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Differences in the Synthesis and Elimination of Phosphatidylethanol 16:0/18:1 and 16:0/18:2 after Acute Doses of Alcohol

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Abstract

Background—The purpose of this study was to examine the synthesis and elimination of phosphatidylethanol (PEth) 16:0/18:1 and 16:0/18:2 following the consumption of alcohol among 56 light and heavy drinkers.

Methods—A transdermal alcohol monitor was used to promote alcohol absence 7 days prior, and 14 days after, alcohol consumption in the laboratory. Participants consumed a 0.4 or 0.8 g/kg dose of alcohol in 15 min. Blood and breath samples were collected before, at various times up to 360 min post-consumption, and 2, 4, 7, 11 and 14 days after alcohol consumption. Initial rates of PEth synthesis, 360 min area under the PEth pharmacokinetic curves (AUC), and elimination half-lives were determined.

Results—(1) Non-zero PEth levels were observed before alcohol dosing for most participants, despite 7 days of alcohol use monitoring; (2) 0.4 and 0.8 g/kg doses of alcohol produced proportional increases in PEth levels in all but 1 participant; (3) the initial rate of synthesis of both PEth homologues did not differ between the two doses, but was greater for PEth 16:0/18:2 than PEth 16:0/18:1 at both doses; (4) the mean AUC of both PEth homologues was higher at 0.8 g/kg than at 0.4 g/kg; (5) the mean AUC of 16:0/18:2 was greater than that of PEth 16:0/18:1 at both alcohol doses; (6) the mean half-life of PEth 16:0/18:1 was longer than that of PEth 16:0/18:2 [7.8 ± 3.3 (*SD*) days and 6.4 ± 5.0 (*SD*) days, respectively]; and (7) there were no sex differences in PEth 16:0/18:1 or 16:0/18:2 pharmacokinetics.

Conclusions—The results of this study support the use of PEth 16:0/18:1 and 16:0/18:2 as biomarkers for alcohol consumption. Because of consistent pharmacokinetic differences, the levels of these two PEth homologues may provide more information regarding the quantity and recentness of alcohol consumption than either alone.

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Keywords

PEth Homologues; pharmacokinetics; blood; ethanol

INTRODUCTION

Phosphatidylethanol (PEth), a direct metabolite of alcohol, is a phospholipid formed in pcell membranes by a phospholipase D catalyzed reaction between phosphatidylcholine and ethanol (reviewed in Isaksson et al., 2011). Human PEth blood levels appear to have unique characteristics as a biomarker for alcohol consumption, including higher sensitivity and specificity, when compared to indirect markers such as γ-glutamyltransferase, aspartate transaminase/alanine transaminase, and carbohydrate-deficient transferrin (Aradottir et al., 2006; Hartmann et al., 2007; Helander et al., 2012; Wurst et al., 2010). PEth also has a longer detection window compared to other direct markers such as blood ethanol, breath alcohol, ethyl glucuronide, whose detection windows range from a few hours to a few days. In contrast, PEth appears to have a half-life of 4 to 10 days and can be detectable for up to 28 days after the last drink (Gnann et al., 2012; Javors et al., 2016; Schrock et al., 2017; Varga et al., 2000; Wurst et al., 2012). Furthermore, unlike other biomarkers, there have been no reports thus far of false positives for PEth (SAMHSA, 2012), including those that could be derived from liver disease (Stewart et al., 2009).

