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Chemico-Biological Interactions 130–132 (2001) 563–571

Chemico-Biological
Interactions

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Involvement of aldose reductase in the metabolism of atherogenic aldehydes

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Abstract

Phospholipid peroxidation generates a variety of aldehydes, which includes free saturated and unsaturated aldehydes, and aldehydes that remain esterified to the phosphoglyceride backbone — the so-called ‘core’ aldehydes. However, little is known in regarding the vascular metabolism of these aldehydes. To identify biochemical pathways that metabolize free aldehydes, we examined the metabolism of 4-hydroxy-*trans*-2-nonenal in human aortic endothelial cells. Incubation of these cells with [³H]-HNE led to the generation of four main metabolites, i.e. glutathionyl HNE (GS-HNE), glutathionyl dihydroxynonene (GS-DHN), DHN and 4-hydroxynonanoic acid (HNA), which accounted for 5, 50, 6, and 23% of the total HNE metabolized. The conversion of GS-HNE to GS-DHN was inhibited by tolrestat, indicating that it is catalyzed by aldose reductase (AR). The AR was also found to be an efficient catalyst for the reduction of the core aldehyde — 1-palmitoyl-2- (5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine, which is generated in minimally modified low-density lipoprotein, and activates the endothelium to bind monocytes. As determined by electrospray mass spectrometry, reduction of POVPC ($m/z = 594$) by AR led to the formation of 1-palmitoyl-2- (5)-hydrovaleryl-*sn*-glycero-3-phosphorylcholine (PHVPC; $m/z = 596$). These observations suggest that due to its ability to catalyze the reduction of lipid-derived aldehydes AR may be involved in preventing inflammation and diminishing oxidative stress during the early phases of atherogenesis. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Aldose reductase; Lipid peroxidation; 4-Hydroxy-*trans*-2-nonenal; Atherosclerosis; 1-Palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine

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PII: S0009-2797(00)00299-4

1. Introduction

Peroxidation of lipoproteins and membrane lipids is a complex, multi-step process that is initiated and mediated by free radical species and intermediates [1,2]. Although multiple intracellular and extracellular antioxidants prevent spontaneous oxidation of lipids *in vivo*, several pathological states enhance the formation of reactive oxygen species resulting in increased lipid peroxidation. Peroxidative reactions not only alter the integrity and functioning of membranes and lipoproteins, they generate reactive intermediates and end products which are toxic to most cells. Moreover, due to inefficient metabolism, the products of lipid peroxidation accumulate in tissues causing long-term oxidative stress. Extensive evidence suggests that increased formation and accumulation of lipid peroxidation products contributes to tissues dysfunction and injury associated with several pathological conditions such as diabetes [3], atherosclerosis [4], Alzheimer's [5,6] and Parkinson's [6] disease.

Lipid peroxidative reactions are initiated by proton abstraction from a double bond of unsaturated fatty acids, causing the formation of peroxy radicals and peroxides. The reaction is propagated by dismutation of peroxides to alkoxy and peroxy radicals. Due to the presence of multiple *bis*-allylic methylenes in polyunsaturated fatty acids, a number of products are generated; of which aldehydes comprise a large fraction [1,2]. Among the free unsaturated aldehydes generated from peroxidation of ω -6 polyunsaturated fatty acids (arachidonate, linolate and linolenate), 4-hydroxy *trans*-2-nonenal (HNE) is formed in highest concentration [2]. In addition to the generation of free aldehydes, lipid peroxidation also generates aldehydes that remain esterified to the phospholipid backbone. One of the most abundant core aldehyde generated during the oxidation of lipoproteins and membrane lipids is 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-3-phosphorylcholine (POVPC). The POVPC is derived from the oxidation of 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine (PAPC) and represents the β -scission product derived from the 'inner most' *bis*-allylic double bond of the arachidonyl chain [7]. It is generated in high concentrations in minimally modified low-density lipoprotein (mmLDL), and in fatty streak lesions of cholesterol-fed rabbits. The phospholipid derivative has been suggested to be responsible, in part, for the ability of mmLDL to activate monocyte binding to the endothelium [8].

