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Lipoproteins secreted by cultured rat hepatocytes contain the antioxidant 1-alk-1-enyl-2-acylglycerophosphoethanolamine

Jean E. Vance

Lipid and Lipoprotein Group and Department of Medicine, University of Alberta, Edmonton (Canada)

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Monolayer cultures of rat hepatocytes have been examined for their ability to secrete ethanolamine plasmalogen as a component of nascent lipoproteins. In culture medium from these cells, ethanolamine plasmalogen comprises approx. 20–30% of total ethanolamine glycerophospholipids when measured either as phospholipid mass or by the incorporation of [1-^{3}H]ethanolamine. An approximately equal distribution of the plasmalogen was found throughout all lipoprotein density fractions. The content of plasmalogen in whole rat serum, was 36% of total ethanolamine glycerophospholipids. In contrast, in rat liver and cultured hepatocytes the amount of ethanolamine plasmalogen was 5-fold lower than in serum or culture medium (approx. 5% of total ethanolamine phospholipids). Normal human plasma also contains ethanolamine plasmalogen in relatively large amounts (approx. 50% of total ethanolamine phospholipids). Thus, a major function of plasmalogen biosynthetic enzymes in liver may be the provision of ethanolamine plasmalogen for secretion into lipoproteins. Previous studies (e.g., Zoeller, R.A. et al. (1988) J. Biol. Chem. 263, 11590–11596) have suggested that ethanolamine plasmalogen may function as an antioxidant for the protection of lipid and protein membrane components against oxidation. Oxidized, but not native, low-density lipoprotein is rapidly taken up by macrophages with the formation of foam cells characteristic of atherosclerotic lesions (Steinbrecher, U.P. et al. (1984) Proc. Natl. Acad. Sci. USA 81, 3883–3887). Thus, the presence of plasmalogen as part of newly secreted lipoprotein particles may prevent their oxidation and subsequent uptake by macrophages.

Introduction

Ethanolamine plasmalogens (1-alk-1-enyl-2-acylglycerophosphoethanolamines) are glycerophospholipids that contain a vinyl ether moiety at the *sn*-1 position and an acyl moiety at the *sn*-2 position. Few specific functions have been ascribed to the ether glycerolipids apart from the potent biological effects of platelet activating factor (1-alkyl-2-acetylglycerophosphocholine) in processes such as inflammation, hypertension and anaphylaxis [1] and the known effects of plasmalogens in altering the physical properties of membranes [2–4]. Recently, using a Chinese hamster ovary mutant cell line deficient in peroxisomes and hence defective in plasmalogen biosynthesis, Zoeller et al. [5] have demonstrated that ethanolamine plasmalogen is apparently necessary for protection of the cells against oxidation by reactive oxygen species. In addition, it has been proposed that the deficiency of plasmalogens might be responsible for at least some of the clinical symptoms of Zellweger's syndrome in which the patients lack peroxisomes [6,7,8].

PtdEtn isolated by traditional thin-layer chromatographic techniques is a mixture of 1-alkyl-2-acyl-, 1-alkenyl-2-acyl- and 1,2-diacylglycerophosphoethanolamine, although in most tissues and cells, the diacyl species is by far the most abundant [9]. In liver, for example, the two types of both ethanolamine and choline ether lipids are minor components of the glycerolipids [9], although the enzymes involved in the synthesis of these lipids are active in this tissue [10].

PtdCho is the major phospholipid constituent of all plasma lipoproteins [11], but these particles also contain smaller amounts of a variety of other phospholipids such as PtdEtn, which comprises approx. 5% of total plasma phospholipids [11]. The biological significance of the presence of PtdEtn in lipoproteins is unknown.

Abbreviations: EtnPm, ethanolamine plasmalogen; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; ChoPm, choline plasmalogen; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Correspondence: J.E. Vance, Lipid and Lipoprotein Group and Department of Medicine, University of Alberta, Edmonton, Alberta T6G 2S2, Canada.

In the present study, the secretion of lipoproteins containing EtnPm by monolayer cultures of rat hepatocytes has for the first time been demonstrated. In addition, EtnPm has been found as a significant proportion (approx. 30%) of rat serum ethanolamine phospholipids. There have been previous reports [12,13] that normal human plasma also contains EtnPm in relatively large amounts (approx. 50% of total ethanolamine phospholipids), although whether or not the plasmalogen was secreted by liver as part of nascent lipoproteins has not been established. A potential role of EtnPm as a natural antioxidant for the protection of plasma lipoproteins against oxidation, and thus a protection against their uptake by macrophages and the development of foam cells, is discussed.

