Published in final edited form as:

Alcohol Clin Exp Res. 2016 December ; 40(12): 2557–2562. doi:10.1111/acer.13254.

Phosphatidylethanol in Postmortem Brain and Serum Ethanol at Time of Death

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Abstract

Background—Phosphatidylethanol (PEth) is a metabolite of ethanol and its concentration in whole blood samples is a direct biomarker of alcohol consumption. Because PEth is also present in the brain and incorporated in lipid membranes, it can be used to classify deceased individuals on alcohol consumption status at the time of death. The purpose of this study was to detect PEth homologs in postmortem brains of individuals known to have had alcohol use disorder (AUD) and to determine the relationship between serum alcohol at the time of death and PEth in the cerebellum and optical frontal cortex.

Methods—Postmortem brain was collected and stored according to standard protocol. Psychiatric symptoms experienced prior to death were obtained by next of kin psychological autopsy to categorize subjects. Thirty male subjects were chosen for analyses: 10 with AUD with positive serum ethanol levels present at time of autopsy (AUD-W), 10 with AUD without positive serum ethanol levels (AUD-WO), and 10 controls. PEth16:0/18:1 and 16:0/18:2 were quantified in 50 mg of cerebellum (CE) and orbital frontal cortex (OFC) of human postmortem brain using HPLC and mass spectrometric detection (triple quadrupole).

Results—Results of this study were: (1) PEth16:0/18:1 and 16:0/18:2 were detected in the CE and OFC of all subjects diagnosed with AUD, (2) PEth16:0/18:1 levels were about 10-fold higher than PEth 16:0/18:2 in all subjects and both areas of brain, (3) AUD-W subjects had higher PEth homolog levels in CE and OFC than controls and AUD-WO subjects, (4) PEth 16:0/18:1, but not PEth 16:0/18:2, levels in CE and OFC of AUD-W subjects correlated significantly with serum ethanol levels at the time of death.

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All components of this study were performed at the Department of Psychiatry, University of Texas HSC, San Antonio, Texas. Dr. Thompson has re-located the Southwest Brain Bank to the Department of Psychiatry, Texas Tech University HSC, in El Paso, Texas, where he is Professor of Psychiatry.

None of the authors have conflicts of interests concerning this manuscript.

Conclusion—Quantification of combined PEth homolog levels in postmortem human brain is a good candidate as a diagnostic factor to classify drinking status, especially for those with AUD at the time of death. For alcohol research studies with postmortem brain, verification of drinking status is essential.

Keywords

PEth; alcoholism; postmortem brain; suicide; orbital frontal cortex; cerebellum

INTRODUCTION

Phosphatidylethanol (PEth) is a non-oxidative metabolite of alcohol quantifiable in uncoagulated, whole blood samples and other tissues. It is an appealing peripheral marker of alcohol consumption in blood samples due to its pharmacokinetic characteristics of a half-life of 4 to 6 days in most humans, and the recent development of a very sensitive HPLC-tandem mass spec bioassay (Gnann et al., 2009, 2010; Jones et al., 2011) which allows for the detection of PEth even after low doses of alcohol (Javors et al., 2016). The synthesis of PEth is catalyzed by the enzyme phospholipase D in the presence of ethanol and phosphatidylcholine. Under normal circumstances phosphatidylcholine is converted to phosphatidic acid, a cellular second messenger (Gustavsson et al., 1991; Kobayashi and Kanfer, 1987). But in the presence of alcohol, PEth is preferentially formed by transphosphatidylation and incorporated into cellular membranes (Alling et al., 1984). Forty-eight PEth homologs have been identified (Gnann et al., 2010), with eight species representing over 90% of the various homologs: 16:0/18:1, 16:0/18:2, 18:0/18:2, 18:0/18:1, 18:1/18:1, 18:0/20:4, 16:0/20:3 and 16:0/16:0 (Gnann et al., 2014).

