Synthesis and secretion of plasmalogens by type-II pneumocytes

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Alveolar surfactant (exposed to air and therefore a prime target of air oxidants) is supplied with antioxidants during its intracellular formation on type-II pneumocytes [Rüstow, Haupt, Stevens and Kunze (1993) *Am. J. Physiol.* **265**, L133–L139]. Plasmalogens can protect animal cells against lipid peroxidation caused by u.v. radiation. It has been suggested that plasmalogens play a direct role in protecting animal cell membranes against oxidative stress [Zoeller, Morand and Raetz (1988) *J. Biol. Chem.* **263**, 11590–11596]. We investigated biosynthesis and secretion of plasmalogens and phospholipids by type-II cells of adult rat lungs. The plasmalogens of type-II cells consist of 93 % ethanolamine plasmalogens (EthPlas) and 7 % choline plasmalogens (ChoPlas). Plasmalogens isolated from alveolar surfactant, however, consist of 36.5 % ChoPlas and 63.5 % EthPlas. The different incorporation rates of [¹⁴C]hexadecanol into both types of plasmalogen by type-II pneumocytes are reflected in the relative proportions of their total cellular plasmalogen content. Type-II cells cultured in the presence of labelled hexadecanol or

labelled hexadecylglycerol and of labelled palmitate secrete labelled ChoPlas and labelled phospholipids, both spontaneously and in response to isoprenaline. The spontaneous and stimulated secretion rates of labelled ChoPlas are 3–6 times higher than those of labelled EthPlas. This higher relative secretion rate of ChoPlas corresponds to its higher proportion in the total plasmalogen content of alveolar surfactant compared with type-II cells. Added extracellular surfactant-specific protein A inhibits the secretion of plasmalogens as well as that of phospholipids by type-II cells. The molecular species of EthPlas and ChoPlas isolated from type-II cells or lung lavage do not differ significantly and consist mainly of molecular species containing polyunsaturated fatty acids. We conclude that ChoPlas are secreted partly as integral constituents of the alveolar surfactant. Type-II cells select between both types of plasmalogens for secretion as a constituent of surfactant. The intramolecular sorting signal presumably is the choline moiety.

INTRODUCTION

Naturally occurring alkyl- and alk-1-enyl (plasmalogen)-linked phospholipids are present in most mammalian cells (Horrocks, 1972; Malins and Varanasi, 1972; Sugiura and Waku, 1987). The alkyl moieties are found in both the phosphatidylcholine (PtdCho) fraction and the phosphatidylethanolamine (PtdEth) fraction, whereas the alk-1-enyl moieties are found mainly in PtdEth. The high content of choline plasmalogens (ChoPlas) in heart phospholipids of several animal species is a well-known exception to this general finding (Sugiura and Waku, 1987).

Type-II pneumocytes contain ChoPlas and ethanolamine plasmalogens (EthPlas) in the same proportions as most mammalian cells. The PtdCho fraction of type-II cells contains about 2 % and the PtdEth fraction nearly 30 % plasmalogens (Rüstow et al., 1992).

The biological function of ether-containing phospholipids is widely unknown. Alkyl acylphospholipids can serve as precursors of platelet-activating factor, which is a potent lipid mediator. Plasmalogens can protect animal cells against lipid peroxidation caused by u.v. radiation. It has been suggested that plasmalogens may play a direct role in protecting animal cell membranes against oxidative stress (Zoeller et al., 1988). As they can function as scavengers of radicals and reactive-oxygen species (Morand et al., 1988), they may be seen to be intracellularly formed lipophilic antioxidants.

Recently, we showed that alveolar surfactant (a prime target of air oxidants) is supplied with antioxidants during its intracellular formation in type-II pneumocytes. The type-II pneumocyte

equips surfactant with vitamin E in an active and regulated manner. Alveolar surfactant and its subfractions also contain vitamin A and plasmalogens, the concentrations of which are in the same range as that of vitamin E (Rüstow et al., 1993).

In the present study we determine content, formation and secretion of plasmalogens by type-II cells. We show that type-II cells contain and synthesize predominantly EthPlas. The relative secretion rate of EthPlas by type-II cells, however, is much lower than that of ChoPlas. The higher relative secretion rate of ChoPlas might lead to a higher proportion of ChoPlas in total lung lavage plasmalogens than in type-II cell plasmalogens.