Materials and Methods

Female Sprague-Dawley rats were used for all experiments. Earle's minimum essential medium was obtained from Gibco Laboratories (Grand Island, NY) and Primaria culture dishes (60 mm) from Becton Dickson (Oxnard, CA). Silica-gel G 60 thin-layer chromatography plates (0.25 mm thickness) were from BDH Chemicals. Cab-O-Sil and butylated hydroxytoluene were purchased from Sigma. The radioactive phospholipid precursors [1-^{3}H]ethanolamine (23 Ci/mmol) and [3-^{3}H]serine (37 Ci/mmol) were from Amersham (Oakville, Ontario, Canada). Phospholipid standards PtdCho, PtdEtn, lyso-PtdEtn and lyso-PtdCho were purchased from Avanti Polar Lipids (Birmingham, AL). Standard EtnPm from pig heart was kindly provided by Dr. Patrick C. Choy, University of Manitoba, Winnipeg, Manitoba.

Monolayer cultures of rat hepatocytes were prepared by the collagenase perfusion technique as previously described [14,15]. Cell viability was > 90% as assessed by Trypan Blue exclusion and leakage of lactate dehydrogenase. Hepatocytes were plated overnight in medium containing 17% fetal calf serum, after which the cells were washed and incubated in serum-free medium for the duration of the experiment. Cells were scraped from dishes in ice-cold phosphate-buffered saline and lipids were extracted as described previously [16], except that the antioxidant butylated hydroxytoluene was present in all organic solvents (50 mg/l). The culture medium containing the secreted lipoproteins was collected and centrifuged at 10000 × *g* for 10 min to remove any dead cells and cell debris.

In some experiments total medium lipoproteins were concentrated by addition of Cab-O-Sil, a form of colloidal silica [15]. In other experiments, medium lipoproteins were first fractionated according to density by single-spin ultracentrifugation on a salt gradient [15], then lipoproteins were concentrated by addition of Cab-O-Sil. The lipoproteins bound to the Cab-O-Sil were pelleted by centrifugation at 10000 × *g* for 15 min, after which the lipids were extracted [15,16]. The ethanolamine and choline glycerophospholipids were separated from total lipids by thin-layer chromatography on Silica-gel G 60 (0.25 mm thickness) in chloroform/methanol/acetic acid/water (50 : 30 : 8 : 3, v/v) containing 50 mg/l of butylated hydroxytoluene. The PtdCho and PtdEtn bands, which contained both diacyl and ether phospholipids, were identified by comparison with standard phospholipids after spraying the plate with Primulin [17]. Immediately, the band was scraped from the plate and the lipid was extracted from the Silica-gel [18]; all solvents contained 50 mg/l of butylated hydroxytoluene. The organic solvents were evaporated under a stream of nitrogen gas and mild acid hydrolysis was performed as follows. The phospholipid residue was exposed for 10 min to a stream of HCl gas generated by dripping concentrated HCl on to concentrated H_2SO_4. The vinyl ether linkage of EtnPm and ChoPm was labile to acid hydrolysis and was cleaved with formation of 1-lyso-PtdEtn and 1-lyso-PtdCho, respectively. Diacyl and alkylacyl phosphoglycerolipids were unaffected by acid treatment. Subsequently, all traces of HCl were removed by exposure of the sample to a stream of nitrogen for 5 min and lyso-phospholipids were separated from the diradyl phospholipids by thin-layer chromatography in chloroform/methanol/acetic acid/water (50 : 30 : 8 : 3, v/v). Lyso-PtdEtn, PtdEtn, lyso-PtdCho and PtdCho were identified by comparison with standard phospholipids. Completeness of the acid hydrolysis was > 95%. The chemical amounts of EtnPm, ChoPm, PtdEtn and PtdCho were determined by phosphorus analysis [19].