Multiple lines of evidence suggest that aldehydes generated from the oxidation of LDL play a critical role in atherogenesis. It has been shown that, *in vitro* derivatization of the nucleophilic side chains of apoB by aldehydes, converts LDL into a ligand for the scavenger receptor [2,9], and high titers of auto-antibodies against protein-aldehyde adducts and lipid aldehydes are present in the sera of animals and humans with atherosclerosis [9,10]. Significantly, monoclonal antibodies raised against oxLDL or antibodies cloned from apoE null mice specifically recognize epitopes generated in oxidized phospholipids or lipid aldehydes derived from phosphatidylcholine [11,12], in particular, POVPC [13]. Interestingly, antibodies recognizing POVPC also bind to apoptotic cells [14] indicating widespread formation of phospholipid aldehydes during oxidative stress, which is an important trigger and contributing factor in atherogenesis.

Despite their well-documented toxicity, the biochemical pathways for the metabolism of atherogenic aldehydes in vascular tissues have not been identified. Therefore, using HNE and POVPC as representatives of free and core aldehydes generated by lipid peroxidation, we examined aldehyde metabolizing processes in vascular endothelial cells, which are the main target of mmLDL and the substratum on which complex atherogenic lesions are elaborated. Our studies show that the aldo-keto reductase-aldose reductase (AKR1B1) plays a central role in the endothelial metabolism of the atherogenic aldehydes, and suggests that metabolism via AR may be an important determinant of the vascular effects of oxidized LDL leading to chronic inflammation and remodeling of the vessel wall.

2. Experimental procedures

Human endothelial cells were purchased from Clonetics, and were grown in cell basal medium containing 0.5 ml of 10 ng/ml human recombinant epidermal growth factor, 0.5 ml of 1 mg/ml hydrocortisone, 0.5 ml of 50 mg/ml gentamycin, 50 µg/ml of amphotericin B, 2 ml of 3 mg/ml bovine brain extract and 10% fetal bovine serum (FBS). The cells were incubated at 37°C in 95% air and 5% CO₂. Cells at the 4th and 5th subcultivation were used. For measurements of HNE metabolism, [³H]-HNE was synthesized as described before [15]. On the day of the experiment, the medium was removed and the cells were suspended in Ringer-HEPES containing [³H]-HNE. After the indicated time of incubation, aliquots of the medium were collected. At the end of the incubation, the remaining medium was removed, and the cells were scraped from the bottom of the dish and treated with 10% trichloroacetic acid. Radioactivity in the acid-insoluble extract was determined as a measure of covalent adducts of HNE with cell constituents. To identify the metabolites, radioactivity recovered in the medium and/or the cell extract were separated by HPLC using a Varian ODS, C₁₈ column, pre-equilibrated with 0.1% trifluoroacetic acid at a flow rate of 1 ml/min as described before [15]. Endothelial metabolites, as well as free unmetabolized HNE, were identified on the basis of the retention time of the reagent metabolites and HNE. Chemical identity of Peak I, that represents GS-HNE and GS-DHN was established by electro-spray ionization mass spectrometry (ESI⁺/MS), and peak II was analyzed by gas chromatography-chemical ionization mass spectrometry (GC/CIMS) as described before [15]. Aldose reductase (AR) was cloned from the human heart cDNA library. The nucleotide sequence of the cloned gene was identical to placental AR. The AR cDNA was ligated to a His-tag leader sequence and inserted into a pET28 vector and expressed in B-21 strain of *E.coli*. The protein was purified on a His-Tag affinity column.