There have been a few studies examining the effects of controlled alcohol administration on PEth synthesis and elimination (Gnann et al., 2012; Javors et al., 2016; Kechagias et al., 2015; Varga et al., 1998). In the first (Varga et al., 1998), healthy volunteers were given a single dose of alcohol to achieve 0.12% blood alcohol concentration (BAC), but PEth was not detected. At that time only a less sensitive analytical method, high-pressure liquid chromatography (HPLC) with evaporative light scattering detection, was available. A more sensitive method was developed to quantify PEth levels: HPLC combined with tandem mass spectroscopic (HPLC/MS/MS, Helander and Zheng, 2009; Gnann et al., 2009; Zheng et al., 2011) and used in subsequent studies. Gnann and colleagues (2012) demonstrated that PEth 16:0/18:1 was detectable in whole blood samples within 1 hour after 11 healthy volunteers consumed 1 g/kg of alcohol with a mean half-life of 4 to 7 days. There was also an obvious inter-individual variability in the rate and capacity of PEth synthesis reported. In a third study, Kechagias et al., (2015) randomized participants to either a group that purportedly abstained from alcohol ($n = 23$) or consumed a prescribed amount of moderate alcohol daily (outside the laboratory) for a three month period ($n = 21$). They reported that PEth 16:0/18:1 was detected after moderate alcohol intake conditions and probably could be used to distinguish between moderate consumption and abstinence. It should be noted, however, that this latter study was not strictly a controlled study and that participants may have consumed more or less alcohol outside the laboratory than they were instructed. In a fourth study (Schrock et al., 2017), the synthesis and elimination of both PEth 16:0/18:1 and 16:0/18:2 were analyzed after 7 women and 9 men consumed enough alcohol in the laboratory to achieve a BAC of 1 g/kg (w/w). Blood samples were collected up to 8 hours after alcohol consumption and then the next 12 days. They found that PEth 16:0/18:2 was formed in lower concentrations in most participants and was eliminated faster compared to PEth

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16:0/18:1. However, all of the aforementioned studies relied on self-reported abstinence outside of controlled alcohol consumption.

Our pilot study (Javors et al., 2016) administered low alcohol doses in the laboratory where transdermal alcohol concentration (TAC) monitoring was used before and after dosing to promote abstinence and monitor possible drinking outside the laboratory. Participants received 0.25 ($n = 16$) or 0.50 g/kg ($n = 11$) oral doses of alcohol. PEth 16:0/18:1 and 16:0/18:2 levels were quantified by HPLC/MS/MS. Even after one week of TAC monitoring, most participants still had positive PEth levels. Nonetheless, administration of single doses of either 0.25 or 0.5 g/kg resulted in an immediate increase in PEth levels in all participants. Similar to Shrock and colleagues (2017), our pilot study showed that PEth 16:0/18:2 was eliminated faster than 16:0/18:1; however in contrast to them, we found that it was formed in higher concentrations in most participants. Nonetheless, all 5 studies showed that PEth 16:0/18:1 is reliably detected for periods spanning 1–2 weeks even after low-level drinking events.

The purpose of the current study, using the same study design as our pilot study, was to characterize dose related effects of 0.4 or 0.8 g/kg alcohol doses on the pharmacokinetics of PEth 16:0/18:1 and 16:0/18:2, and to evaluate possible sex differences. Thus far, however, there have not been any systematic studies to examine if differences in the synthesis and elimination of PEth exist between men and women. There has been one study, in alcohol-dependent inpatients (9 women and 48 men) where there were no sex differences in PEth levels measured over time (Wurst et al., 2010). For both PEth homologues, we examined the *in vivo* rate of synthesis, area under the concentration-time curves (over six hours after alcohol consumption), and their elimination rates during the next 14 days.

MATERIALS AND METHODS

Recruitment and Initial Screening

Participants responded to community advertisements. Individuals who met basic criteria during the initial phone screen were invited for an in-person interview and to give written informed consent prior to study participation. Additional screening included a detailed a history of alcohol consumed within the last 28 days using the Timeline Follow-back interview method (Sobell and Sobell, 1992), a psychiatric screening method using the Structured Clinical Interview for DSM-IV-TR Axis I Disorders (research version; First et al., 2001), substance abuse history, urine drug and pregnancy tests, and a medical history and physical examination by a physician's assistant.

Inclusion criteria were healthy men and women who: (1) were 21 to 54 years old; (2) were height and weight proportionate (body mass index 30; WIN, 2006) because alcohol doses were calculated based on weight; (3) reported having had at least one alcohol drinking episode in the last month where BAC would have been comparable to those expected in the study. In order to assure a range of drinking patterns, additional criteria recruited similar numbers of males and females whose TLFB interview indicated they were above or below a heavy drinking criteria. Light drinkers were defined as 3 for women, 4 for men per drinking day and 7 for women, 14 for men average drinks per week, and heavy drinkers

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exceeded those limits (i.e., > 3 for women, > 4 for men per drinking day and > 7 for women, > 14 for men average drinks per week).

