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Metabolic Fate of [^{14}C]-Ethanol into Endothelial Cell Phospholipids Including Platelet-Activating Factor, Sphingomyelin and Phosphatidylethanol

Roberta M. Magai Shivendra D. Shukla

Department of Pharmacology, University of Missouri School of Medicine, Columbia, Mo., USA

Key Words

Phospholipids · Ethanol · Platelet-activating factor · Sphingomyelin · Phosphatidylethanol · Endothelial cells

Abstract

The metabolic fate of ethanol into the phospholipid pool of calf pulmonary artery endothelial cells was studied. [^{14}C]-ethanol was incorporated into various endothelial cell phospholipids including phosphatidylethanol (PEth), which may represent a substantial fraction in microdomains of membrane phospholipids. The incorporation into phospholipids was reduced in the presence of pyrazole and cyanamide, inhibitors of ethanol metabolism. Wortmannin, the phosphatidylinositol 3-kinase inhibitor, increased [^{14}C]-PEth formation. [^{3}H]-acetate was also incorporated into endothelial cell phospholipids but in a different pattern. Distribution of [^{3}H]-acetate and [^{14}C]-ethanol into the fatty acyl moiety versus the glycerophosphoryl backbone of the phospholipids was also different. Stimulation of the endothelial cells with ATP increased [^{3}H]-acetate incorporation into platelet-activating factor (PAF) and ethanol decreased it. Ethanol exposure increased ATP-stimulated [^{3}H]-acetate incorporation into sphingomyelin. However, ATP had no effect on the incorporation of [^{14}C]-ethanol into any phospholipids. The results suggest that the two precursors contribute to a separate acetate pool and that the sphingomyelin cycle may be sensitized in ethanol-treated cells. Thus, metabolic conversions of ethanol into lipids and the effect of ethanol on specific lipid mediators, e.g PAF, PEth and sphingomyelin, may be critical determinants in the altered responses of the endothelium in alcoholism.

Early research suggested that ethanol strongly interacts with biological membranes by partitioning into the lipid bilayer [19]. More recently, ethanol has been shown to interact with membrane-associated signal transduction mechanisms that rely on the reaction of phospholipases with their phospholipid substrates in the membrane [9, 20]. However, it is not known whether and how much of this ethanol is distributed into the cellular lipid. The main pathway for ethanol metabolism involves hepatic alcohol dehydrogenase (ADH), an enzyme that catalyzes the formation of acetaldehyde. Acetaldehyde is converted to acetate through the activity of another enzyme, acetaldehyde dehydrogenase. Acetate is in turn converted into acetyl-coenzyme A (CoA), which can enter a number of path-

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Dr. S.D. Shukla
Department of Pharmacology
School of Medicine, 1 Hospital Drive
University of Missouri-Columbia, Columbia, MO 65212 (USA)
Tel. +1 573 882 2740, Fax +1 573 884 4558, E-Mail shuklasd@health.missouri.edu

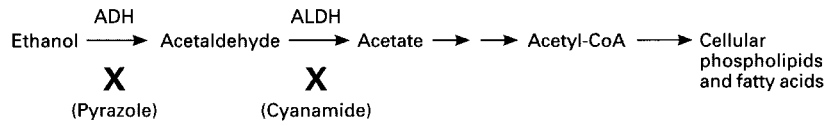


Fig. 1. Metabolism of ethanol. Ethanol is metabolized primarily in the liver, first to acetaldehyde by ADH, and then to acetate by aldehyde dehydrogenase (ALDH). Acetate is then converted to pyruvate, which is converted to acetyl-CoA by the pyruvate dehydrogenase complex. Acetyl-CoA is an important component of several biosynthetic pathways, one of which can lead to the formation of cellular phospholipids and fatty acids. ADH and aldehyde dehydrogenase are inhibited (shown by X) by pyrazole and cyanamide, respectively.

ways including phospholipid synthesis (fig. 1) [15]. The presence and activity of different isoforms of ADH in rat and in human blood vessels have been studied, thus suggesting that blood vessels themselves may contribute to extrahepatic ethanol metabolism [1].

Vascular endothelial cells (ECs) play an important role in mediating the effects of various substances that are introduced into the body through the circulation. Quiescent EC have the important physiological function to facilitate blood flow by providing an antithrombotic, profibrinolytic surface that inhibits adhesion of various types of circulating blood components. EC contribute to vasoregulation by releasing compounds such as nitric oxide, prostacyclin (PGI_2), and platelet-activating factor (PAF) in response to changes in their external cellular environment [6]. Vascular EC are continuously exposed to ethanol circulating in blood; therefore, ethanol can profoundly affect ECs. One pertinent issue is the incorporation of this ethanol into specific phospholipid pools, some of which have roles in cellular signalling responses.

It is well known that ethanol affects fatty acids and glycerolipid metabolism in liver. The formation of the phospholipid phosphatidylethanol (PEth) during ethanol exposure has been implicated in the pathogenesis of alcohol-induced organ damage [7]. Hepatic triacylglycerol accumulations have been described in chronic alcoholism [12]; however, reports on the effects of ethanol administration on the biosynthesis of cellular phospholipids are few and contradictory [4, 10, 16]. Several researchers have found that ethanol affects the incorporation and distribution of various exogenously supplied substrates in different ways. To our knowledge, there has been no study of the metabolic fate of ethanol into individual phospholipids. We have addressed this issue using EC.