Little is known about the synthesis of PEth in the human brain and published information about the relationship of alcohol consumption to central nervous system PEth levels is limited. PEth 16:0/18:1 was detected and quantified in postmortem human organs, including the brain (cortex and cerebellum), of individuals with known alcoholism during their lifetime who also had detectable ethanol in their blood at the time of death (Aradottir et al., 2004). The purpose of the current study was to detect PEth homologs (16:0/18:1, 16:0/18:2) in postmortem brains of individuals known to have had alcohol use disorder (AUD; based on psychiatric and physical symptoms, and information from next of kin and close friends) and to determine the relationship between serum alcohol at the time of death and PEth in the brain. Postmortem brains from 10 male subjects with AUD with positive serum ethanol levels present at time of autopsy (AUD-W), 10 with AUD that without positive serum ethanol levels (AUD-WO), and 10 controls were analyzed for PEth 16:0/18:1 and 16:0/18:2 levels in the cerebellum and optical frontal cortex.

METHODS

Postmortem Tissue

Tissue was obtained by the University of Texas Health Science Center Southwest Brain Bank (SWBB) with consent from the next-of-kin. The collection of postmortem tissue for research in Texas is under the jurisdiction of the State of Texas Anatomical Board. To

prepare tissue, the cerebrum was hemisected and cut into 1 cm-thick coronal blocks starting at the frontal pole, immediately frozen in isopentane (2-methylbutane, Fisher) chilled with dry ice to -60°C , and then stored at -80°C . A neuropathologist examined all tissue and there was no confounding gross and microscopic neuropathology noted. The brains used in this study were chosen based on the classification of Alcohol Use Disorder without (AUD-WO) or with (AUD-W) detectable serum ethanol levels and a control, non-AUD group. In this study we used brain tissue from the lateral orbital frontal cortex (OFC) and cerebellar vermis (CE) from normal, non-alcohol use disorder controls ($n=10$), and from an alcohol use group [$n = 10$ subjects with detectable serum ethanol levels (AUD-W), and $n = 10$ without (AUD-WO)]. All subjects were male. The demographics of the subjects whose brain tissue was used in this study are presented in Table 1. One donation used in this analysis was noted to have a small old lacunar infarct in the contralateral hemisphere from what was used in this analysis. Toxicology results, including serum ethanol levels for all subjects, were obtained from the Medical Examiners Toxicology Laboratory or from NMS Laboratory, Willow Grove Pennsylvania. Control tissue was toxicologically free of drugs of abuse. Typical tissue characteristics used to confirm the integrity of postmortem tissue are presented in Table 1. Two of the 30 subjects whose brains were used were known to have diabetes. Tobacco was used by 3, 6, and 9 subjects in CON, AUD-WO, and AUD-W, respectively, but the level and time frame of use were not known. Metabolic syndrome and liver function indicators were not available.

Postmortem Psychological Autopsy

The next of kin interview about the life of the individual whose tissue was donated (psychological autopsy) is an exempt protocol per the UTHSCSA IRB. A trained clinician interviewed the next of kin about the donor to provide diagnostic information and information about mood state (lifetime and in the last week of life). Psychiatric clinical diagnoses, including AUD diagnoses, was performed using the semi-structured Mini-International Neuropsychiatric Interview (M.I.N.I.; Sheehan et al., 1998) with DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, American Psychiatric Association) criteria. Information from these interviews plus a review of all available medical records was presented to the diagnostic consensus group.