Rat serum was obtained from blood collected by cardiac puncture of rats fasted overnight. Lipids were extracted from serum by addition of three volumes of chloroform/methanol (2 : 1, v/v) containing 2% acetic acid and 50 mg/l of butylated hydroxytoluene and the lower phase was washed three times with two volumes of methanol/water (1 : 1, v/v). In other experiments, VLDL, LDL and HDL were first isolated from serum by sequential centrifugation [20] before the lipids were extracted. Lipids were extracted from a homogenate of rat liver and subcellular membrane fractions by basically the same method as for cultured hepatocytes. The content of EtnPm, ChoPm, PtdEtn and PtdCho in blood and/or liver lipids was measured as described above for hepatocyte cells and medium. Protein was determined by the method of Lowry et al. [21].

Results

The EtnPm content of rat serum and liver was measured (Table I). In agreement with the results of others [9], the percentage of ethanolamine phosphoglycerolipid in liver that was EtnPm was low (5.8%). On the con-

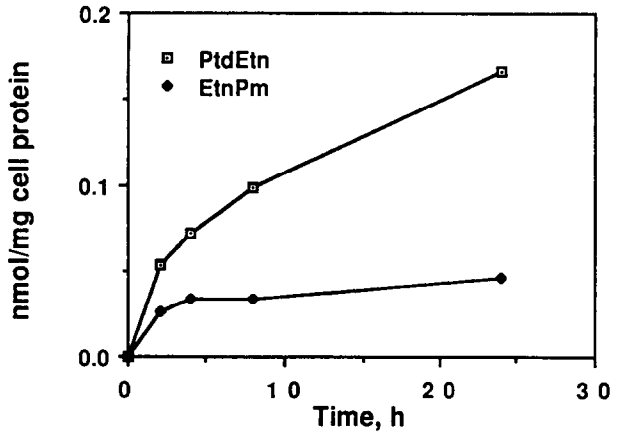

Fig. 1. Secretion of PtdEtn and EtnPm by monolayer cultures of rat hepatocytes. The culture medium from 48 dishes of hepatocytes was collected after the indicated times of incubation. The ethanolamine-containing phospholipids were extracted and isolated by thin-layer chromatography. EtnPm was converted into lyso-PtdEtn by mild acid hydrolysis and the content of PtdEtn and EtnPm (lyso-PtdEtn) was determined by phosphorus analysis. The data are from one of three similar experiments.

trary, in rat serum EtnPm was a major component of total ethanolamine phospholipids (36.1%). Since the percentage of total ethanolamine phospholipid that was EtnPm was 6-fold lower in liver than in serum, the origin of serum plasmalogen was not clear. EtnPm could have arisen from several possible sources: (a) by secretion from the liver; (b) by secretion from the intestine; and/or (c) from a non-secreted source, such as diet or blood cell membranes. Thus, monolayer cultures of rat hepatocytes were examined for their ability to secrete EtnPm directly into nascent lipoproteins. As shown in Table I, the EtnPm content of hepatocytes was comparable to that in liver, however, approx. 30% of total ethanolamine glycerophospholipid in lipoproteins from culture medium was EtnPm - a percentage similar to that in serum. The techniques used for measurement of EtnPm and PtdEtn did not separate 1-alkyl-2-acylglycerophospholipid from PtdEtn. However, the content of alkyl ethers in rat liver and human serum has been reported to be very low (1–3% [9], and 8% [13], respectively).