Methods

Materials

Fetal bovine serum, pyrazole, cyanamide, wortmannin and the NAD-ADH assay kit were purchased from Sigma (St. Louis, Mo.). Minimum essential media, penicillin, streptomycin and glutamine were purchased from Gibco BRL (Grand Island, N.Y.). The [^{14}C]-ethanol and [^{3}H]-acetic acid were purchased from American Radiochemicals (St. Louis, Mo.). Propranolol and Ro-31-8220 were purchased from BioMol (Plymouth Meeting, Pa.). The compound UO126 was purchased from Calbiochem (San Diego, Calif.). Precoated silica gel G thin-layer chromatography (TLC) plates were purchased from Analtech (Newark, Del.).

Cell Culture

The experiments were conducted using calf pulmonary artery endothelial (CPAE) cells (ATCC CCL289), a bovine (calf) pulmonary artery EC line, between passages 17 and 27. Stock cultures were routinely maintained in 75-cm^2 flasks in a 37°C incubator with an atmosphere of 95% air/5% CO_2. The cells were grown to confluence in 60-mm culture dishes in a medium of 20% fetal bovine serum and 80% minimum essential medium supplemented with 100 U/ml penicillin, 10 μg/ml streptomycin and 2 m*M* *L*-glutamine.

Metabolism of Ethanol by CPAE Cells

CPAE cells (2.5×10^5) were seeded and grown to confluence ($\sim 1 \times 10^6$ cells) in 60-mm culture dishes in modified Eagle's medium (MEM) supplemented with 20% FBS. The 20% FBS MEM was aspirated, the cells were washed with PBS, and 1% FBS MEM containing 100 m*M* ethanol was added to the plates. Cell-free dishes with the ethanol-containing media were used as controls. The dishes were sealed with parafilm to prevent loss of ethanol due to evaporation. Media (10 μl) were collected at the specified time points and the concentration of ethanol was determined using an NAD-ADH assay kit.

Incorporation of [^{3}H]-Acetate into CPAE Cell Phospholipids

Prior to cell stimulation with agonists, the 20% FBS MEM was aspirated, the cells were washed with PBS, and 1% FBS MEM containing ethanol was added to the dishes. The dishes were then sealed with parafilm to prevent evaporation of ethanol. After 8 h of treat-

ment with ethanol, the media were again aspirated and the cells were washed with PBS. Next, 1% FBS MEM containing 100 μCi [^{3}H]-acetate was added to each dish for 10 min. ATP (10 μ*M*) was added to each dish for 10 min. Control dishes were incubated for 10 min without ATP. Cell stimulation was stopped by washing the cells with ice-cold PBS, adding ice-cold methanol containing 50 m*M* acetic acid, and placing the dishes on ice. The cells were scraped from the dishes and transferred to glass tubes maintaining ice-cold conditions.

Incorporation of [^{14}C]-Ethanol into CPAE Cell Phospholipids

The [^{14}C]-ethanol was acquired from ARC (St. Louis, Mo.) in a pressure-sealed glass tube. The tube was kept cold with dry ice when it was opened in order to minimize evaporation. The [^{14}C]-ethanol was then diluted with ice-cold 3% BSA-saline for a final activity of 1 μCi/μl. For the [^{14}C]-ethanol experiments, 5 μCi of [^{14}C]-ethanol was added along with varying amounts of unlabelled ethanol to bring the final concentration of ethanol to 0, 50 or 100 m*M*. The dishes were sealed with parafilm during the incubation period with ethanol. After 8 h of treatment, 10 μ*M* ATP was added to each dish for 10 min. Control dishes were incubated for 10 min without ATP. Cell stimulation was stopped by washing the cells with ice-cold PBS, adding ice-cold acidified methanol as described above, and placing the dishes on ice. The cells were scraped from the dishes and transferred to glass tubes as above.

Extraction and Analysis of Phospholipids

The phospholipid samples were extracted from the cells using a chloroform-methanol-water system [3]. The samples were centrifuged and the organic phase was collected and dried under N_2 stream. Ten percent of the extracted total lipids were subjected to base catalyzed methanolic hydrolysis to analyze the moiety where the radiolabel was incorporated. The remaining 90% of the extracted total lipids were fractionated by TLC on precoated silica gel G using several different systems and visualized by spraying the plates with 2-*p*-toluidinylnaphthylene 6-sulfonate (TNS) and their positions compared to known R_f values of standard lipids run in parallel. The phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol/phosphatidylserine (PI/PS) and sphingomyelin (Sph) were fractionated using either chloroform/methanol/water (65:35:6; R_f values PC = 0.77, PE = 0.90, PI/PS = 0.67, Sph = 0.63) or chloroform/methanol/water/concentrated NH_4OH (65:35:4:2; R_f values PC = 0.82, PE = 0.86, PI/PS = 0.72, Sph = 0.59). PAF was fractionated using two sequential TLC systems. First, it was separated in a system of chloroform/methanol/water (65:35:6). The band comigrating with the PAF standard (R_f PAF = 0.4) was cut and scraped off the plate, the phospholipid extracted from the silica and dried. The PAF was then purified from the eluted sample using a second TLC system of water and methanol (80:40). PEth and phosphatidic acid (PA; R_f values 0.13 and 0.53, respectively) were fractionated using benzene/chloroform/pyridine/formic acid (45:38:4:2.2). The appropriate bands were then cut and scraped off the plates, eluted, dried, and the radioactivity of each band was determined by liquid scintillation in a Beckman LS-1801 counter. Incorporated radioactivity is reported as cpm per 1 × 10^6 cells.

