aldehyde. In hepatocytes from young animals, on the contrary, the additional efficiency of other pathways affords a higher HNE consumption at the given times. In fact, pyrazole-inhibited alcohol dehydrogenase, aldehyde reductase and glutathione-S-transferase are also implicated, being responsible for about 35% of the total HNE metabolism by the hepatocyte (12). As regards conjugation with glutathione via glutathione transferases, HNE is an elective substrate of one of these isoenzymes (24) and an age-related decrease of total enzyme activity has been described in the liver of male Fischer rats (25). In male Wistar rats we did not find any change of hepatic GSH-transferase activity using HNE as substrate. An age-related change in the enzyme affinity for substrates seems more likely than a different expression of the isoenzyme pattern; at any rate, an accurate enzymologic approach to this matter deserves attention. The same can be said as regards HNE reductive metabolism via alcohol dehydrogenase and aldehyde reductase, whose behaviour with age has not yet received much attention.

Finally, the overall metabolism of HNE analysed in liver homogenates consistently shows a higher efficiency in the young than in the old male rat (Table III). Indeed, aldehyde consumption, especially in homogenates from young males, is remarkably faster than in hepatocytes from rats of the same age, indicating the certain contribution of cells other than hepatocytes.

In conclusion, the data reported demonstrate that hepatocytes from old rats are readily susceptible to suitable prooxidant stimuli, thus being able to increase the production of various toxic aldehydes. Among these compounds, HNE easily accumulates in the aged hepatocytes, because of the significantly slowed enzymatic metabolism of the aldehyde. It is interesting that such metabolic behaviour is also evident in kidney from old rats, while heart, lung and brain do not show diversity in this respect. Hence the possible contribution of HNE to lipofuscin composition appears quantitatively different in the various organs.

ACKNOWLEDGMENTS

The authors wish to thank the Ministero dell'Universita' e della Ricerca Scientifica e Tecnologica (MURST), National Project "Fisiologia e Patofisiologia dell'Invecchiamento" and the National Research Council (CNR), Project "Invecchiamento" contract n. 93.00439.PF40, and Project "Applicazioni Cliniche della Ricerca Oncologica" contract n° 93.02223.PF39, for supporting this research.

REFERENCES

1. Harman, D. (1983) Age 6, 86-94.
2. Wolff, S.P. and Dean, R.T. (1986) Biochem. J. 234, 399-403.
3. Davies, K.J.A. (1987) J. Biol. Chem. 262, 9895-9901.
4. Slater, T.F. (1972) Free Radical Mechanisms in Tissue Injury, Pion Press Ltd., London.
5. Poli, G., Albano, E. and Dianzani, M.U. (1987) Chem. Phys. Lipids 45, 117-142.

6. Kasai, H., Crain, P.F., Kuchino, Y., Nishimura, S., Ootsuyama, A. and Tanooka, H. (1986) Carcinogenesis 7, 1849-1851.
7. Richter, C., Park, J.W. and Ames, B.N. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 6465-6467.
8. Hunt, J.V., Dean, R.T. and Wolff, S.P. (1988) Biochem. J. 256, 205-212.
9. Esterbauer, H. (1982) In Free Radicals, Lipid Peroxidation and Cancer (D.C.H. McBrien and T.F. Slater, Eds.), pp. 101-128, Academic Press, London.
10. Davies, K.J.A. (1988) In Lipofuscin-1987: state of the art (Y. Nagy, Ed.) pp. 109-133, Elsevier, Amsterdam.
11. Esterbauer, H., Zollner, H. and Lang, J. (1985) Biochem. J. 22, 363-373.
12. Esterbauer, H., Schaur, R.J. and Zollner, H. (1991) Free Rad. Biol. Med. 11, 81-128.
13. Esterbauer, H., Koller, E., Slee, R.G. and Koster, J.F. (1986) Biochem. J. 239, 405-409.
14. Poli, G., Gravela, E., Albano, E. and Dianzani, M.U. (1979) Exp. Mol. Pathol. 30, 116-127.
15. Bernheim, F., Bernheim, M.L.C. and Wilbur, K.M. (1948) J. Biol. Chem. 174, 257-264.
16. Poli, G., Dianzani, M.U., Cheeseman, K.H., Slater, T.F., Lang, J. and Esterbauer, H. (1985) Biochem. J. 227, 629-638.
17. Biasi, F., Albano, E., Chiarpotto, E., Corongiu, F.P., Pronzato, M.A., Marinari, U.M., Parola, M., Dianzani, M.U. and Poli, G. (1991) Cell Biochem. Funct. 9, 111-118.
18. Carini, R., Mazzanti, R., Biasi, F., Chiarpotto, E., Marmo, G., Moscarella, S., Gentilini, P., Dianzani, M.U. and Poli, G. (1988) In Alcohol Toxicity and Free Radical Mechanisms, Advances in the Biosciences (R. Nordmann, C. Ribiere and H. Rouach, Eds.), Vol. 71, pp. 61-64, Pergamon Press, Oxford.
19. Esterbauer, H., Cheeseman, K.H., Dianzani, M.U., Poli, G. and Slater, T.F. (1982) Biochem. J. 208, 129-140.
20. Warholm, M., Guthenberg, C., Von Bahr, C. and Mannervik, B. (1985) In Methods in Enzymology (A. Meister, Ed.) Vol. 113, pp. 499-504, Academic Press, London.
21. Di Monte, D., Ross, D., Bellomo, G., Eklöw, L. and Orrenius, S. (1984) Arch. Biochem. Biophys. 235, 334-342.
22. Peterson, G.L. (1977) Anal. Biochem. 83, 346-356.
23. Mitchell, D.Y. and Petersen, D.R. (1987) Toxicol. Appl. Pharmacol. 87, 403-410.
24. Mannervik, B. and Danielson, U.H. (1988) CRC Crit. Rev. Biochem. 23, 283-337.
25. Spearman, M.E. and Leibman, K.C. (1984) Biochem. Pharmacol. 33, 1309-1313.