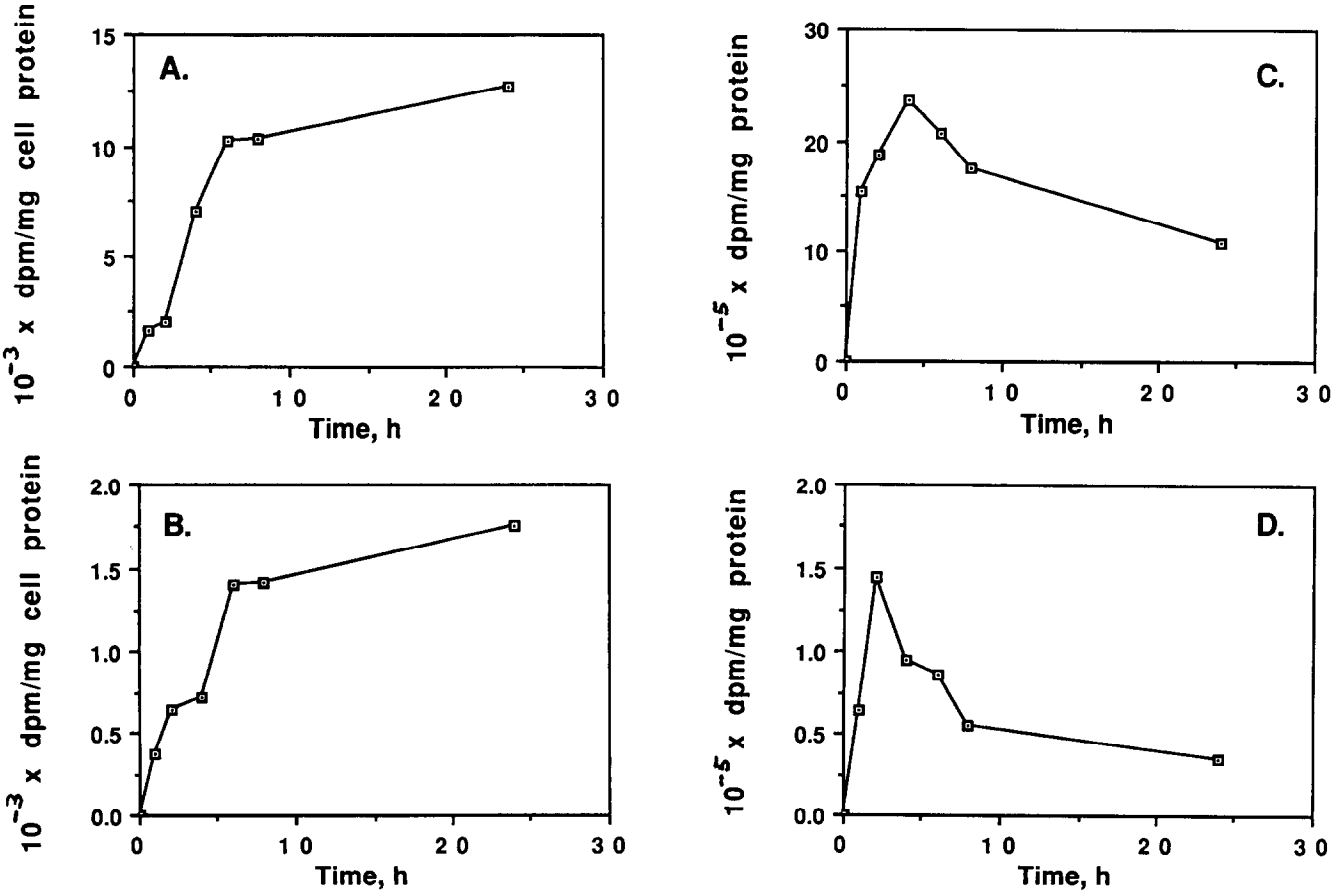

Fig. 2. Secretion of [1-^{3}H]ethanolamine-labeled PtdEtn and EtnPm by cultured rat hepatocytes. Monolayer cultures of rat hepatocytes were incubated for various times up to 24 h with 10 μCi/dish of [1-^{3}H]ethanolamine. Cells and medium were harvested (24 dishes combined) and the incorporation of [^{3}H] into PtdEtn and EtnPm was determined. (A) PtdEtn from culture medium; (B) EtnPm from culture medium; (C) PtdEtn from cells; (D) EtnPm from cells. The data are from one of two similar experiments.

TABLE I

Concentration of EtnPm and PtdEtn in rat liver, rat serum, monolayer cultures of rat hepatocytes and culture medium

	nmol phospholipid		% of total ethanolamine glycerophospholipid as EtnPm
	PtdEtn	EtnPm	
Rat liver [a]	1175 ±288	72.4 ±17.7	5.8±0.4
Rat serum [b]	5.6 ± 1.1	3.2 ± 0.4	36.1±2.0
Cultured hepatocytes [c]	16.3 ± 2.9	1.3 ± 0.5	5.7±2.8
Culture medium [d]	0.48± 0.22	0.19± 0.10	28.2±1.5

[a] Data from four independent experiments; units are nmol/g liver.
[b] Data from four independent experiments; units are nmol/ml serum.
[c] Data from six independent experiments; units are nmol/mg cell protein.
[d] Data from four independent experiments; units are nmol/mg cell protein per 6 h.

A time-course for secretion of EtnPm and PtdEtn by cultured rat hepatocytes is shown in Fig. 1. At early time-points, the amount of EtnPm relative to PtdEtn was higher than at later times. The most likely explanation for this observation is that the vinyl ether linkage of EtnPm is readily oxidized (see Discussion).