Exclusion criteria were: (1) body mass index > 30 kg/m^2; (2) a current Axis I psychiatric disorder; (3) pregnancy or currently breastfeeding; (4) a current medical health condition; (5) a positive urine-drug test for the metabolites of drugs of abuse (cocaine, opiates, methamphetamines, barbiturates, benzodiazepines) during study entry or leading up to the day of alcohol administration in the laboratory; (6) evidence of alcohol withdrawal (Clinical Institute Withdrawal Assessment for Alcohol scores > 10; Sullivan et al., 1989); (7) pending criminal charges threatening incarceration or otherwise involving alcohol-related offenses; (8) a court mandate to not consume alcohol; or (9) consuming alcohol or tampering with the alcohol monitor more than once during the course of the study. The Institutional Review Board at The University of Texas Health Science Center at San Antonio reviewed and approved the experimental protocol.

Participants were randomly assigned to receive either 0.40 or 0.80 g/kg ethanol. Randomization was stratified on sex, body weight (i.e., above or below 63.5 kg for women or 83.9 kg for men), and type of drinker (light vs. heavy).

Study Design (see Supplemental Fig. 1)

Similar to our previous study (Javors et al., 2016) participants came in to complete Screening (always on a Monday), and were fitted with a TAC monitor (described below). Participants were told to abstain from alcohol use outside of the laboratory, which would be monitored by TAC readings on an ankle monitor worn continuously for the 22 days of the study. One week after screening, participants reported to the laboratory for the Alcohol Administration Day (see below), which was also always on a Monday. Participants were instructed to begin fasting at 12 AM on the alcohol consumption day, where blood and breath samples were collected for up to 360 min after alcohol consumption (see below). Follow-up visits occurred at 2 (Wednesday), 4 (Friday), 7 (Monday), 11 (Friday) and 14 (Monday) days post-alcohol consumption during which participants were expected to remain abstinent. During the follow-up visits, a single blood sample was collected by standard phlebotomy techniques for the quantification of PEth.

Participants received $20 for screening, $75 for the alcohol consumption day, $25 for each laboratory follow-up, and $10 per day for wearing the TAC monitor. Also, a bonus of $300 was also given to those who successfully completed the study.

Alcohol Administration Day

On the Alcohol Administration Day, starting breath alcohol concentration (BrAC) levels of zero g/dL were verified, and pregnancy and urine toxicology tests were obtained. Also, data from the TAC monitor were downloaded to monitor abstinence during the previous 7 day period. Participants were excluded from the study if any positive TAC readings or monitor tampering was detected. An intravenous catheter was placed in the antecubital fossa of the participant and a baseline blood sample was obtained prior to alcohol consumption. Participants then received either 0.4 g/kg or 0.8 g/kg of alcohol (Everclear; Luxco, Inc., St. Louis, Missouri), which was divided into 3 cups with juice (8 oz per cup). The 0.4 g/kg dose

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provided between 2 and 3 standard alcohol drinks and the 0.8 g/kg dose provided between 4 and 6 standard alcohol drinks (depending on the weight of the participant), where a "standard drink" as defined in the United States is equal to 14.0 grams (0.6 ounces) of pure alcohol. Participants, monitored by research staff, completed each cup within 5 min so that the entire dose of alcohol was consumed during a 15 min period. Blood and breath samples were collected pre-consumption (baseline) and at 15, 30, 45, 60, 90, 120 and 360 min post-consumption. A meal was provided 4 h post-alcohol consumption and participants remained in the laboratory until their BrAC was 0.030 g/dL.