Measurement of PEth 16:0/18:1 and 16:0/18:2 in Human Postmortem Brain

The procedure for quantification of PEth 16:0/18:1 and PEth 16:0/18:2 in uncoagulated blood samples has been previously described (Javors et al., 2016). This procedure for brain tissue has been modified slightly. Briefly, PEth 16:0/18:1 (1-palmitoyl-2-oleoyl-phosphatidylethanol), PEth 16:0/18:2 (1-palmitoyl-2-linoleoyl-phosphatidylethanol), and d5-PEth 16:0/18:1 (1-palmitoyl-2-oleoyl-phosphatidyl-d5-ethanol) were purchased as pure analytical standards from Avanti Polar Lipids (Alabaster, AL) and Echelon (Salt Lake City, UT). Fifty mg of whole brain samples from the CE and the OFC were homogenized in 600 μl isopropanol and 5 μl of the internal standard solution (deuterated (d5) PEth 16:0/18:1), then 1 ml of hexane was added with mixing followed by shaking for 15 minutes. The samples were centrifuged for 20 minutes at $3,200 \times g$ at 4°C . The supernatants were evaporated to residue with a stream of nitrogen at 30°C and then the residues were dissolved in 100 μl mobile phase A (20:80; 10 mM ammonium acetate in water:acetonitrile). These

samples were placed in 300- μ l glass autosampler tubes, and then 10 μ l were injected into the HPLC/MS/MS system (Shimadzu SCL controller, 2 LC-20AD pumps with a DGU20A degassing unit and mixing chamber, SIL-20ACHT autosampler (Shimadzu Scientific Instruments, Columbia, MD), and an AB Sciex API 4000 Q-trap mass spectrometer with turbo ion spray (AB Sciex LLC, Framingham, MA)). The analytical column was a HyPurity C4 column (Thermo Scientific, Waltham, MA) and mass spectrometer parameters were: ESI (electrospray ionization) interface operated in negative ion mode using selected reaction monitoring to detect the major product ions from the deprotonated molecules of PEth 16:0/18:1 (m/z 701.7 to $\rightarrow$ 281.5), PEth 16:0/18:2 (m/z 699.7 to $\rightarrow$ 279), and PEth 16:0/18:1 (m/z 707 to $\rightarrow$ 288). The ratio of peak areas of PEth 16:0/18:1 and PEth 16:0/18:2 to d5-PEth 16:0/18:1 were compared against a linear regression of ratios of calibrators from 0 to 4,000 ng/ml. PEth 16:0/18:1 and PEth 16:0/18:2 concentrations were expressed in μ g/g total tissue.

Statistical Analysis

Differences among the means of PEth homolog levels in control, AUD-WO, and AUD-W subjects were determined using non-parametric t tests. Differences between PEth homolog levels were determined using Mann-Whitney tests. Correlations were determined using the equations for linear regression in Prism7 software (Graph-Pad).

RESULTS

PEth16:0/18:1 and 16:0/18:2 were detected in the CE and OFC of all subjects diagnosed with AUD and all control subjects. PEth 16:0/18:1 levels were about 10-fold higher than PEth 16:0/18:2 levels in CE and OFC of all three subject groups, AUD-W (Fig. 1A), AUD-WO (Fig. 1B), and controls (Fig. 1C). and The means and standard deviations of PEth homolog levels in both brain areas and among subject groups are in table 1.

AUD subjects with measurable serum ethanol levels at the time of death (AUD-W) had higher mean PEth 16:0/18:1 levels in CE and OFC than controls and AUD-WO subjects (Fig. 1A; Table 1). The serum ethanol levels in AUD-W subjects ranged from 0.02 to 0.26 g/dL with mean (SD) of 0.144 (0.094) g/dL (Table 1). PEth 16:0/18:2 levels were also higher in AUD-W subjects than control and AUD-WO subjects. Serum ethanol levels were not detected in control and AUD-WO subjects.

To evaluate the possibility that the concentration of ethanol in the brain at the time of death was directly related to PEth levels in CE and OFC, we compared serum ethanol concentrations, which were only detectable in AUD-W subjects, versus PEth homolog levels. For AUD-W subjects only, PEth 16:0/18:1 levels in both CE and OFC correlated significantly with serum ethanol levels at the time of death (Fig. 2A). PEth 16:0/18:2 did not correlate with serum ethanol levels at the time of death in either the CE or OFC (Fig. 2B).

Finally, 8 of the 30 subjects in this study committed suicide, 7 were AUD-W subjects and 1 was in the AUD-WO group. All other subjects were reported to have died of natural causes based on the coroner's reports.