Statistical Analysis

ANOVA followed by Student's t test was used to determine statistical significance at $p < 0.05$.

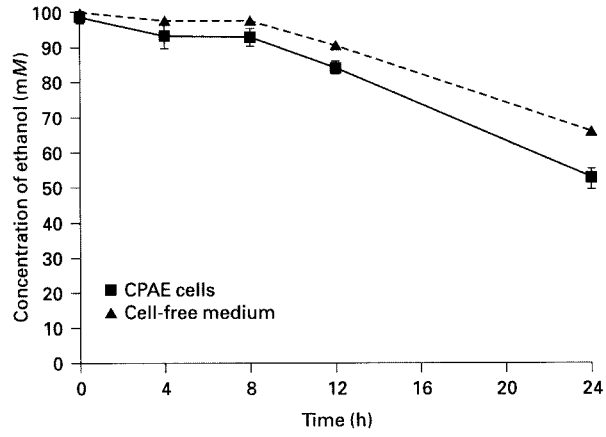


Fig. 2. Metabolism of ethanol by CPAE cells. CPAE cells were grown to confluence in 60-mm culture dishes in 20% FBS media. Cells were incubated in 100 m*M* ethanol for a total of 24 h. Media were collected at specific time points and the concentration of ethanol was determined using an NAD-ADH assay kit. Cell-free dishes were used as a control to correct for loss due to evaporation of ethanol. Values presented are means ± SEM of two experiments.

Results

Metabolic Fate of [^{14}C]-Ethanol in CPAE Cells

One of the first questions to assess in our study was whether EC could metabolize ethanol, and the extent of its incorporation into lipids. Confluent CPAE monolayers were incubated with 100 m*M* ethanol and media were sampled at the indicated time points and analyzed for alcohol content using an NAD-ADH assay kit. Cell-free control dishes were used to correct for loss of ethanol due to evaporation. As shown in figure 2, between 0 and 12 h, only 5% of the ethanol was metabolized by the CPAE cells. However, by 24 h, the cells had metabolized 15% of the ethanol in the media. Figure 3 shows incorporation of [^{14}C]-ethanol into CPAE cell total lipids. In one set, cells were stimulated with ATP (10 min) after 8 h of labelling. The graph indicates that with an increasing concentration of unlabelled ethanol, incorporation of the [^{14}C]-ethanol was decreased, suggesting that ethanol was incorporated into the lipid pool and the observed decrease in incorporation was due to dilution of the [^{14}C]-ethanol with unlabelled ethanol. Stimulation with ATP had no effect on the lipid radioactivity.

Next, incorporation of [^{14}C]-ethanol was monitored under stimulated conditions using ATP as an agonist.

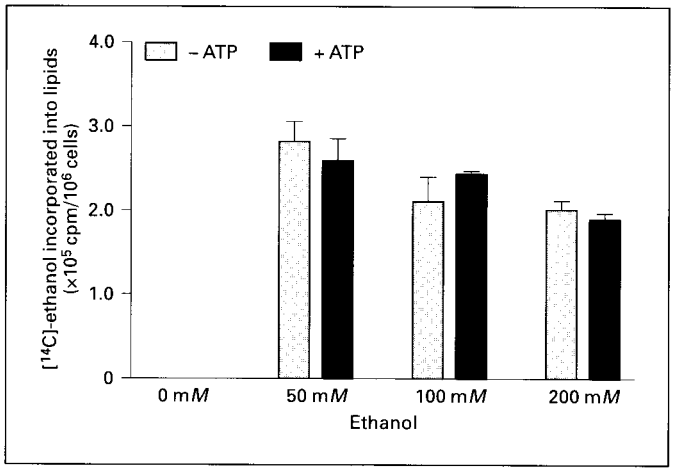


Fig. 3. Total [^{14}C]-ethanol incorporated into CPAE cell lipids. CPAE cells were incubated with the indicated concentrations of cold ethanol +5 μCi of [^{14}C]-ethanol for 8 h. In one set, cells were stimulated with ATP for 10 min at the end of labelling. Lipids were extracted from the cells and the amount of [^{14}C]-ethanol incorporated into total lipids was determined by liquid scintillation counting. The values are presented as cpm ± SEM of three experiments.