Blood Sample Collection

All blood samples were collected into 4 mL vacutainer tubes containing 7.2 mg of K_2EDTA (Becton Dickinson Vacutainer Franklin Lakes, NJ USA) and immediately stored away from light at 4°C. PEth concentrations have been shown to not be affected when stored for up to 3 weeks at 4°C (Aradottir et al., 2004; 2005; Helander et al., 2009; Isaksson et al., 2011). Within 24 h, blood samples were aliquoted into 1.7 mL cryotubes and stored at −80°C, which is the recommended temperature for long term storage (Aradottir et al., 2002; 2004; Isaksson et al., 2011), until analysis for PEth (see below).

BrAC Monitoring

BrAC was measured using portable breathalyzers (Dräger Alcotest 6810 portable, Dräger Safety Diagnostics Inc., Irving, TX). The Dräger breathalyzer uses an electrochemical sensor that reacts specifically to alcohol and has a 365 day "Calibration Test lockout" feature that ensures that each breathalyzer is sent to the manufacturer for calibration. Each breath sample reading was acquired using a new disposable mouthpiece and participants rinsed their mouths with water twice to prevent residual alcohol contamination. Results displayed on the device are estimated % BAC and recorded by study personnel at screening, and 0, 15, 30, 45, 60, 90, 120 and 360 min post-alcohol consumption (the same time as the blood draws) during the Alcohol Administration Day, and during the 5 Follow-up visits.

Measurement of Transdermal Alcohol Concentrations (TAC)

Secure Continuous Remote Alcohol Monitors [SCRAM-CAM™, Alcohol Monitoring Systems Inc. (AMS), Highlands Ranch, CO)] TAC monitors were used to promote abstinence during the entire 22 day study period. This device measures TAC levels every 30 min continuously (Marques and McKnight, 2009). Our laboratory has used it in previous research studies to monitor alcohol use (e.g., Dougherty et al., 2015; Javors et al., 2016). The device records infrared signals and skin temperature to ensure no device disruption occurs. TAC monitoring results were downloaded at the laboratory on alcohol consumption day and during each of the 5 follow-up visits after alcohol administration day, using SCRAM Direct Connect™. Newly re-calibrated monitors were used for each participant.

For the current study, we used AMS confirmed events for alcohol use detection before and after the alcohol administration day. The details of AMS procedures for the resolution and confirmation of drinking events are proprietary. In brief, AMS determines whether there is evidence of environmental contamination or tampering and confirms a possible drinking event only when (1) 3 consecutive TAC readings exceed 0.02 g/dl; (2) the absorption rate

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0.05 g/dl//hr; and (3) the elimination rate > 0.003 g/dl/hr and 0.025 g/dl/hr if peak TAC < 0.15 g/dl or 0.035 g/dl/hr if peak TAC 0.15 g/dl. More discussion of the AMS criteria are provided elsewhere (Barnett et al., 2011; 2014).

Measurement of PEth 16:0/18:1 and 16:0/18:2 in Whole Blood

As described previously (Javors et al., 2016), PEth 16:0/18:1 and PEth 16:0/18:2 were quantified in uncoagulated, whole blood using HPLC/MS/MS. All solvents and reagents were HPLC grade and purchased from either Fischer or Sigma Chemical. Milli-Q Plus water was used to prepare solutions used in the experiments. 1-palmitoyl-2-oleoyl-phosphatidylethanol (PEth-16:0/18:1), 1-palmitoyl-2-linoleoyl-phosphatidylethanol (PEth-16:0/18:2), and deuterated 1-palmitoyl-2-oleoyl-phosphatidylethanol (dPEth-16:0/18:1) were purchased from Avanti Polar Lipids (Alabaster, USA).