DISCUSSION

The principal finding of this study is that detectable levels of PEth 16:0/18:1 and 16:0/18:2 were found in the CE and OFC brain regions of deceased alcoholic and control subjects, with higher levels in AUD subjects with serum alcohol levels versus AUD and control subjects without detectable serum alcohol levels, providing for the use of this measurement in postmortem brains to confirm the alcohol drinking status prior to death. The concentration of PEth 16:0/18:1 was several-fold higher than PEth 16:0/18:2 in all subjects and both areas of brain, suggesting that synthesis and elimination of individual PEth homologs in brain differed from that in red blood cells. Finally, PEth 16:0/18:1, but not PEth 16:0/18:2, levels in CE and OFC correlated significantly with serum ethanol levels at the time of death, a finding that supports an earlier report that PEth turnover is much faster in brain than in red blood cells (Lundqvist et al., 1994). These findings are important to scientists who use postmortem brain tissue to study the effects of alcohol on brain structure and function as a confirmation of recent alcohol consumption prior to death.

It has long been known that rats acutely or chronically treated with ethanol have high concentrations of PEth in the brain, and other organs (Alling et al., 1984; Aradottir et al., 2002, 2004; Lundqvist et al., 1994). For example, after a single injection of ethanol, PEth has been shown to be quickly synthesized in several brain regions (hippocampus, cerebellum, and cerebrum) of the rat, it reached a maximum at 2 hours, and remained detectable for up to 14–24 hours (Lundqvist et al., 1994). In contrast, there is very little known about PEth levels in the human brain. To our knowledge, there has been only one report of PEth 16:0/18:1 levels in human organs (Aradottir et al., 2004). This was a small study, and consisted postmortem samples from 6 adult alcoholics (2 women and 4 men) with positive blood alcohol levels at the time of death and 2 controls (2 girls less than a year old) with no measurable blood alcohol levels. In the alcoholics, PEth was found in the kidneys, lungs, spleen, liver, heart, skeletal muscle, small intestine, fat tissue, and brain cortex and cerebellum. In the control samples, PEth was not detected in these organs, except in the kidneys of 1 control. In the current study, we have demonstrated that PEth 16:0/18:1 and PEth 16:0/18:2 are present in the cerebellum (CE) and orbital frontal cortex (OFC) in all three groups of subjects, including the controls (albeit at much lower levels). Detectable PEth levels in the controls may be due to the recent development of a more sensitive HPLC-tandem mass spec bioassay (Gnann et al., 2009, 2010; Jones et al., 2011) as compared to that used by Aradottir and colleagues (2004).

Our results also show that PEth 16:0/18:1 levels were at least 10-fold higher than PEth 16:0/18:2 levels in all subjects and in both areas of the brain. This result was surprising because, by comparison, the relative levels of PEth 16:0/18:1 and PEth 16:0/18:2 are similar in whole blood samples from human participants who consumed oral doses of alcohol in a clinical lab study (Javors et al., 2016). However, it appears that PEth 16:0/18:2 is synthesized and eliminated in whole blood samples (red blood cells) at higher rates than PEth 16:0/18:1 (Gnann et al., 2014; Javors et al., 2016 (Schrock et al., 2016)). Thus, a possible reason for the limited presence of PEth 16:0/18:2 in human brain samples might be that the half-life of PEth 16:0/18:2 is short in human brain.

PEth 16:0/18:1 and PEth 16:0/18:2 levels were higher in CE and OFC of AUD-W subjects versus AUD-WO and control subjects. This finding is consistent with the speculation that a faster turnover rate of PEth exists in brain than in blood samples. This notion is based on the report of the half-life of PEth in rat brain to be 17 hours (Lundqvist et al., 1994) and the half-life of PEth in blood to be from 4 to 7 days (Javors et al., 2016). But information about drinking in control and AUD-WO subjects during the last few days of life is not known, so our results are not conclusive. Other possibilities are that there is a reduced level of the precursor phosphatidylcholine 16:0/18:2 in the brain or that the phospholipase D in brain does not catalyze its conversion to PEth 16:0/18:2.