Table 1. Distribution of [^{14}C]-ethanol into CPAE cell phospholipids

[^{14}C]-labelled lipid	Ethanol		
	50 m*M*	100 m*M*	200 m*M*
PC	56.2±0.377	47.5±2.73	48.0±2.30
Neutral lipids	16.0±0.586	17.4±0.312	18.3±0.417
Sph	14.7±0.279	15.6±0.294	15.1±0.320
PI/PS	3.6±0.226	8.8±2.59	8.3±1.89
PE	5.0±0.125	5.0±0.202	4.9±0.082
PEth	4.0±0.707	5.3±0.255	4.9±0.271
PAF	0.5±0.040	0.4±0.065	0.6±0.133

Confluent CPAE monolayers were incubated for 8 h with ethanol at the concentration shown +5 μCi of [^{14}C]-ethanol, and then incubated with or without 10 μ*M* ATP for 10 min. Total cell lipids were extracted and then fractionated by TLC. Bands corresponding to comigrating standards were scraped and radioactivity determined by liquid scintillation counting. Results are presented as the level of [^{14}C] radiolabel in a particular fraction as a percentage of total radioactivity recovered from each sample lane on the TLC plate. Values are the mean percentages ± SEM of two experiments.

Table 1 shows the relative distribution of [^{14}C]-ethanol into various phospholipids as percentages of the total recovered [^{14}C] radioactivity from TLC fractionation and purification. At each concentration of ethanol, approximately half of the recovered radioactivity was found in the PC fraction. Incorporation of [^{14}C]-ethanol into neutral lipids and Sph was approximately 17 and 15%, respectively. About 5% of the radioactivity was in the [^{14}C]-PEth fraction. Other phospholipids had a much lower incorporation. Interestingly, a small but reproducibly significant fraction, 0.5%, of the [^{14}C]-ethanol was incorporated into PAF (table 1). However, there was no change in the distribution of cellular lipids with ATP stimulation (data not shown).

Figure 4a shows the incorporation of [^{14}C]-ethanol into total CPAE cell lipids after pretreatment of the cells with pyrazole (2 m*M*) and cyanamide (200 μ*M*), inhibitors of ethanol metabolism (see fig. 1). In the presence of each of these inhibitors, incorporation of [^{14}C]-ethanol into CPAE cell total lipids was reduced by about 30%. However, when these inhibitors were used combined at the above concentrations, the CPAE cells detached from the culture plates during the incubation period, indicating that such treatment was toxic (data not shown). Inhibitors of various intracellular signalling components [wortmannin for PI-3 kinase, Ro-31-8220 for protein kinase C (PKC), UO126 for mitogen-activated protein kinase, propranolol for phosphatidate phosphohydrolase] were also used. These inhibitors individually had no effect on the incorporation of [^{14}C]-ethanol into total CPAE lipids (data not shown). However, incorporation of PEth was increased in the presence of wortmannin (fig. 4b). Again, ATP stimulation had no effect on the incorporation.

Effect of Ethanol on [^{3}H]-Acetate Incorporation in CPAE Cell Phospholipids

Ethanol is metabolized to acetaldehyde, then acetate, which is subsequently incorporated into cell phospholipids. In the case of PAF biosynthesis, exogenous acetate can be used for acetyl-CoA production, which then transfers its acetate to the *sn2*-acetyl group on PAF via an acetyl-CoA transferase enzyme. We, therefore, investigated whether directly supplied [^{3}H]-acetate would also be incorporated into phospholipids and PAF in these cells. Duplicate samples of CPAE cells were incubated for 10 min in low serum medium with 100 μCi [^{3}H]-acetate followed by 10 min stimulation with 10 μ*M* ATP. Control cells, which were not exposed to ATP, were incubated for a total of 20 min with [^{3}H]-acetate. Total cell lipids were then isolated and radioactivity was determined. Figure 5 shows that with increasing concentration of ethanol, there was a decrease in the incorporation of [^{3}H]-acetate into CPAE cell phospholipids, likely due to dilution of the [^{3}H]-acetate pool by the acetate derived from the ethanol

