Oxidation of Plasmalogens Produces Highly Effective Modulators of Macrophage Function

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Model derivatives of plasmalogens and chemically synthesized oxidative degradation products as found e.g. during oxidation of low density lipoproteins show strong effects on phagocytosis induced secretion of reactive oxygen species of macrophages which was measured by luminol-enhanced chemiluminescence. Whereas a plasmalogen epoxide showed enhancing effects in submicromolar range, inhibition was found with higher concentrations as well as with α-hydroxyaldehydes. The substances showed only little effects on the non-cellular ROS-dependent chemiluminescence of the reaction between hydrogen peroxide and opsonized zymosan and no cytotoxic effects under the assay conditions used. These results show that oxidative modification and degradation of plasmalogens occuring also under pathophysiological situations *in vivo* produces effective modulators of macrophage function which could be important; e.g. during inflammation or atherogenesis.

Introduction

Plasmalogens are ubiquitously occurring lipid compounds present in the glycerophospo-lipid fraction (Horrocks and Sharma, 1982). Although they form a considerable proportion of cell membranes and plasma lipoproteins their biological functions are not well established.

Besides their distinct effect on the physicochemical properties of cell membranes, influences on activities of ion channels and enzymes as well as the HDL-mediated cholesterol-efflux were described (Lohner *et al.,* 1991; Groß, 1984; Horrocks and Fu, 1978; Mandel *et al.,* 1998; Williams and Ford, 1997; Yang *et al.,* 1996). Plasmalogens were also discussed to play a role as a reservoir of prostaglandin and thromboxane precursors (Groß, 1984; Horrocks and Fu, 1978), however, a main function is seen in their antioxidative action (Zoeller *et al.,* 1999; Brosche and Platt, 1998).

Especially it was shown, that plasmalogens are scavengers for singlet oxygen (Zoeller *et al.*, 1988) and inhibitors of metal-catalyzed lipid peroxidation (Zommara *et al.,* 1995). Furthermore, they are preferentially decomposed during oxidation of LDL and other unsaturated lipids (Engelmann *et al.,* 1994; Reiss *et al.,* 1997; Khaselev and Murphy, 1999).

Alterations in plasmalogens were found under various pathological conditions: e.g. in atherosclerotic vessel wall, in plasma lipoproteins of patients with cerebrovascular diseases, or in the cell membrane of erythrocytes of patients with chronic renal failure decreased contents of plasmalogens were observed (Miller *et al.,* 1964; Hoerrmann *et al.,* 1991; Stenvinkel *et al.,* 1998).

Similarly, a decrease of plasmalogens but an increase in plasmalogen epoxides and in the corresponding hydrolysis products, α-hydroxyaldehydes, respectively, was determined in LDL and HDL during oxidative modification (Felde and Spiteller, 1995; Jira and Spiteller, 1996). Formation of α hydroxyaldehydes was also found in subcellular fractions of bovine liver after epoxidation of plasmalogens (Loidl-Stahlhofen *et al.,* 1995), and in the aged human brain (Weisser *et al.,* 1997).

Since oxidation and modification of LDL are regarded as key events in atherogenesis (Steinberg

Abbreviations: DMSO, dimethylsulfoxide; LDH, lactate dehydrogenase; LDL, low density lipoproteins; PSS, physiological salt solution; ROS, reactive oxygen species; HDL, high density lipoproteins.

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et al., 1989; Witztum, 1994), oxidation products of plasmalogens could be involved in the underlying mechanisms of lesion formation. Therefore we were interested in the effects of several chemically synthetized oxidized model derivatives of plasmalogens on macrophage function as determined by measuring phagocytosis induced release of reactive oxygen species (ROS).

Experimental procedures

The following model compounds were used for the experiments described below:

I, 1-0-(hexadec-1-enyl)-2,3-diacetylglycerol (synthesis in (Knörr and Spiteller, 1990), corresponding to a model plasmalogen)
II, 1-0-tetradecyl-2,3-diacetylglycerol (synthesis in (Knörr, 1989), corresponding to a "plasmalogen" with saturated side chain at C1)
III, 1-0-(1',2'-epoxyhexadecyl)-2,3-diacetylglycerol (synthesis in (Meyer and Spiteller, 1993), a plasmalogen epoxide)
IVa, 2-acetoxyheptanal
IVb, 2-acetoxynonanal
(synthesis in Lutz (1991), α-hydroxyaldehydes as formed by oxidative decomposition of plasmalogens)
V, 1,2,3-trihexadecylglycerol (tripalmitin/Sigma, München/FRG)

Stock solutions were prepared using dimethylsulfoxide (DMSO). The final concentrations are given in the results.