From these results we postulate that type-II cells select between both types of plasmalogens for secretion. Because the patterns of the molecular species of ChoPlas and EthPlas isolated from type-II cells and from lung lavage do not differ significantly, and contain shorter-chain fatty acids only as minor components, we suggest that the choline moiety represents an important intramolecular signal for the secretion of phospholipids.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade and purchased from different suppliers. ¹⁴C-labelled hexadecanol (specific radioactivity: 10.4 mCi/mmol) was from Sigma and ³H- and ¹⁴C-labelled palmitate (specific radioactivities: 54 Ci/mmol and 59 mCi/mmol respectively) were obtained from Amersham. [³H]Hexadecylglycerol was a generous gift of Dr. F. Paltauf (Graz, Switzerland).

Table 1 Relative proportion of ChoPlas and EthPlas in total plasmalogens and total phospholipids isolated from lavage and type-II pneumocytes of rat lung

Source...	ChoPlas		EthPlas	
	Plasmalogens (% of total)	Phospholipids (% of total)	Plasmalogens (% of total)	Phospholipids (% of total)
Type-II pneumocytes	7.2 (0.8)	1.0 (0.3)	92.8 (0.8)	5.4 (0.8)
Lung lavage	36.5 (1.0)	0.43 (0.20)	63.5 (1.0)	0.66 (0.11)

Values are given as means and S.D. values are given in parentheses. Results are from three independent experiments.

Preparation of lung lavage

Male Wistar rats (body wt 150–180 g) were anaesthetized by an intraperitoneal injection of 30 mg of pentobarbital. The lungs were lavaged five times with 8 ml of ice-cold 0.9% NaCl. The individual lavages were immediately centrifuged at 150 g for 10 min.

Type-II cell preparation

Type-II cells were isolated by digestion of lung tissue from one or two animals (Wistar rats, body wt 130–150 g) with elastase and purified by differential adherence on immunoglobulin-coated plates (Dobbs et al., 1986). Viability and purity were 85–93%. The cells were then plated at a density of $(0.8\text{--}1.0) \times 10^6$ cells per 35-mm-diam. tissue-culture dish. Plating efficiency after 18 h was 85–95%.

Plasmalogen synthesis

For each time point 3×10^6 type-II cells were cultured for from 5 min to 18 h with 2 ml of Eagle's minimum essential medium (MEM) containing 10% fetal-calf serum and 2.5 μCi of [^{14}C]hexadecanol and 1 μCi of [^3H]palmitate. The cells were washed and the lipids extracted according to Bligh and Dyer (1959).

Secretion of phospholipids and plasmalogens by type-II cells

For double-labelling experiments 6×10^6 cells were cultured for 18 h with 2 ml of MEM containing either 5 μCi of [^{14}C]hexadecanol and 2 μCi of [^3H]palmitate, or 5 μCi of [^3H]hexadecylglycerol and 2 μCi of [^{14}C]palmitate. Thereafter the cells were washed and 1.5 ml of fresh MEM containing 3 mg/ml albumin was added to each dish. For secretion experiments, isoprenaline (final concentration 1×10^{-6} M) was added after 30 min and the culture was incubated for a further 2 h. The spontaneous and stimulated secretion, as well as the effect of surfactant-specific protein A (SP-A) on secretion, were measured as previously described (Rüstow et al., 1993).

Lipid analysis

Lung lavages, type-II cells or MEM medium were extracted according to Bligh and Dyer (1959). For quantification of the plasmalogen content the lipid extracts were separated by two-dimensional t.l.c. (Rüstow et al., 1987). After methanolysis of the isolated PtdCho and PtdEth fractions the fatty acid methyl ester and the dimethylacetals (representing the plasmalogens) were separated by g.c. The g.c. analyses were carried out using an HP 5890/Series II equipped with a DB 225 column (30 m $\times$ 0.25 mm; 0.25 μm pore diam.), slit/splitless injection at 240 °C and flame ionization detection. The oven programme was: 140–180 °C at

10 °C/min, 180 °C for 8 min; 180–220 °C at 10 °C/min, 220 °C for 8 min; 220–240 °C at 20 °C/min, 240 °C for 18.5 min. For quantification we used margaric acid (C_{17}) as internal standard added to the fraction before methanolysis.