A similar result was obtained when hepatocytes were labeled with [1-^{3}H]ethanolamine (Fig. 2) which would be expected to label all ethanolamine phospholipids made via the CDP-ethanolamine pathway. The secretion of labeled PtdEtn and EtnPm increased with time (Figs. 2A and B). In hepatocytes the percentage of total labeled ethanolamine phospholipids that was EtnPm was much lower than in medium lipoproteins (Figs. 2C and D), in agreement with mass measurements (Fig. 1 and Table I).

In rat hepatocytes, PtdEtn is derived from both the CDP-ethanolamine pathway [22] and the decarboxylation of phosphatidylserine [23]. Thus, in cells incubated with [3-^{3}H]serine, PtdEtn will be labeled in the ethanolamine head-group moiety. In lipoproteins secreted by these hepatocytes, we have demonstrated that there is a preference for secretion of PtdEtn derived from phosphatidylserine decarboxylation and a discrimination against secretion of PtdEtn biosynthesized from CDP-ethanolamine [16]. The biosynthesis of EtnPm involves formation of 1-alkyl-2-acylglycerol from dihydroxyacetone phosphate (the first two steps of which occur in peroxisomes) and the subsequent transfer of the phosphoethanolamine moiety from CDP-ethanolamine [1]. Thus, indirectly, the ethanolamine head-group of EtnPm might be labeled from [^{3}H]serine by one of the following routes: (a) degradation of serine-labeled PtdEtn to (phospho)ethanolamine which might be incorporated into CDP-ethanolamine and hence into EtnPm; (b) base exchange of serine-labeled PtdEtn with an EtnPm molecule; or (c) decarboxylation of serine plasmalogen to EtnPm. When hepatocytes were incubated with [3-^{3}H]serine, in a protocol parallel to that used for the [^{3}H]ethanolamine labeling experiment, 0.5 to 3% of the labeled cellular ethanolamine glycerophospholipid was present as EtnPm (for example, after 8 h there were 219 dpm/mg cell protein in EtnPm vs. 31118 dpm/mg cell protein in PtdEtn). EtnPm from secreted lipoproteins was labeled to a similar extent (at 8 h, 2.9 and 98.0 dpm/mg cell protein were in EtnPm and PtdEtn, respectively). Thus, there was no pronounced preference for secretion of serine-derived EtnPm.

The distribution of EtnPm among individual lipoproteins was determined in both rat serum (Table II) and

TABLE II

EtnPm and ChoPm content of lipoproteins isolated from rat serum

Lipoprotein fractions were separated on the basis of density by ultracentrifugation of rat serum. The amounts of EtnPm, ChoPm, PtdEtn and PtdCho were determined in each fraction. The data are averages ± S.D. from three different samples of blood.

Lipoprotein fraction	nmol/10 ml serum		% of total ethanolamine phosphoglycerolipid as EtnPm	% of total choline phosphoglycerolipid as ChoPm [a]
	PtdEtn	EtnPm		
VLDL	18.9±1.7	3.5±2.5	14.7± 7.9	1.6±0.1
LDL	3.1±0.6	1.3±0.3	32.2± 5.7	1.9±0.2
HDL	25.1±2.2	21.7±4.2	46.3±12.4	2.4±1.2

[a] Total nmol of choline-containing phospholipids/10 ml serum in VLDL, LDL and HDL were: 568, 208 and 1406, respectively.

TABLE III

EtnPm and ChoPm content of lipoproteins isolated from culture medium of isolated rat hepatocytes

Monolayer cultures of rat hepatocytes were incubated in serum-free medium for 18 h and lipoprotein fractions were isolated by ultracentrifugation on the basis of their densities. The content of PtdEtn, PtdCho, EtnPm and ChoPm in each fraction was determined. The data are the averages of two experiments in which the individual values did not differ from the mean by more than 12%.