Alveolar macrophages were isolated by alveolar lavage (Heinle *et al.*, 1992) from normal fed male New Zealand rabbits (2.5–3.5 kg body weight) sacrificed for other investigations. Shortly, 4 volumes of 50 ml of isotonic NaCl solution were gently pressed into the lung via an intratracheal tube. The washing fluid of each animal was collected, the cells were washed twice, counted and finally suspended in physiologic salt solution (PSS) (composition (mM): NaCl 119, KCl 4, $CaCl_2$ 1.5; $MgSO_4$ 1.2, NaH_2PO_4 1.5, N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (Hepes) 10, glucose 5, pH 7.4) at a density of $2x10^6$ cells/ml.

Phagocytosis was induced by incubation of macrophages with zymosan (Sigma, München/FRG) opsonized by incubation with plasma as described and secretion of ROS was detected by luminol- or lucigenine-enhanced chemiluminescence (Heinle *et al.*, 1992; Bruchelt and Schmidt, 1984). $4x10^5$ cells were transferred into a thermostated (37 °C) cuvette of a photon counter (Biolumat LB 9500, Berthold, Wildbad/FRG) together with 200 µl PSS and luminol or lucigenine (Sigma, München/FRG) ($2x10^{-4}$ M) dissolved in DMSO. After a preincubation time of 10 min opsonized zymosan (0.5 mg) was added to the cells. Chemiluminescence was measured during the preincubation time and for 10 min after induction of phagocytosis which includes about 85–90% of the zymosan induced reaction. The actual light emission was recorded, and the photon counts per 10 min were integrated separately during preincubation (A) and phagocytosis (B), respectively.

The plasmalogen derivatives were added to the cuvettes together with the cells (i.e. the cells were preincubated in the presence of these compounds), the controls received the vehicle solution alone.

The stimulation of release of ROS was quantified by calculating the ratios (B-A)/A. The results were related to the corresonding controls (= 100%) and mean values ± S. D. were calculated from 3–5 identical experiments using cell preparations from different animals.

In order to characterize pro- or antioxidative effects independent from the cellular action substances **III** and **IVa** were also tested in a radical-generating system which consists in the reaction of hydrogen peroxide with opsonized zymosan and detection by luminol-enhanced chemiluminescence (Heinle and El-Dessouki, 1995). Shortly, opsonized zymosan is incubated with 0.5–2 mM H_2O_2 in the presence of luminol. The effects on the generated chemiluminescence which is mainly due to the occurence of superoxide anions can be quantified using a photon counter.

Furthermore, in separate incubation experiments cytotoxicity was evaluated by determining the release of lactate dehydrogenase (LDH) from 10^7 cells in each case into the incubation medium (0.4 ml) (Okech, 1999). The activity of the enzyme found after 25 min at 37 °C in the medium was related to that which could be released from the same number of cells by 0.1% Triton X 100.

Results

In alveolar macrophages obtained form normal fed rabbits the luminol-enhanced chemiluminescence can be increased approximately 4-fold with interindividual variations by addition of opsonized zymosan. Using this parameter which indicated phagocytosis-stimulated production of ROS effects on macrophage function can be elucidated.

The results of these experiments are given in Table I. It is obvious that the saturated derivative **II** and tripalmitin (**V**) showed slight activating effects on ROS production within the concentration range used which might be caused by an unspecific effect of the glycerol compounds on the cell membrane. Incubation of the cells with the unsaturated derivative **I** (corresponding to a natural plasmalogen) showed at low concentrations (<5 μM) a similar increasing effect, however, higher concentrations reduced remarkably the secretion of ROS. This dual effect is even more pronounced with the epoxide (substance **III**). At 0.2 μM a strong activation of ROS release was found whereas increasing concentrations had inhibitory effects. At 200 μM a complete loss of zymosan induced activation was seen.

Exclusively inhibition of radical secretion of macrophages was found in the experiments with two model compounds of α-hydroxyaldehydes (substance **IVa** and **b**). Even in submicromolar concentrations (0.5 μM) these compounds could attenuate macrophage activation by zymosan.

In representative experiments using lucigenine, which indicates specifically superoxide anions, similar effects of the test substances on the activation of macrophages were obtained (results not shown).

In order to exclude direct enhancing or suppressing effects of the tested compounds with the chemiluminescence reaction, the substances **III** and **IVa** were also tested in a cell-free radical generating system consisting of H_2O_2 and opsonized zymosan. The results in Table II show that luminol-enhanced chemiluminescence itself is not affected by both substances.

Furthermore, in order to rule out the possibility that the inhibition of radical secretion as found with the aldehydes or the epoxide could be due to injury to the cells, cytotoxicity was determined for substances **III** and **IVa** by determining the LDH release into the incubation medium. The results show that both substances applied in concentrations of 10 and 100 μM, respectively, induce LDH release which amounts to 6 and 10% of that evoked by treatment with 0.1% Triton X100. Since under control conditions 4–6% were found, the cytotoxic effect of the substances seems to be negligible under the conditions used.

Discussion

Although superoxide anions and myeloperoxidase-dependent products were found, the whole spectrum of the radicals secreted by phagocytes and detected by luminol-enhanced chemilu-

Table I. Influence of derivatives of plasmalogens on zymosan-induced release of ROS of alveolar macrophages.