In brief, immediately after removal from the −80°C freezer, the blood samples were thawed to room temperature on the workbench for about 15 min before the addition of isopropanol, which quenched the activity of PLD, similar to studies by us and others (Andreassen et al., 2017; Javors et al., 2016; Wang et al., 2017). It has been previously shown that when venous blood is collected in a tube containing EDTA and not centrifuged, the sample is stable for 24 h at room temperature (Isaksson et al., 2011). While ethanol was in some of the samples (as determined by BrACs measured at the same time blood samples were collected), the synthesis of PEth would have been negligible. Immediately after the samples have thawed to room temperature, 300 μl of each sample was added with constant mixing into 600 μL isopropanol and 5 μL of the internal standard solution (dPEth-16:0/18:1). After thorough mixing, the organic layer was evaporated to dryness and the pellets dissolved in HPLC mobile phase for injection into the HPLC/MS/MS. The ratio of peak areas of PEth-16:0/18:1 and PEth 16:0/18:2 to dPEth-16:0/18:1 were compared against a linear regression of ratios of calibrators from 0 to 4000 ng/mL. PEth-16:0/18:1 and PEth 16:0/18:2 concentrations were expressed in ng/mL. The lower limit of detection was estimated to be 5 ng/ml for both homologues. The imprecision of quantification ranged between 6 and 11% for control samples spiked with 42 (PEth 16:0/18:1) and 187 (PEth 16:0/18:2) ng/mL, respectively.

Statistics

BrAC and the levels of two PEth homologues were examined for 360 min after alcohol administration to measure the rate and extent of PEth formation after consumption of two alcohol doses. The decline of levels of PEth 16.0/18.1 and PEth 16.0/18.2 were used to measure PEth half-lives over 14 days thereafter. SAS Proc Mixed (SAS Release 9.3) employed analysis of variance (ANOVA) models to examine the effects of sex (M vs. F) and dose (0.4 vs. 0.8 g/kg). Though randomization was stratified on heavy vs. light patterns of drinking, only % heavy drinking days was considered as a covariate in statistical models and this became pertinent only for the prediction of the baseline starting point of PEth level monitoring. Time-course analyses employed mixed models ANOVA's accounting for the repeated measure of minutes (for the Alcohol administration day) and days for the 14 day follow-up period. BrAC areas under the curve were determined for samples collected during the Alcohol Administration Day. Owing to the potentially high levels and long half-life of PEth, and despite 7 days of TAC monitoring to promote abstinence, we observed detectable

levels of PEth in 47 of the 56 participants (84%) prior to any alcohol administration. Therefore, we adjusted statistical models for baseline PEth levels and used the difference from time zero (baseline) in PEth levels to calculate the areas under the concentration-time curves (AUCs) during 360 min after alcohol doses. AUCs were quantified using the trapezoid rule. The 360 minute BrAC and PEth homologue AUCs, initial rates of *in vivo* PEth homologue synthesis, and half-lifes were tested using a 2-way ANOVA. The relationship between BrAC and PEth homologue 360 min AUCs was tested using linear regression analysis (Prism 7.03, Graphpad Software, La Jolla, CA). Half-lives of PEth homologue levels were determined (Prism 7.03 software) using samples collected during the 14 day period after the Alcohol Administration Day. Useful PEth homologue data were not available for two female participants. Laboratory errors caused loss of PEth level determinations for one woman and only negligible PEth levels were observed for a second woman who started from 0.0 ng/ml at baseline and never increased above 12 ng/ml after alcohol administration. Therefore, data from only $n = 54$ participants were available for analysis.

RESULTS

Participants

Demographics of research participants stratified by dose and sex are shown in Table 1. Again, it should be noted that for one women PEth was 0.0 ng/ml at baseline and never increased above 12 ng/ml after alcohol administration, so her data was excluded from the study, therefore, data from only $n = 54$ participants are presented. The p values in Table 1 are for the differences between the two ethanol dose groups. Data from 27 males and 27 females aged 21 to 52 ($M = 27.6 \pm 6.32$ years) are presented. There were sex differences in: age ($p = 0.03$), $M = 25.7$ ($SD = 3.88$) years for men and $M = 29.5$ ($SD = 7.68$) years for women; weight ($p < 0.001$), $M = 81.1$ ($SD = 9.5$) kg for men and $M = 63.3$ ($SD = 8.22$) kg for women; the number of drinking days (out of 28 days prior to study entry; $p < 0.001$), $M = 13.2$ ($SD = 4.83$) days for men and $M = 8.30$ ($SD = 3.12$) days for women, the number of standard drinks per week ($p < 0.001$), $M = 17.9$ ($SD = 10.8$) for men and $M = 8.18$ ($SD = 4.13$) for women, and percent heavy drinking days ($p = 0.02$), $M = 22$ ($SD = 18$)% for men and $M = 13$ ($SD = 8$)% for women. There were no sex differences in the standard drinks per drinking day ($p = 0.10$ $M = 5.32$ ($SD = 3.17$) and $M = 4.10$ ($SD = 2.05$) for men and women, respectively.