Lipoprotein fraction	nmol/10 mg cell protein		% of total ethanolamine phospholipid as EtnPm	% of total choline phospholipid as ChoPm [a]
	PtdEtn	EtnPm		
VLDL	3.02	0.86	22.2	2.4
LDL	0.78	0.36	31.6	5.1
HDL	1.05	0.43	29.1	5.4

[a] Total nmol of choline-containing phospholipids/10 mg cell protein in VLDL, LDL and HDL were: 47.7, 11.8 and 24.3, respectively.

in hepatocyte culture medium (Table III). Lipoproteins were separated by ultracentrifugation according to the following densities: VLDL < 1.006, LDL 1.006–1.063, HDL 1.063–1.18 g/ml [15,20]. Tables II and III demonstrate that EtnPm was present in all classes of both serum and nascent lipoproteins. In serum HDL, the percentage of total ethanolamine glycerolipids that was EtnPm was higher than in VLDL (46.3% vs. 14.7%). In addition, there was a higher percentage of EtnPm in serum HDL than in newly-secreted HDL (46/3% vs. 29.1%), probably due to the complex metabolism of lipoproteins that occurs in the circulation, and/or the contribution of intestine-derived lipoproteins to the total serum lipoproteins.

The ChoPm content of rat liver and human serum has been reported to be 1.3% [9] and 3.6% [13], respectively, of the choline-containing glycerophospholipids. Rat serum lipoproteins and hepatocyte medium lipoproteins were examined for their ChoPm content. ChoPm comprised only 2% and 2 to 5%, respectively, of total choline-containing glycerophospholipids (Tables II and III). The amount of alkyl glycerophosphocholine was not measured in these experiments, but in human serum and rat liver this ether lipid is a minor component, 3.4% [13] and 1 to 3% [9], respectively, of the total choline-containing glycerophospholipids].

Discussion

These experiments demonstrate for the first time that EtnPm is present in VLDL and HDL secreted by cultured rat hepatocytes in quantities that probably account for the level of EtnPm in rat plasma. Since human plasma also contains significant quantities of EtnPm (approx. 50% of total ethanolamine glycerophospholipids [12,13]) one may anticipate that this also originates from hepatic secretion. Paradoxically, the levels of EtnPm in liver are low (Table I) so that the reason for the relatively high enzyme activities for biosynthesis of ether lipids in liver has always been an enigma. It now seems likely that a major function of EtnPm biosynthetic enzymes in liver may be the provision of EtnPm for secretion into lipoproteins.

The present experiments do not exclude the possibility that the PtdEtn and/or EtnPm of nascent lipoproteins in the culture medium are acquired from the cell surface. Previous experiments, however, have demonstrated the presence of PtdEtn in nascent VLDL particles isolated from rat liver Golgi [24,25] at a concentration higher than that in circulating lipoproteins [26]. The level of EtnPm in rat liver and hepatocytes is approx. 5.8% of total ethanolamine phospholipids, whereas in newly secreted lipoproteins EtnPm is much more abundant - approx. 28% of ethanolamine glycerophospholipid. Therefore, if EtnPm were acquired by lipoproteins from the cell surface, after secretion of the particles, the transfer would have to be highly selective for EtnPm. Such a transfer might occur if the plasma membrane of hepatocytes were enriched in EtnPm. Thus, subcellular membrane fractions were isolated from rat liver and characterized for their purity, as described previously [27]. The PtdEtn and EtnPm content of the different organelle membranes (endoplasmic reticulum, Golgi, mitochondria and plasma membrane) was determined as described under Materials and Methods. In the plasma membrane fraction, the content of PtdEtn and EtnPm was 79.4 and 6.0 nmol/mg protein, respectively. The EtnPm content of the plasma membrane (7.0% of ethanolamine phospholipid) was, therefore, very similar to that of the liver homogenate (5.8%) and the endoplasmic reticulum (5.1%). Thus, the plasma membrane does not appear to be enriched in EtnPm relative to other subcellular membranes. Consequently, there is no evidence for the post-secretory acquisition of EtnPm by nascent lipoproteins.

The secretion of VLDL apoproteins, triacylglycerols and phosphatidylcholine by cultured rat hepatocytes is approximately linear with time for at least 24 h [15,16]. As shown in Fig. 1, the secretion of PtdEtn mass continued for the 24 h duration of the experiment. On the contrary, after 4 h there was no further accumulation of EtnPm in the medium, whether measured as mass (Fig. 1) or incorporation of [^{3}H]ethanolamine (Fig. 2B). The most likely explanation for the lack of EtnPm accumulation is not that EtnPm secretion had ceased, but that EtnPm secretion continued and that EtnPm, which is highly sensitive to oxidation [5] was oxidized in the medium.