3. Results and discussion

To examine the metabolism of HNE, endothelial cells ($3-4 \times 10^6$) were incubated with 5 µM [³H]-HNE for 30 min at 37°C, and aliquots of the medium were

withdrawn at various time intervals. The HNE concentration of the medium was determined by quantification of the radioactive peak corresponding to reagent HNE. Approximately, 95% of HNE was metabolized under these conditions. After 30 min of incubation, the total radioactivity recovered in the incubation medium was $\sim 78\%$. Upon HPLC separation, the percentage distribution of the radioactivity was; peak I (glutathione conjugates) 53%, peak II (dihydroxynonene, DHN) 6%, Peaks III and IV (unidentified) 5 and 9%, respectively, and peak V (4-hydroxy nonanoic acid; HNA) 23%.

Peak I, with a retention time corresponding to the glutathione conjugates, was further analyzed by ESI⁺/MS. As shown in Fig. 1, the ESI⁺/MS spectrum of Peak I shows a predominant peak at m/z 466.1, which was assigned to glutathionyl-1, 4-dihydroxynonene (GS-DHN). A minor ion with m/z 463.9 was also evident, along with a relatively weak signal at 446.1. These ions were assigned to the molecular ion of GS-HNE and its ions arising from dehydration (M-18) of the precursor ion. The total concentration of GS-HNE estimated from the intensity of these ions was $< 10\%$ of GS-DHN, indicating that the glutathione conjugate is extruded predominantly, ($> 90\%$) as GS-DHN. The other major peak (peak V) which accounted for $\sim 23\%$ of the radioactivity was analyzed by GC-CI/MS, and was found to display a fragmentation pattern identical to that of the reagent HNA (data not shown), indicating that peak V is due to HNA.

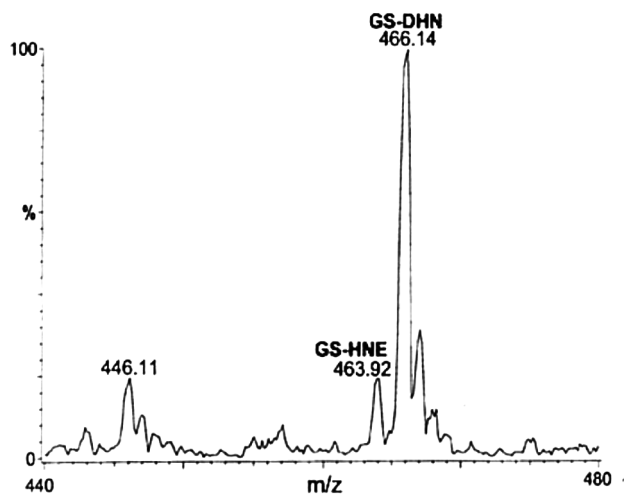


Fig. 1. Formation of glutathione conjugates by endothelial cells exposed to HNE. Cultured endothelial cells were incubated with [³H]-HNE in Ringer-HEPES and aliquots of the incubation medium were removed after 30 min. Radioactivity in the medium was separated by HPLC, and peak I, corresponding to the retention time of glutathione conjugates, was collected and analyzed by ESI⁺/MS. The predominant ion at m/z 466.14 was ascribed in GS-DHN. Additional ions due to GS-HNE and its dehydration product with m/z 463.92 and 446.11 were also observed as indicated. For details see the text.