For determination of the secretion rate of ChoPlas and EthPlas as components of surfactant, cells and medium were extracted and the extracts were separated by t.l.c. using CHCl_3 /methanol/water (65:25:4; by vol.) as solvent. Thereafter an acid mixture [2% (v/v) trichloroacetic acid in 8% (v/v) HCl] was dropped on the area of the plate containing the separated phospholipids. After 10 min at room temperature the plates were developed in the second dimension using hexane/acetone (4:1, v/v) as solvent. By this procedure the alk-1-enyl moieties of the plasmalogens were cleaved off as aldehydes. The lipids on the plates were detected using iodine vapour. The areas containing the aldehydes and those areas containing the phospholipids were scraped off and their radioactivities measured. The areas of PtdCho and PtdEth contain, beside the ^3H label of the palmitic acid, ^{14}C label also. However, it was not differentiated what proportion of [^{14}C]hexadecanol was incorporated as the alkyl acyl subclasses, or what proportion was incorporated as palmitic acid after oxidation. The aldehyde fractions cleaved off from plasmalogens contain less than 0.3% of the total ^3H label incorporated into the individual phospholipids.

When the patterns of the molecular species of the ChoPlas and EthPlas had to be determined, the PtdCho and PtdEth fractions isolated by t.l.c. were derivatized to naphthylisocyanates as previously described (Rüstow et al., 1987). For the separation of the alkenyl acyl naphthylisocyanate derivatives of the PtdCho and PtdEth fractions by t.l.c. we used benzene/hexane/ether (52:48:3; by vol.) as solvent. The molecular-species patterns were determined by h.p.l.c. as previously described (Rüstow et al., 1987).

Measurement of radioactivities

The radioactivities were measured in 8 ml of BCS scintillation fluid (Amersham) using a Philips liquid-scintillation counter PW 4700 equipped with automatic compensation for quenching.

RESULTS

Table 1 shows that the plasmalogens of type-II cells consist of 93% EthPlas and 7% ChoPlas. The plasmalogens isolated from alveolar surfactant, however, consist of 36.5% ChoPlas and 63.5% EthPlas. Compared with total phospholipids both types of plasmalogens are minor components of type-II pneumocytes and of lung lavage (Table 1). The different incorporation rates of [^{14}C]hexadecanol into both types of plasmalogen by type-II pneumocytes are reflected in the proportion of the cellular content of each plasmalogen (Table 2). Table 2 demonstrates

Table 2 Incorporation of hexadecanol into ethanolamine plasmalogens and choline plasmalogens by type-II pneumocytes

Adherent type-II pneumocytes (3×10^6) were incubated with $5 \mu\text{Ci}$ of [^{14}C]hexadecanol (sonicated in MEM containing 10% fetal-calf serum) for the time indicated. The values of three independent experiments are given.

Incubation time (min)	Target...	Increase of label incorporated (fold)*		Amount of label incorporated (%)†	
		EthPlas	ChoPlas	EthPlas	ChoPlas
5		1.0	1.0		
10		1.2 (0.3)	0.7 (0.1)	84 (5)	16 (5)
20		2.9 (0.3)	0.5 (0.3)	84 (5)	16 (5)
90		5.0 (2.7)	0.7 (0.4)	93 (2)	7 (2)
180		15.8 (8.6)	1.3 (0.4)	96 (0)	4 (0)
18 h		91.3 (6.7)	9.1 (1.4)	93 (1)	7 (1)

* The label incorporation into EthPlas or ChoPlas after 5 min [EthPlas: 158, 112 and 192 d.p.m. per 3×10^6 type-II cells; ChoPlas: 47, 40 and 85 d.p.m. per 3×10^6 type-II cells] was fixed as 1.

† The sum of label incorporated into EthPlas and ChoPlas was 100%.

Table 3 Secretion of phospholipids and plasmalogens by type-II pneumocytes

Type-II cells were prelabelled for 18 h. Thereafter the cells were washed and incubated with medium containing isoprenaline or $1 \mu\text{g}$ of SP-A/ml medium or both. Values of three to five independent experiments are expressed as label measured in the medium as percentage of the total (label in cells plus label in medium) after incubation for 2 h. S.D. values are given in parentheses.