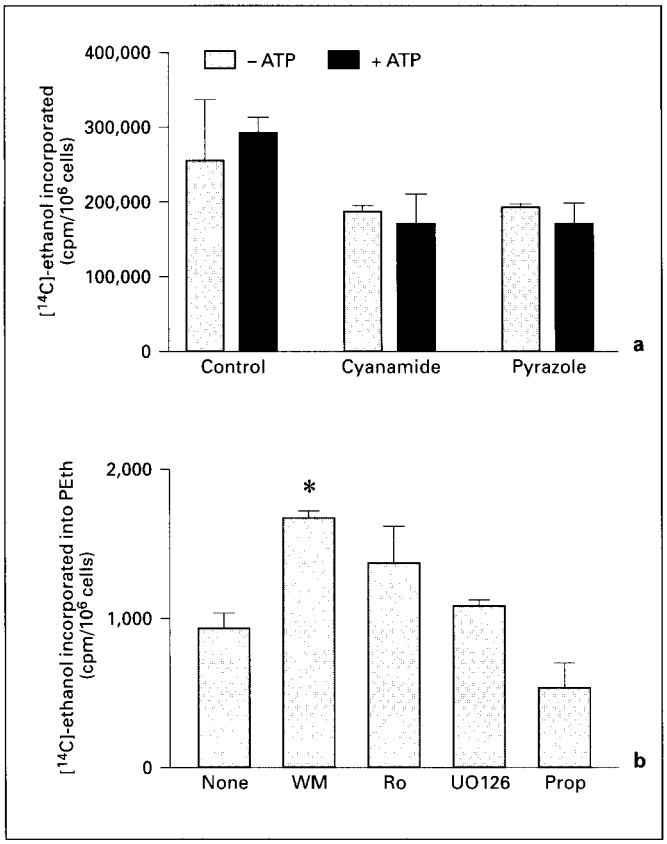


Fig. 4. Effect of inhibitors on the incorporation of [^{14}C]-ethanol into CPAE cell lipids. Confluent monolayers of CPAE cells were pretreated with either 200 μ*M* cyanamide or 2 m*M* pyrazole (**a**) or 10 μ*M* wortmannin (WM), 10 μ*M* Ro-31-8220 (Ro), 10 μ*M* UO126 or 100 μ*M* propranolol (Prop) (**b**) for 1 h before incubation in 50 m*M* ethanol +5 μCi ^{14}C-ethanol for 8 h. Total cell lipids were extracted and incorporation of [^{14}C]-ethanol was determined by liquid scintillation counting. Data are represented as cpm ± SEM of two experiments. *$p < 0.05$.

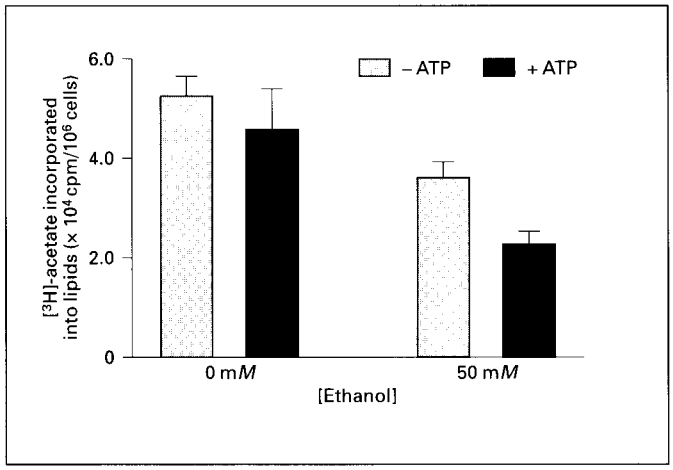


Fig. 5. Effect of ethanol on the incorporation of [^{3}H]-acetate into CPAE cell lipids. CPAE cells were incubated with the indicated concentrations of ethanol for 24 h. The cells were then incubated in media containing 100 μCi [^{3}H]-acetate for 10 min, and incubated for an additional 10 min with or without 10 μ*M* ATP. Lipids were extracted from the cells and the amount of [^{3}H]-acetate incorporated into phospholipids was determined by liquid scintillation counting. The values are presented as cpm ± SEM of three experiments.

metabolism. Thetotal cell lipids were then analyzed by TLC fractionation to determine incorporation of [^{3}H]-acetate into individual phospholipids (table 2). In contrast to the observed distribution of [^{14}C]-ethanol into phospholipid classes, a majority (55%) of the [^{3}H]-acetate was incorporated into neutral lipids and 35% into PC. The other phospholipids showed relatively small percentages of distribution. Again, a small fraction of [^{3}H]-acetate was incorporated into PAF. Ethanol treatment did not affect percent incorporation of [^{3}H]-acetate into phospholipids. Stimulation of the CPAE cells with ATP decreased the incorporation of [^{3}H]-acetate into neutral lipids and increased the incorporation into Sph.

Analysis of Incorporation of [^{14}C]-Ethanol or [^{3}H]-Acetate in Fatty Acid and Glycerophosphoryl Moieties

We next investigated the extent of incorporation of [^{14}C]-ethanol or [^{3}H]-acetate into the phospholipid head group-containing glycerol backbone and into the fatty acid side chains. Table 3 shows the results of methanolic hydrolysis of total cell lipids. The basal level of distribution of [^{3}H]-acetate was 13% in the water-soluble fraction and 87% in the fatty acid moiety. Stimulation of the cells with ATP slightly increased incorporation into the water-soluble fraction with a concomitant decrease in distribution into the lipid-soluble fraction. Increasing the concentration of alcohol also increased the incorporation of [^{3}H]-acetate into the water-soluble fraction. However, in the case of [^{14}C]-ethanol, approximately 95% of the [^{14}C]-ethanol was incorporated into the fatty acid side chains. Neither increasing the concentration of alcohol nor stimulation of the cells with ATP significantly altered the pattern of distribution of [^{14}C]-ethanol between the water-soluble and lipid-soluble fractions.

Production of PAF by CPAE Cells

Since PAF is a potent lipid mediator involved in EC interactions with other cells (e.g. neutrophils), we investigated more closely the PAF production by these cells. The

Table 2. Effect of ethanol and ATP on distribution of [^{3}H]-acetate into lipids in CPAE cells

[^{3}H]-labelled lipid	No ethanol		50 m*M* ethanol	
	–ATP	+ATP	–ATP	+ATP
Neutral lipids	55.6±0.4	49.5±2.4	56.3±0.8	42.9±4.4
PC	35.1±2.7	39.9±2.0	35.3±2.3	32.5±1.9
PE	4.4±2.5	2.6±0.5	2.2±0.1	2.1±0.1
PI/PS	2.1±0.1	1.65±0.7	1.9±1.5	1.1±0.5
Sph	1.5±0.2	4.6±1.2	3.3±0.2	17.0±6.8*
PAF	1.4±0.1	1.7±0.6	0.9 ±0.1	4.4±3.6

Confluent CPAE cell monolayers were incubated for 24 h in ethanol at the concentrations shown, incubated for 10 min with 100 μCi [^{3}H]-acetate and then for an additional 10 min with or without 10 μ*M* ATP. Total cell lipids were extracted and subjected to TLC fractionation. Each lipid band was identified by its R_f value, scraped, and incorporated [^{3}H]-acetate was determined by liquid scintillation counting. Results are reported as the [^{3}H]-acetate activity in a particular TLC fraction as a percentage of the total [^{3}H]-acetate activity recovered from each sample lane on the TLC plate. Values are percent distribution ± SEM for two experiments. * $p < 0.05$.