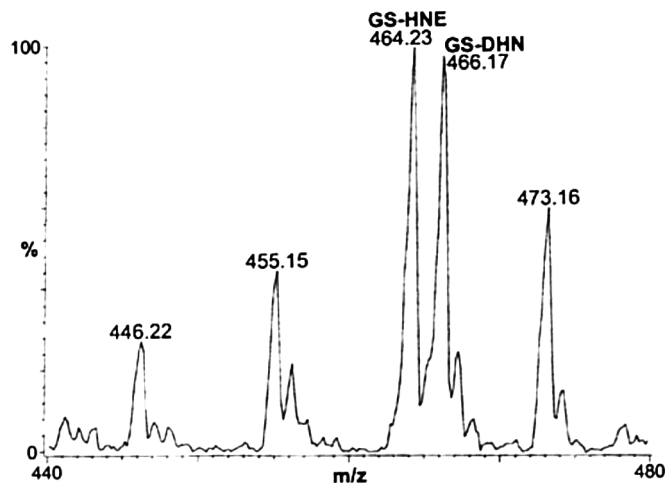


Fig. 2. Role of AR in catalyzing the reduction of glutathione conjugate of HNE. Endothelial cells were incubated with [^3H]-HNE in Ringers solution containing 10 μM tolrestat. The glutathione conjugates in the medium was separated by HPLC and analyzed by ESI + /MS. The spectrum shows two major ions with m/z 464.23 and 466.17 corresponding to GS-HNE and GS-DHN. Additional ions with m/z 446.22 and 455.15 due to dehydration products of GS-HNE are also evident. The ion with m/z 473.16 was not identified. AN increase in the ratio of GS-HNE/GS-DHN (cf; Fig. 1) in the presence of tolrestat suggests involvement of AR in the conversion of GS-HNE to GS-DHN.

To test the involvement of AR in the formation of GS-DHN, the endothelial cells were incubated with AR inhibitor-tolrestat. After pre-incubation with 10 μM tolrestat, 50 nmoles of [^3H]-HNE, were added to the medium, and the incubation was carried out for an additional 30 min, after which the metabolites were separated by HPLC as described above. The ESI⁺/MS spectrum of peak I obtained from cells treated with tolrestat, showed a predominant ion of GS-HNE with m/z 464, and ions with m/z 446 and 455 due to dehydrated daughter ions (Fig. 2). The intensity of these ions was much more than that obtained in the absence of tolrestat (cf; Fig. 1). An additional ion with m/z 473.1 was also observed. The identity of this peak is currently under investigation. Nevertheless, the data obtained so far clearly show that inhibition of AR decreases the extent to which the glutathione conjugate is converted to GS-DHN, suggesting that in endothelial cells, reduction of GS-HNE to GS-DHN is catalyzed by AR.

The results described above indicate that AR participates in the metabolism of HNE by catalyzing the reduction of GS-HNE. Because HNE is an unsaturated aldehyde, it readily combines with glutathione. Thus, in most glutathione competent cells, the primary role of AR in the metabolism of unsaturated aldehydes may be the reduction of their conjugates. However, the metabolism of saturated aldehydes may be different. These aldehydes do not form stable Michael adducts with glutathione and are thus, expected to be directly reduced by AR. To assess the efficacy of AR in reducing saturated atherogenic aldehydes, we examined the

reduction of POVPC. For these experiments, recombinant human AR was prepared as described under Experimental Procedures. The expressed protein was purified by His-Tag affinity column and digested by thrombin to remove the His-Tag. The digested protein was reduced by incubating with 0.1 M DTT at 37°C for 1 h, and desalted over a PD-10 column, equilibrated with 10 mM ammonium acetate. The protein was then suspended in 50:50:0.5 (v/v/v) methanol:water:acetic acid. As shown in Fig. 3, the molecular mass of the protein, estimated from the distribution of charge states was 36 135 Da, which is in excellent agreement with the expected molecular mass of 36 134 Da (AR + His-Ser-Gly = 35 853 + 281 = 36 134 Da).

The POVPC prepared as described elsewhere [8], was provided as a generous gift by Dr A.D. Watson (University of California, Los Angeles). The aldehyde was dissolved in chloroform, and stored at -20°C . An aliquot of the solution was removed, and evaporated under nitrogen and dissolved in methanol:water:acetic acid (50:50:0.1 v/v/v) and injected into the electrospray. As shown in Fig. 4, the aldehyde formed a well-resolved $[\text{M}^{+1}]$ ion with a m/z of 594.4. For measurement of enzyme activity, an aliquot of the aldehyde was dried under nitrogen to remove the chloroform, and reconstituted in 0.15 M potassium phosphate, pH 7.0, containing 0.1 mM NADPH, and recombinant AR. The enzyme activity was determined by following oxidation of NADPH. The enzyme activity obtained with POVPC was comparable to that observed with HNE. After 30 min of incubation, the enzyme protein was removed by ultrafiltration and the reaction mixture was desalted using a pipette tip packed with C_{18} resin (Zip-Tip; Millipore). The desalted sample was suspended in methanol:water:acetic acid solution and analyzed by ESI^{+}/MS . As