Although there was a continual increase in PtdEtn mass in the culture medium for at least 24 h (Fig. 1), [^{3}H]ethanolamine-labeled PtdEtn did not accumulate in the medium after 6 h (Fig. 2A). A plausible explanation for this observation is that secreted PtdEtn, labeled from [^{3}H]ethanolamine, reflects the labeling of PtdEtn in the cells (Fig. 2C). Ethanolamine was rapidly incorporated into cellular PtdEtn (Fig. 2C) and EtnPm (Fig. 2D) but after 6 h there was a rapid decline in radiolabel in the two phospholipids, most likely as a result of rapid degradation of PtdEtn and depletion of [^{3}H]ethanolamine as substrate. In support of this explanation, when hepatocytes were labeled with [3-^{3}H]serine, the amount of [^{3}H]labeled PtdEtn in the medium (synthesized via decarboxylation of phosphatidylserine) increased approximately linearly with time for at least 24 h (data not shown). Thus, PtdEtn and, most likely, EtnPm continue to be secreted into the medium for the duration of the experiment.

EtnPm is apparently distributed among all individual lipoprotein classes in both rat serum (Table II) and hepatocyte culture medium (Table III). Whether or not the EtnPm is originally secreted in both nascent VLDL and HDL has not been established. It is possible that

EtnPm is secreted as part of a single lipoprotein class (e.g., VLDL) and that other lipoproteins (e.g., HDL) acquire their EtnPm by exchange from VLDL. Such a possibility cannot be excluded at present but since the concentration of lipoproteins in the culture medium is very low, a rapid exchange and equilibration of EtnPm among the different lipoprotein classes does not seem likely.

A potential biological role of EtnPm is as an antioxidant. Raetz and co-workers [5,28] have recently examined a line of Chinese hamster ovary mutant cells which, when compared to wild type cells, had a 10-fold reduced content of plasmalogen. The deficiency was most likely the result of a defect in the biogenesis of peroxisomes [5], which are involved in the first two steps of plasmalogen biosynthesis. The mutant cells were several orders of magnitude more sensitive than wild-type cells to killing by oxidation conditions that generated singlet oxygen and various reactive radical species [5,24]. When mutant cells were supplemented with 1-*O*-hexadecyl-*sn*-glycerol their EtnPm content was restored, without restoration of peroxisome biogenesis, and the increased sensitivity to oxidation was abolished [5]. It appears, therefore, that the vinyl ether linkage enabled EtnPm to act as a scavenger of reactive oxygen species and thereby protect against oxidation. There is additional evidence that the vinyl ether linkage of ethanolamine plasmalogen renders the molecule extremely sensitive to oxidation [28–31].

In the context of lipoprotein metabolism, the presence of EtnPm as an antioxidant may be of great importance. The majority of lipid-laden foam cells of atherosclerotic lesions are believed to be derived from macrophages [32]. However, macrophages in culture do not readily take up native LDL by the classical LDL receptor [33]. Instead, macrophages have an additional lipoprotein receptor, commonly called the scavenger receptor or the acetyl-LDL receptor [33,34]. This receptor specifically recognises certain chemically modified forms of LDL, causing accumulation of cholesterol ester in a process which is not down-regulated by the cells' cholesterol level [33]. Consequently, modified LDL uptake continues until macrophages morphologically resemble foam cells [33]. One form of modification of LDL that allows its uptake via the macrophage scavenger receptor is oxidation, which can be achieved by several different mechanisms [35], since native LDL is highly sensitive to oxidation. During oxidation there are extensive changes in the structure of apoprotein B, including oxidation of lysine residues and degradation of the apo B molecule [35]. In addition, peroxidation of polyunsaturated fatty acyl moieties occur at the *sn*-2 position of PtdCho and subsequently, PtdCho is hydrolysed to lyso-PtdCho through the action of a specific phospholipase A_2 contained in LDL [35,36]. All of these oxidative changes in LDL, as well as the enhanced uptake of LDL by macrophages, could be blocked by antioxidants added to the culture medium [35,36].

Thus, the biological function of EtnPm in nascent lipoproteins may be as an antioxidant for the protection of lipoproteins against oxidation. Consequently, their uptake by the scavenger receptor of macrophages would be prevented. A lack of EtnPm in lipoproteins may be responsible for their atherogenic potential by allowing macrophages to accumulate cholesterol ester-containing lipoproteins which have become oxidized. Experiments designed to test this hypothesis are currently underway.

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