Table 3. Effect of ethanol on distribution of [^{3}H]-acetate or [^{14}C]-ethanol into fatty acyl or water-soluble fraction of phospholipids

Ethanol	^{3}H-acetate incorporation				^{14}C-ethanol incorporation			
	water-soluble		lipid-soluble		water-soluble		lipid-soluble	
	–ATP	+ATP	–ATP	+ATP	–ATP	+ATP	–ATP	+ATP
0	13.4	15.1	86.6	84.9	–	–	–	–
50 m*M*	14.6	17.1	85.4	82.9	4.8	5.2	95.2	94.8
100 m*M*	–	–	–	–	3.6	2.7	96.4	97.3
200 m*M*	–	–	–	–	2.6	3.5	97.4	96.5

Distribution in CPAE cell phospholipids into fractions after methanolic hydrolysis. Confluent CPAE cell monolayers were incubated with or without ethanol and with either 100 μCi ^{3}H-acetate or 5 μCi ^{14}C-ethanol. The cells were then incubated for 10 min with or without ATP and then total cell lipids were extracted. Ten percent of the extracted total lipids were subjected to base catalyzed methanolic hydrolysis to analyze the moiety where the [^{3}H]-acetate or [^{14}C]-ethanol was incorporated.

PAF pool was labelled by both [^{14}C]-ethanol and [^{3}H]-acetate. Figure 6 indicates that treatment of the [^{3}H]-acetate-exposed cells with ATP for 10 min stimulated a 4-fold increase in formation of [^{3}H]-PAF. A 24-hour pretreatment of the cells with either 50 or 100 m*M* ethanol decreased basal [^{3}H]-PAF; however, stimulation of the ethanol-treated cells with 10 μ*M* ATP for 10 min stimulated only a 1- and 2-fold increase, respectively.

We also investigated the pattern of [^{14}C]-ethanol incorporation into PAF. Figure 7 shows that increasing time of incubation with 50 m*M* [^{14}C]-ethanol resulted in increased [^{14}C]-PAF. However, unlike the incorporation of [^{3}H]-acetate, treatment of the cells with 10 μ*M* ATP for 10 min did not result in increased incorporation of [^{14}C]-ethanol into [^{14}C]-PAF.

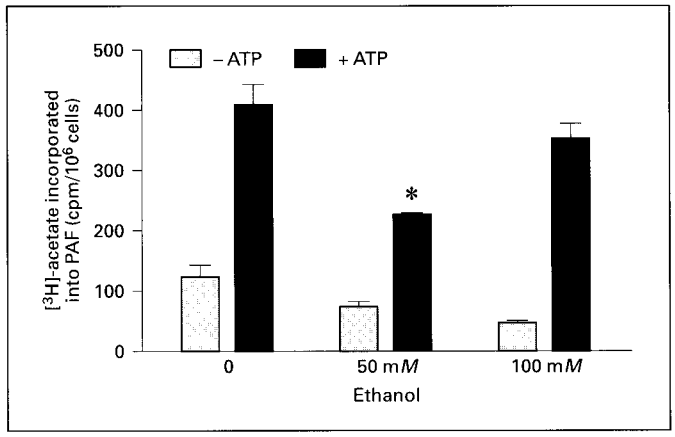
Discussion

As far as it can be ascertained, this is the first report of metabolic incorporation of [^{14}C]-ethanol into various cellular phospholipids of EC. Bioactive lipids in EC play a central role in vascular homeostasis and the response to injury. Studies suggest that alcohol acts mainly on the

acyltransferase activities involved in the remodeling of membrane phospholipids. Since the incorporation of radioactivity into each phospholipid is an index of the metabolic turnover of its acyl chains and head groups, we determined the effect of ethanol on the distribution of radioactivity among different phospholipids (PA, PAF, PE, PEth, PS, PC, Sph) as well as in neutral lipids. In examining the incorporation of [^{14}C]-ethanol and of [^{3}H]-acetate into phospholipids, there was a striking contrast in the pattern of distribution. Whereas [^{14}C]-ethanol was mostly incorporated into PC with neutral lipids accounting for much less, [^{3}H]-acetate was incorporated mostly into neutral lipids with PC being second. Also, the effect of increased concentration of ethanol caused a dramatic decrease in the total incorporation of [^{3}H]-acetate while showing little effect on the distribution of [^{14}C]-ethanol. It can be inferred that even though the [^{14}C]-ethanol is being metabolized to acetate, it is possibly contributing to a separate acetate pool than that of the directly supplied [^{3}H]-acetate. Also, cyanamide and pyrazole caused decreases in [^{14}C]-ethanol incorporation into lipids and this is consistent with the metabolic conversion of ethanol into acetate and then to lipids.