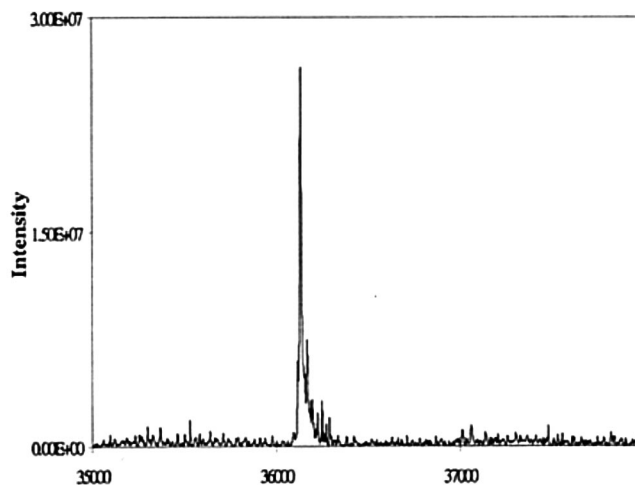


Fig. 3. Transformed ESI^{+}/MS spectrum of recombinant AR. The AR was cloned from the human heart cDNA library, and the AR cDNA was ligated to a His-tag leader sequence and expressed in *E. coli*. The expressed protein was purified by His-tag affinity column and digested to remove all but three amino acids (His-Ser-Gly) residues of the His-tag. The spectrum corresponds to a molecular mass of 36, 135 Da close to the expected mass of AR containing His-Ser-Gly (= 36, 134 Da).