BrAC and Baseline PEth Homologue Levels

After 0.4 and 0.8 g/kg doses of ethanol were consumed, proportional increases in BrAC were observed in all participants. Peak BrAC levels were observed within 45–60 min. Highly significant dose and time main effects and interactions (all $p < 0.0001$) were seen in the ANOVA model and there were no significant effects of sex alone or as interactions with dose or time. The AUCs for BrAC were higher in the 0.8 g/kg group [$M = 0.334 \pm 0.0708$ (SD)] than for those participants who consumed the 0.4 g/kg dose [$M = 0.110 \pm 0.0266$ (SD), $p < 0.0001$] and there was no overlap.

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Non-zero PEth homologue levels were observed for most participants before alcohol dosing on the Alcohol Administration day. PEth 16:0/18:1 levels were detectable for 47/56 participants, ranging from 8.0 to 787 ng/mL, and PEth 16:0/18:2 levels were detectable for 46/56 participants, ranging from 5.5 to 778 ng/mL. These positive PEth levels were observed despite 7 days of TAC monitoring to promote abstinence prior to alcohol administration. The 28-day drinking patterns of participants within the top quartile of baseline PEth levels is presented in Supplemental Table 1. None of the participants had any AMS-confirmed drinking events during the 7-day pre-study monitoring period. However, 37.5% of participants had a positive TAC event greater than 0.01 g/dl during that week suggesting possible low level drinking using lower threshold TAC criteria compared to standard AMS criteria (Dougherty et al., 2015; Karns-Wright et al., 2017; Roache et al, 2015). There were no significant ($p > 0.05$) differences in baseline PEth levels among those who had low level TAC readings and those who did not; and even among those for whom TAC levels were absolutely zero for all 7 days, the average baseline PEth levels were 105.2 ± 31.6 and 94.1 ± 19.9 for the PEth 16:0/18:1 and 16:0/18:2 homologues, respectively. However, baseline PEth levels observed were highly correlated with self-reported drinking over the 28 days preceding the study and the % heavy drinking days was the strongest predictor (see Fig. 1). Because men (22.1 ± 14.5 (*SD*) % heavy drinking days) drank heavily more often than the women (12.8 ± 8.35 (*SD*) % heavy drinking days), there were significant main effects of sex ($F_{1,50} = 4.1$, $p < 0.05$) on baseline levels of PEth 16.0/18:1 but not 16.0/18:2. However, inclusion of % heavy drinking days in the ANOVA models predicting baseline PEth levels were highly significant ($p < 0.004$ for both homologues) and removed the significance of sex as a predictor for either homologue.

Synthesis of PEth Homologues on Alcohol Administration Day

Because of non-zero baseline levels of PEth and possible sex differences in those baselines, statistical ANOVA models were adjusted for baseline PEth levels to reveal the levels of PEth 16.0/18.1 and PEth 16.0/18.2 induced by alcohol consumption (see Fig. 2). PEth homologue levels increased immediately after alcohol consumption and reached a maximum at about 60–120 min (Figs. 2A, 2B), suggesting up to a one hour time lag after the rising BrAC levels. As with the BrAC analyses, there were no significant effects of sex in any of the ANOVA models. All analyses showed highly significant effects of time (minutes, both $p < 0.0001$) but importantly, we observed a significant dose by time interaction for PEth 16.0/18.2 [$F_{7,350} = 2.26$, $p < 0.03$] and less so for PEth 16.0/18.1 [$F_{7,350} = 1.9$, $p < 0.09$]. The amount of PEth homologues synthesized as measured by AUCs proved to be more variable among the 2 alcohol doses than BrAC levels; that is, there was considerable overlap of AUCs of both PEth homologues (See Table 2 and Supplemental Fig. 2). Again, the ANOVA models detected no significant main effects or interactions of sex [all $p > 0.30$], but significant main effects of dose [$F_{1,50} = 5.5$, $p < 0.03$, and $F_{1,50} = 3{,}8$, $p < 0.05$] were found for PEth 16.0/18.1 and PEth 16.0/18.2, respectively. We also found that PEth 16.0/18.2 AUCs were higher than for PEth 16.0/18.1 (See Table 2 and Supplemental Fig. 2) in an ANOVA where only dose [$F_{1,50} = 5.7$, $p < 0.03$] and homologue [$F_{1,50} = 30.9$, $p < 0.0001$] were significant and the mean AUC for PEth 16:0/18:2 was higher than the mean AUC for PEth 16:0/18:1 at each dose individually.