PAF is not constitutively synthesized nor is it stored in resting, unactivated cells [17]. Rather, it is rapidly synthesized in response to agonists [5, 11, 14]. Previous research has demonstrated that EC synthesize PAF in response to stimulation with ATP [13]. In our present studies, we investigated the effect of alcohol on ATP-induced PAF accumulation. The acetate arising from ethanol metabolism was subsequently incorporated into various EC lipids. There was a marked difference in the response of the cells. Cells incubated with [^{3}H]-acetate showed increased incorporation of [^{3}H]-acetate into PAF upon cell stimulation with ATP. In the cells incubated with [^{14}C]-ethanol, treatment with ATP did not result in increased incorporation of [^{14}C]-ethanol into PAF. These data suggest that the acetate (directly supplied [^{3}H]-acetate or [^{14}C]-ethanol-derived acetate) may exist in distinct pools that are differently affected by ATP stimulation.

PC is the preferred phospholipid hydrolyzed by phospholipase D (PLD) to produce PA and choline. In the presence of ethanol, the enzyme PLD catalyzes transphosphatidylation to form PEth [18]. In [^{14}C]-ethanol-treated cells, PEth constituted approximately 5% of the recovered activity in phospholipids. This value is much higher than that reported before where PEth concentration was found to reach 0.5–1% of total cellular lipids [7]. In EC, this may represent a substantial fraction in microdomains of membrane phospholipids and may effect changes seen in intracellular signalling in ethanol-exposed cells. Inhibition of PI-3 kinase, PKC, mitogen-activated protein kinase, and modulation of PLD activity had no effect on the incorporation into lipids other than PEth. In this case, it can be proposed that in EC, inhibition of the PI-3 kinase path-



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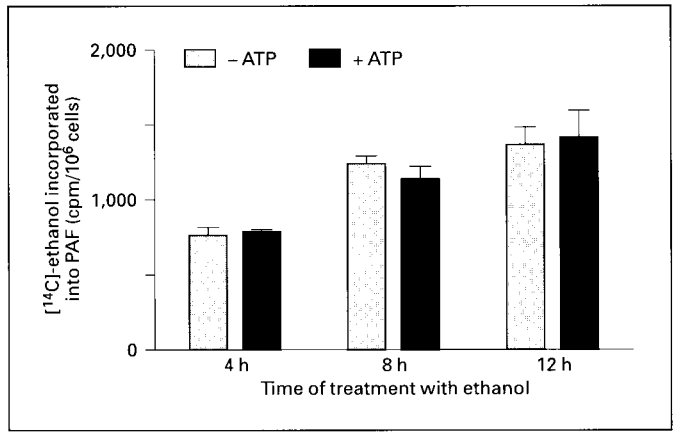

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Fig. 6. Effect of ethanol and ATP on the incorporation of [^{3}H]-acetate into PAF in CPAE cells. Confluent CPAE cells were incubated in ethanol containing media for 24 h. Following that incubation period, the cells were then incubated in media containing 100 μCi [^{3}H]-acetate for 10 min and then stimulated for 10 min with 10 μ*M* ATP. Total cell lipids were extracted and fractionated in two sequential TLC steps to purify PAF. The amount of [^{3}H]-acetate incorporated into PAF was determined by liquid scintillation counting of the eluted lipid. Results are presented as cpm ± SEM of two experiments. *$p < 0.05$.

Fig. 7. Time course of incorporation of [^{14}C]-ethanol into PAF in CPAE cells. Confluent CPAE cells were incubated in media containing 50 m*M* ethanol and 5 μCi [^{14}C]-ethanol for the times indicated. Following this incubation, the cells were then incubated with or without 10 μ*M* ATP for 10 min. Total cell lipids were extracted and fractionated in two sequential TLC steps to purify PAF. The amount of [^{14}C]-ethanol incorporated into PAF was determined by liquid scintillation counting of the eluted lipid. Results are presented as cpm ± SEM of two experiments.

way, in some manner, has a negative influence on basal PEth formation in the presence of ethanol. The formation of PEth brings to bear its effect on membrane characteristics. It has been demonstrated that for at least one PKC isoform, PEth can specifically take the place of PS in activating PKC [2]. This observation raises the interesting question of the role of PEth, in addition to PS, in EC surface interactions with neutrophils and platelets [6].

In the CPAE cells incubated with [^{3}H]-acetate, there were changes seen in Sph formation both with ethanol treatment and upon stimulation with ATP. There is accumulating evidence that sphingolipids can affect steady state concentrations of choline-containing glycerolipids such as PC. It is well established that glycerophospholipids and their metabolic products such as diacylglycerol, inositol 1,4,5-trisphosphate (IP_3), PAF and eicosanoids function as mediators in signal transduction and cellular responses. More recently it has been hypothesized that membrane sphingolipids could serve in signal transduction pathways. A Sph cycle has been described in which activation of a sphingomyelinase leads to the breakdown of Sph and the generation of phosphocholine and ceramide [8]. This cycle is thought to be analogous to the generation of IP_3 and diacylglycerol from the phospholipase C-mediated hydrolysis of inositol phospholipids. Ceramide has been shown to modulate a number of downstream events such as protein phosphorylation, phosphatase activation, downregulation of the c-myc protooncogene, and apoptosis. The changes seen in incorporation of [^{3}H]-acetate into Sph with ethanol treatment, as well as under stimulated conditions (+ATP), suggest that activation of the Sph cycle may be involved in the altered signal transduction observed in cells exposed to ethanol.