Substance **I**	conc. [μmol/l]	0.05	0.5	5	50	500		
plasmalogen	rel.activ.(%)	115	136*	137	76*	19**		
(n = 3)	± S. D.	20	18	27	11	20		
Substance **II**	conc. [μmol/l]	0.1	1	10	100			
saturated deriv.	rel.activ.(%)	112	120	106	176*			
(n = 3)	± S. D.	6	25	25	42			
Substance **III**	conc. [μmol/l]	0.02	0.2	2.0	20	100	200	400
epoxide	rel.activ.(%)	105	160*	128*	72*	11**	0	0
(n = 4)	± S. D.	12	21	18	22	15		
Substance **IVa**	conc. [μmol/l]	0.02	0.2	2	20	200		
2-acetoxyheptanal	rel.activ.(%)	105	75	70*	59**	23**		
(n = 4)	± S. D.	30	26	20	15	8		
Substance **IVb**	conc. [μmol/l]	0.05	0.5	5	50	500		
2-acetoxynonanal	rel.activ.(%)	115	72*	57**	48**	21**		
(n = 5)	± S. D.	32	19	16	28	14		
Substance **V**	conc. [μmol/l]	0.1	1	10	100			
tripalmitin	rel.activ.(%)	111	109	115	144*			
(n = 4)	± S. D.	18	11	12	26			

The untreated controls in each series obtained with cells of different animals are 100%.
For statistical calculations (t-test) the absolute values were used: significance of the effects.
* $p<0.05$; ** $p<0.01$.

Table II. Influence of oxidized derivatives of plasmalogens on zymosan-induced production of ROS by hydrogen peroxide.

	Conc. [μmol/l]	0.2	2.0	20	100
Substance **III** expoxide n=2	rel. chemiluminescence	108	122	124	120
Substance **IVa** 2-acetoxyheptanal n=2	rel. chemiluminescence	102	102	120	130

The values are mean values of 2 independent determination which differed not more than 8% in each case. Chemiluminescence of the control assay (measured as counts per 10 min) was related to 100.

minescence is still not completely described (Johansson and Dahlgren, 1989; Vilim and Wilhelm, 1989). However, since similar results were found in experiments with lucigenine which indicates specifically O_2^- (Okech, 1999) the effects of the substances tested here seem to be mostly attributed to the secretion of this reactive oxygen species.

Whereas the unsaturated model plasmalogen and the epoxide reveal biphasic effect with stimulation at low, inhibition with high concentrations, the α-hydroxyaldehydes show exclusively inhibitory effects. Since one can assume that the macrophages are able to metabolize the test substances added, it seems possible that primary oxidation products as e.g. epoxides, but also hydroperoxides are responsible for stimulation of radical secretion, probably by mechanisms mediated by cytoplasmic Ca^{2+} ions. This possibility is supported by the fact, that neutrophiles and lung macrophages are able to produce epoxides of linoleic acid which show strong cellular activity (Hayakawa *et al.*, 1986; Ozawa *et al.*, 1986).

The decreasing effect produced by the α-hydroxyaldehydes seems to be related with inhibition of the NADPH-oxidase reaction. In parallel experiments with the reconstituted enzyme of granulocytes a direct interaction with similar aldehydes suppressing the enzyme activity was found (Okech, 1999). Again, suspecting oxidative metabolism of compounds **I** and **III** in macrophages, their inhibitory action could also be due to α-hydroxyaldehydes formed during the incubation time.

Whether the α hydroxyaldehydes used in the present experiments were active in the acetylated or in the hydroxy form cannot be answered. However, since acetylated α hydroxyaldehydes were found to be hydrolysed in a cell proliferation test probably due to secreted hydrolases (Kern *et al.*, 1992), the formation by macrophages of the deacetylated form can be assumed.

Similarly, there are open questions concerning the concentrations of plasmalogen epoxide or α hydroxyaldehydes which can be expected under in vivo conditions. With respect to human arterial intima, which contains in the interstitial fluid approx. twice the plasma concentration of LDL (Smith and Ashall, 1983), a simplified calculation shows that during oxidation of LDL μM concentrations of hydroxyaldehydes can be expected. (Under *in vitro* oxidation 25 μg hydroxyaldehyd per g LDL were determined (Felde and Spiteller, 1995)).

In conclusion, the presented results show that plasmalogens cannot be regarded to function only as antioxidants but that their epoxidation and oxidative degradation produce compounds wich are very effective modulators of ROS release of macrophages during phagocytosis. Depending on the chemical structure activation as well as inhibition of ROS release were found with submicromolar concentrations. Therefore similar effects can be expected when plasmalogens originating either from lipoproteins or cell membranes are oxidized *in vivo* thus contributing essentially to different pathophysiologic mechanisms, e.g. in atherogenesis, inflammation or immun responses.

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