There were not sufficient numbers of female brains to include in this study, so the effect of gender was not testable. Nevertheless, the difference in the absorption, distribution, and metabolism of ethanol between males and females is well known. About 10% higher blood levels of ethanol occur in women who consume the same dose of alcohol as men (Jones, 2011), which might result in a higher rate of synthesis of PEth in red blood cells and brain. Conversely, the considerably faster rate of turnover of PEth in brain than in RBCs (Javors et al., 2016, Aradottir et al., 2004) as well as the sizable variability of ethanol absorption within groups of males and females might make these sex differences indistinguishable. Finally, it should be noted that previous studies have not been able to demonstrate unequivocal gender differences of PEth levels (Wurst et al., 2010, Bajunirwe et al., 2014). Future studies should include female brains when possible.

PEth levels in CE and OFC were dramatically higher in AUD-W than AUD-WO subjects' brains, suggesting that alcohol consumption immediately prior to death played an important role. This conclusion is based on the previous reports that PEth half-life in brain is less than 24 hours. Therefore, abstinence from alcohol for even a few days would reduce PEth levels significantly. Nevertheless, detectable PEth levels in postmortem brain tissue appears to confirm alcohol use within the few days immediately prior to death and very high levels of PEth might indicate excessive, harmful drinking.

Quantification of PEth 16:0/18:1 and PEth 16:0/18:2 levels in postmortem brain as indicators of recent alcohol consumption could be combined with other specific biological and clinical characteristics to more accurately diagnose drinking status before death. For example, acute intoxication and alcoholism have been shown to be associated with high suicide rates, and alcohol is found in the blood of most individuals who killed themselves (Caetano et al., 2013). We now show a direct association of higher serum alcohol levels with higher brain PEth levels, a family history of mental illness, and suicide. These suicides do not appear to be due to current depression. Individuals whose brains were used in this study and who killed themselves had more secondary diagnoses of mood disorders, but they did not have higher levels of mood symptoms around the time of death (data not shown).

There is the possibility of PEth synthesis in situ in the brain after death in the presence of ethanol. It's likely that ethanol existed in the brains of AUD-W subjects based on its presence in their serum and the known distribution of ethanol in total body water (Jones, 2011). Previous studies showed that PEth synthesis in the presence of 100 mM ethanol does not occur in blood and brain samples stored at 4°C for up to 72 h or after freezing in liquid

nitrogen for up to 144 h (Aradottir et al., 2004). The temperature at which the bodies of the AUD-W group were stored could affect PEth synthesis. Post-mortem interval is the time from death until the brain is frozen at the brain bank. Refrigeration time is the time after the medical examiner picks up the body until autopsy when the body is stored in a refrigerator at 0 to 4°C. The exact refrigeration time for our samples is not known, but all bodies were refrigerated prior to autopsy by the Medical Examiner. Since the post mortem interval average was 31 hours and at most 43.7 hours, we believe that the percent increase of PEth levels during our post-mortem interval would be minimal. It should be noted that the synthesis as well as the degradation of PEth in the brain in the presence of ethanol would occur at ambient temperature during the period between death and the time the medical examiner picks up the body. Therefore, the percent increase of PEth levels in human post-mortem during the post-mortem interval would be minimal.

This study used a well characterized cohort of postmortem donations, but all retrospective informant-derived information has some appreciable variance associated with it. To limit these errors, brains of subjects' matched for age and tissue quality and variability were selected. Another limitation was the use of brain tissue from only male subjects, which limits the generalizability of the findings. Future studies should include tissue from female subjects with and without AUD. Also, the relatively small sample size of 30 subjects, 10 in each of 3 study groups, should be increased in future studies. Finally, studies including non-AUD subjects with positive blood alcohol levels at the time of death would also provide additional information to elucidate the relationship between recent drinking immediately prior to death and the turnover of PEth levels in brain tissue.