Prelabelling	Secretion (%)	
	Spontaneous	Isoprenaline-stimulated
(1) ^3H - or ^{14}C -labelled palmitic acid		
Total phospholipid		
without SP-A	2.3 (0.2)	5.1 (0.5)
with SP-A	1.8 (0.4)	1.8 (0.6)
(2) With [^{14}C]hexadecanol		
EthPlas		
without SP-A	2.0 (1.5)	2.7 (1.2)
with SP-A	1.5 (0.8)	1.8 (0.8)
ChoPlas		
without SP-A	10.0 (3.8)	18.4 (6.4)
with SP-A	7.8 (2.8)	8.9 (1.9)
(3) With [^3H]hexadecylglycerol		
EthPlas		
without SP-A	3.2 (1.6)	4.1 (2.4)
with SP-A	1.7 (0.8)	1.7 (0.9)
ChoPlas		
without SP-A	9.0 (2.1)	14.6 (5.4)
with SP-A	4.4 (2.0)	6.5 (3.6)

that there is no, or very little, incorporation of label into ChoPlas between 5 and 180 min of incubation. The incorporation of [^{14}C]hexadecanol into ChoPlas depends on the incorporation rate into EthPlas, which represent the direct precursors for the synthesis of ChoPlas. Increased amounts of hexadecanol used for incubation did not have a linear correlation with the formation of labelled EthPlas and ChoPlas. The incorporation rate into both types of plasmalogens was the same regardless of the precursor (^3H]glycerol or [^3H]hexadecylglycerol) used. Also,

Table 4 Molecular species of EthPlas and ChoPlas isolated from type-II pneumocytes and alveolar surfactant

Molecular species	EthPlas and ChoPlas content (% of total)			
	Type-II pneumocytes		Alveolar surfactant	
	EthPlas	ChoPlas	EthPlas	ChoPlas
16:0-18:1				
16:0-16:0	1.2 (0.8)	2.8 (0.7)	0.6 (0.4)	1.0 (0.3)
16:0-18:2				
18:1-20:4	12.0 (1.8)	12.3 (0.3)	12.0 (1.5)	8.1 (1.1)
16:0-20:4				
16:0-22:6	40.5 (2.4)	38.9 (5.3)	35.9 (2.7)	45.2 (4.3)
16:0-20:5				
18:2-20:4	3.2 (0.8)	1.4 (0.3)	4.6 (1.0)	2.5 (0.2)
18:2-22:6				
18:0-18:2	2.0 (0.6)	3.5 (0.7)	6.5 (2.0)	1.1 (0.3)
18:0-20:4	16.8 (0.3)	13.5 (3.1)	18.9 (3.5)	10.8 (1.2)
18:0-20:5				
18:1-18:2	4.6 (1.0)	3.9 (0.9)	4.5 (0.8)	4.3 (0.6)
18:0-22:6	12.1 (0.5)	14.1 (2.1)	4.1 (0.4)	8.0 (1.8)
Not identified	6.6 (2.6)	9.6 (2.4)	13.8 (2.5)	14.2 (2.0)

Values are given as percentages of total of $n = 3$ experiments; S.D. values are given in parentheses.

the results were similar for adherent cells, as for cells in suspension (results not shown).

Table 3 shows the spontaneous and isoprenaline-stimulated secretion rates of palmitate-labelled phospholipids and of hexadecanol- or hexadecylglycerol-labelled plasmalogens, as well as the inhibitory effect of SP-A on the secretion rates. In each case the experiments were carried out as double-labelling experiments with [^3H]palmitate and [^{14}C]hexadecanol, or with [^{14}C]palmitate and [^3H]hexadecylglycerol. The spontaneous and isoprenaline-stimulated secretion of ChoPlas exceeds that of EthPlas by a factor between three and six. SP-A inhibits the secretion of surfactant phospholipid and both types of plasmalogens. The variability of the relative secretion rates of the plasmalogens is relatively high. It has to be taken into account that the measurement of the low level of radioactivity secreted into the medium is linked to a high variation, although at each individual measurement the blank was carefully corrected and the time of counting was extended to 10 min. But it cannot be excluded that the complicated time-consuming analysis, in combination with the instability of plasmalogens due to oxidation, is an additional reason for this variability. However, in each individual experiment the secretion rate of ChoPlas was clearly higher than that of EthPlas.

Table 4 shows the patterns of the molecular species of ChoPlas and EthPlas isolated from type-II pneumocytes or from lung lavage respectively. As in other tissues, the plasmalogens of type-II cells and alveolar surfactant consist mainly of species containing polyunsaturated fatty acids. The short-chain or saturated fatty acid-containing species are minor components at most.

DISCUSSION

As in most mammalian cells, plasmalogens of type-II pneumocytes consist mainly of EthPlas (Table 1). The *de novo* synthesis of ChoPlas and of EthPlas (as measured by the incorporation of labelled hexadecanol, hexadecylglycerol or glycerol) reflects the cellular content of both types of plasmalogens (Table 2). The higher relative proportion of ChoPlas in

lung lavage compared with that in type-II cells (Table 1) could be due to differential secretion into, or differential clearance from, the extracellular compartment. Nothing is known about a preferred enzymic or oxidative degradation of ChoPlas in the alveolar space, but we did find a higher relative secretion rate of ChoPlas (Table 3). Therefore we suggest that the different proportion of ChoPlas in total plasmalogens in type-II cells versus lung lavage is caused by a preferential secretion, into the alveolar space, of this type of plasmalogen as a component of surfactant.