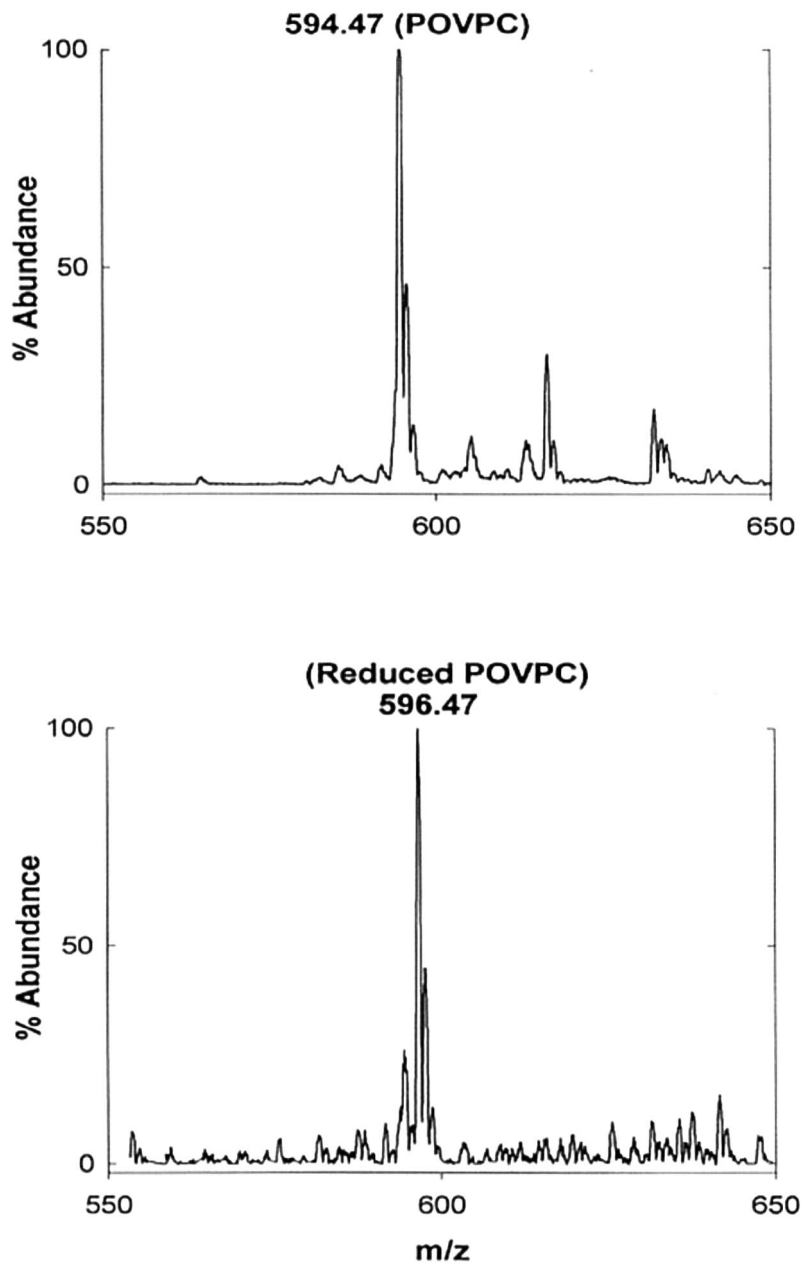


Fig. 4. Reduction of POVPC by AR. The phospholipid derived aldehyde POVPC was incubated with recombinant AR in 0.1 M K-phosphate, pH 7.0 and 100 μ M NADPH. The aldehyde and its products were removed from the media by Zip-Tips. Spectra show POVPC before (upper panel) and after (lower panel) incubation with AR. An increase in the molecular mass of the aldehyde by 2 Da is consistent with its conversion to an alcohol in the presence of AR.

shown in Fig. 4, incubation with AR led to a complete reduction of POVPC, as evinced by an increase in its m/z ratio by 2.

The reduction of GS-HNE and POVPC by AR, suggests that the enzyme may be involved in protecting the endothelium from the effects of aldehydes generated during the oxidation of LDL. These aldehydes could directly diffuse to the endothelium or may be taken up by the endothelial cells via scavenger receptors. In both cases, these aldehydes are likely to profoundly affect the functioning of the endothelium. Previous studies show that core aldehydes such as POVPC, generated in mmLDL, enhance the expression of surface adhesion molecules on the endothelial cells, thereby triggering the recruitment of monocytes [16]. However, when POVPC was reduced chemically by sodium borohydride, it was unable to enhance monocyte binding to the endothelial cells, suggesting that the aldehyde, but not the alcohol derivative of the lipid, enhances monocyte adhesion [8]. Thus, by catalyzing the reduction of aldehydes such as POVPC, AR may be able to curtail immune responses to the oxidized phospholipid which are associated with several autoimmune diseases as well as atherosclerosis. The AR may have a similar protective function in HNE metabolism. However, in the case of HNE, the glutathione conjugate, rather than the free aldehyde, is reduced. Although, because of removal of unsaturation at C-3, the glutathione conjugate is likely to be chemically less reactive than free HNE, toxicological studies suggest that the formation of GS-HNE may not, by itself, be sufficient for detoxification. When delivered to animals, the GS-acrolein conjugate was found to be markedly nephrotoxic [17], and the GS-conjugates of hexenal and nonadienal have been shown to induce DNA damage [18]. In addition, in cell-free systems, GS-acrolein is a more potent stimulator of oxygen radical formation than acrolein [19]. Therefore, reduction of the glutathione-aldehyde conjugates by AR may be necessary to minimize the reactivity of the conjugates and to diminish their toxicity and participation in free radical generation. Finally, because both HNE and POVPC are generated under several conditions other than oxidation of LDL [2,14,20], it is likely that regulation of their metabolism may be a significant physiological function of AR.

Acknowledgements

This work was supported in part by NIH grants HL55477, HL59378 (to A. Bhatnagar), DK36118 (to S.K. Srivastava) and grants from the American Heart Association — Ohio Valley Affiliate (to S. Srivastava and S.Q. Liu).

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