In conclusion, quantification of PEth homolog levels in postmortem human brain is a good candidate as a diagnostic factor to classify drinking status prior to death, especially for those with AUD at the time of death. For alcohol research studies with postmortem brain, verification of drinking status is essential. This study supports future studies to characterize PEth levels in postmortem human brain.

Acknowledgments

Sources of Support: This publication was supported by the National Institute on Alcohol Abuse and Alcoholism of the National Institutes of Health [R01AA022361]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Figure 1.

PEth Homolog Levels in Postmortem Brain of Control, AUD-WO, and AUD-W Subjects.

Error bars are the means and standard deviations of PEth 16:0/18:1 (circles) and PEth 16:0/18:2 (squares) in CE and OFC. Dark grey symbols are PEth levels in CE and light grey symbols are PEth levels in OFC. P values are statistical significance between PEth groups using two-tailed Mann Whitney tests. (A) AUD-W, (B) AUD-WO, (C) Controls.

Figure 2.

Correlations between Serum Ethanol Levels at Time of Death and PEth Homolog Levels in CE and OFC. Symbols are PEth 16:0/18:1 (circles in Fig. 2A) and PEth 16:0/18:2 (squares in Fig. 2B) in CE (dark grey) and OFC (light grey). P values represent statistical significance for the correlations, which was determined using the equations for correlation in Prism 6 software and is represented by the p values on the graphs. (A) Serum ethanol versus PEth 16:0/18:1, (B) Serum ethanol versus PEth 16:0/18:2.

Table 1

Demographics, Brain Tissue Characteristics, Brain Phosphatidylethanol Homolog Levels, and Serum Ethanol Levels

		CON	AUD-WO	AUD-W
N		10	10	10
Sex		male	male	male
Ethnicity	Hispanic	3	4	3
	White	7	6	7
Age (years)	Range	20–68	42–64	25–66
	Average	46	51	48
PMI (hrs)	Mean (SD)	25.1 (6.4)	27.7 (10.6)	30.9 (7.3)
	Range	15.4–34.7	13.2–41.7	20.1–43.7
pH	Mean (SD)	6.33 (0.30)	6.33 (0.27)	6.54 (0.18)
	Range	5.88–6.66	5.90–6.68	6.21–6.88
RIN	Mean (SD)	7.75 (1.1)	7.3 (1.5)	8.23 (0.63)
	Range	6.00–8.90	4.20–8.70	7.20–9.30
CE	PEth 16:0/18:1 [μg/g (SD)]	0.294 (0.21) ^f	0.463 (0.46) ^d	14.8 (9.6) ^{a,h}
	PEth 16:0/18:2 [μg/g (SD)]	0.019 (0.02) ^f	0.021 (0.02) ^d	0.51 (0.5) ^{a,c,j}
OFC	PEth 16:0/18:1 [μg/g (SD)]	0.302 (0.29) ^g	2.130 (1.9) ^e	11.6 (8.2) ^{b,i}
	PEth 16:0/18:2 [μg/g (SD)]	0.030 (0.02) ^g	0.093 (0.01) ^e	0.16 (0.2) ^{b,c,k}
Serum [EtOH]	g/dL (SD)	ND	ND	0.144 (0.09) ^{h,i,j,k}

Mann-Whitney Comparison Tests:

^a, $p < 0.0001$;

^b, $p < 0.0001$;

^c, $p < 0.036$ - Fig. 1A

^d, $p = 0.0039$;

^e, $p = 0.0054$ - Fig 1B

^f, $p < 0.0001$;

^g, $p < 0.023$ - Fig. 1C

Correlations:

^h, $r^2 = 0.89$, $p = 0.0001$;

ⁱ, $r^2 = 0.79$, $p = 0.0006$ (Fig 2A);

^j, $r^2 = 0.21$, ns;

^k, $r^2 = 0.25$, ns (Fig 2B)

CE = cerebellum, CON = Control, AUD-WO = alcohol use disorder without serum ethanol levels, AUD-W = alcohol use disorder with serum ethanol levels, OFC = orbital frontal cortex, PMI = postmortem interval, RIN = RNA integrity, ND = not detectable, ns – not significant