The vascular endothelium is not only a structural barrier between circulation and organs, it also plays a pivotal role in responding quickly to environmental changes that influence the regulation of blood flow. In this regard, the maintenance of adaptive processes in the EC is of great importance in both normal and disease states. We have shown in this study that vascular EC actively metabolize alcohol and that a portion of this alcohol is incorporated into different cell lipids, some of which are biologically active, such as PAF. Signalling by PAF is closely linked to adhesive reactions between cells involved in inflammatory responses and the vascular endothelium [6]. Thus the effect of alcohol on PAF and other lipids (e.g. PEth, Sph) may be critical determinants in the pathophysiological responses of the alcoholic endothelium.

References

1 Allali-Hassani A, Martínez SE, Peralba JM, Vaglenova J, Vidal F, Richart C, Farrés J, Parés X. Alcohol dehydrogenase of human and rat blood vessels: Role in ethanol metabolism. FEBS Lett 405:26–30;1997.

2 Asaoka Y, Kikkikawa U, Sekiguchi K, Shearman MS, Kosaka Y, Nakano Y, Satoh T, Nishizuka Y. Activation of brain-specific protein kinase C subspecies in the presence of PEth. FEBS Lett 231:221–224;1988.

3 Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917;1959.

4 Carrasco MP, Sanchez-Amate MC, Marco C, Segovia JL. Evidence of differential effects produced by ethanol on specific phospholipid biosynthetic pathways in rat hepatocytes. Br J Pharmacol 119(2):233–238;1996.

5 Chap H, Mauco G, Simon MF, Benveniste J, Douste-Blazy L. Biosynthetic labelling of platelet activating factor from radioactive acetate by stimulated platelets. Nature 289:312–314; 1991.

6 Cines DB, Pollak ES, Buck CA, Loscalso J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McRae KR, Hug BA, Schmidt A-M, Stern DM. Endothelial cells in physiology and pathophysiology of vascular disorders. J Am Soc Hematol 91(10):3527–3561;1998.

7 Gustavsson L, Lundqvist C, Hansson L, Rodríguez D, Simonsson P, Alling C. Ethanol induced changes in signal transduction via formation of phosphatidylethanol. In: Alling C, Sun G, eds. Alcohol, Cell Membranes and Signal Transduction in Brain. New York, Plenum Press, 63–74;1993.

8 Hannun YA, Bell RM. The sphingomyelin cycle: A prototypic sphingolipid signalling pathway. Adv Lipid Res 25:27–41;1993.

9 Hoek, JB, Rubin E. Alcohol and membrane associated signal transduction. Alcohol Alcohol 25:143–156;1990.

10 Le Petit-Thevenin J, Nobili O, Vérine A, Boyer J. Differential in vitro effects of ethanol on glycerolipid acylation and biosynthesis in rat reticulocytes. Biochim Biophys Acta 1257: 103–110;1995.

11 Lee T-C. Biosynthesis of platelet activating factor. J Biol Chem 260(20):10952–10955;1985.

12 Lieber CS. Alcohol and the liver: 1994 update. Gastroenterology 106:1085–1105;1994.

13 McIntyre TM, Zimmerman GA, Satoh K, Prescott SM. Cultured endothelial cells synthesize both platelet-activating factor and prostacyclin in response to histamine, bradykinin, and adenosine triphosphate. J Clin Invest 76:271–280;1985.

14 Prescott SM, Zimmerman GA, McIntyre TM. Human endothelial cells produce platelet-activating factor (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) when stimulated with thrombin. Proc Natl Acad Sci USA 81:3534–3538: 1984.

15 Rang HP, Dale MM, Ritter JM, Gardner P. Drug dependence and drug abuse. In: Pharmacology, ed 3. New York, Churchill Livingstone, 645–664;1995.

16 Sanchez-Amate MC, Zurera JM, Carrasco MP, Segovia JL, Marco C. Ethanol and lipid metabolism. Differential effects on liver and brain microsomes. FEBS Lett 293(1,2):215–218; 1991.

17 Shukla SD. Platelet activating factor and platelets. In: Rao HR, ed. Handbook of Platelet Physiology and Pharmacology. Boston, Kluwer Academic Publishers, 120–141;1999.

18 Shukla SD, Halenda SP. Phospholipase D in cell signalling and its relationship to phospholipase C. Life Sci 48:851–866;1991.

19 Taraschi TF, Rubin E. Effects of ethanol on the chemical and structural properties of biologic membranes. Lab Invest 52:120–131;1985.

20 Thurston AW, Shukla SD. Ethanol modulates epidermal growth factor-stimulated tyrosine kinase and phosphorylation of PLC-γ_1. Biochem Biophys Res Commun 185:1062–1068: 1992.