The results of the double-labelling experiments argue in favour of the hypothesis that ChoPlas are at least in part secreted as a constituent of lung surfactant. They show that ChoPlas are secreted as components of surfactant phospholipids and that SP-A, a specific surfactant protein known to inhibit the secretion of surfactant phospholipids (Dobbs et al., 1987), also inhibits the secretion of these plasmalogens to nearly the same extent as it inhibits surfactant secretion (Table 3). The difference between the spontaneous secretion rate of ChoPlas and that of surfactant phospholipids may be explained by assuming that ChoPlas are also secreted independently from lung surfactant.

The content of EthPlas in alveolar lavage is twice that of ChoPlas despite their lower relative secretion rate. Isoprenaline does not stimulate secretion of EthPlas to the same extent as it does with surfactant or ChoPlas. This would suggest constitutive secretion of EthPlas, probably independent of surfactant secretion. Different turnover kinetics might also be possible.

We suggest that type-II cells select between both types of plasmalogens for secretion as constituents of surfactant. Recently Longmuir and Haynes (1991) suggested that the chain length of the fatty acids of phospholipids represents the major intramolecular signal for the selection of phospholipid species to be secreted as constituents of surfactant by type-II cells. To apply this hypothesis to plasmalogen secretion, we determined the patterns of the molecular species of ChoPlas and EthPlas isolated from type-II cells and from lung lavage (Table 4). The species patterns show, however, that, as in other sources, ChoPlas as well as EthPlas in type-II cells and in lung surfactant consist predominantly of molecular species containing long-chain polyunsaturated fatty acids. The biosynthetic pathways for diacylphospholipids and ChoPlas are totally different (Lee et al., 1991). Nevertheless type-II cells preferentially secrete both choline-containing phospholipids although the content of ChoPlas in type-II cells is clearly below the content of EthPlas. Therefore the intramolecular sorting signal for preferential secretion of ChoPlas as a constituent of surfactant by type-II cells cannot be chain length. The only difference between both types of plasmalogens is their polar head group. Thus we suggest that the choline moiety represents the most important intramolecular sorting signal for secretion as a constituent of the alveolar surfactant by type-II cells.

The chain length of the fatty acids of phospholipids might represent another less important signal. That would explain why we find higher proportions of the dipalmitoyl species of PtdCho, phosphatidylglycerol and phosphatidylinositol in the alveolar surfactant synthesized *de novo* in lung or present in these phospholipid fractions isolated from type-II cells (Rüstow et al., 1985, 1988; Schlame et al., 1988).

Plasmalogens are thought to be intracellularly synthesized antioxidants. One could speculate that in cases of a limited

supply of vitamin E in the lung, the antioxidative protection of alveolar surfactant can be augmented by an increased formation and secretion of plasmalogens by the type-II cell. Another possible function of plasmalogens in the alveolar surfactant should also be considered. The monolayer on the air-liquid interface of the alveoli is generated from tubular myelin, a structure with a complex morphology consisting of a lattice-like network of long tubes, the walls of which consist mainly of a phospholipid bilayer (Williams, 1982). To generate the monolayer, this structure must be rendered unstable. It has been proposed that discontinuities in the bilayer of surfactant lipids or, if present, non-bilayer structures in this material could serve as initiation sites for the transfer of lipids to the air-liquid interface. Metcalfe et al. (1980) noted that non-bilayer structures in tubular myelin seem to be indeed involved in this process of film formation. In artificial surfactant suspensions lipids promoting H_{II} phases increase the adsorption of lipids to the air-liquid interface (Yu et al., 1984). It is interesting that plasmalogens promote and stabilize H_{II} structures of phospholipids (Lohner et al., 1991). Therefore it might be speculated that plasmalogens, which themselves can form non-bilayer structures and often make phospholipids do so, may have an important role in the generation of the alveolar monolayer.

Whatever might be the main function of plasmalogens in the alveolar surfactant they are secreted in a regulated manner, at least partly as an integral constituent of the alveolar surfactant. Understanding the function of plasmalogens would be helpful to obtaining a deeper insight into the causes of lung surfactant dysfunction.

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