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Initial Rate of PEth Homologue Synthesis

During the first 45 min of blood sample collection, which included 15 min for alcohol consumption and then another 30 min, the increase of PEth homologue levels was linear at both doses of alcohol (Figs. 2A, 2B). These data were used to calculate the initial rate of synthesis of both PEth homologues. The overall initial rate of PEth homologue synthesis showed only a statistically significant effect of homologue [$F_{1,50} = 25.13$, $p < 0.0001$] (Table 2 and Supplemental Fig. 3). There were no significant main effects or interactions with dose or sex (all $p > 0.2$) in the model indicating that the rate of synthesis was greater for 16.0/18:2 than 16.0/18:1. This effect was seen within each dose, and within each sex in separate analyses (not shown). Inclusion of baseline PEth value in the model showed a significant positive correlation [$F_{1,50} = 6.52$, $p < 0.02$] with initial synthesis rate, but no change in any of the other dose, sex, or homologue effects.

Elimination of PEth 16:0/18:1 and PEth 16:0/18:2

The elimination time course of the two PEth homologues is shown in Figs. 3a and b. The ANOVA model for PEth 16.0/18.1 showed significant effects of dose [$F_{1,50} = 7.7$, $p < 0.008$], day [$F_{4,200} = 24.6$, $p < 0.0001$], and a dose*day interaction [$F_{4,200} = 4.6$, $p < 0.0015$] and the same findings were obtained for PEth 16.0/18.2 [dose: $F_{1,50} = 5.5$, $p < 0.03$; day: $F_{4,200} = 50.5$, $p < 0.0001$, and dose*day: $F_{4,200} = 4.0$, $p < 0.004$], respectively. These results reflect the fact that the higher alcohol dose tended to start higher and have a steeper decline over days than the lower dose. For the PEth 16.0/18.1 homolog only, there also was a sex*day interaction [$F_{4,200} = 4.0$, $p < 0.004$] and a tendency ($p < 0.07$) for a sex*dose*day interaction which was largely driven by one male participant in the high dose group which started at very high PEth levels and showed a steep decline over days (data not shown). Separately, we calculated the terminal elimination half-lives of PEth (see Table 2) which are shown as scatter plots in Supplemental Fig. 4. There were no significant effects of dose or sex (both $p > 0.6$) in the ANOVA model but there was a significant effect of homologue [$F_{1,50} = 11.1$, $p < 0.002$] indicating that the mean half-life of the 16:0/18:1 homologue (7.8 ± 3.3 days, range 0.79 to 20.8) was greater than the half-life for 16:0/18:2 (6.4 ± 5.0 days, range 0.73 to 18.1). Although all participants wore TAC monitors to monitor alcohol use, 8 of them had an AMS confirmed drinking event above 0.02 g/dl over the 2-week monitoring period. Another 13 participants showed signs of 1–4 days of low-level drinking defined by us as TAC levels between 0.01–0.02 that were consistent with low level drinking (Roache et al., 2015). However, drinking was seemingly infrequent enough or at low enough levels that there was no evidence that any of these drinking criterion events affected the apparent half-life calculations. In fact the only significant effect observed in any exploration of these effects was that the 8 participants with AMS confirmed positive TAC readings had shorter (not longer) half-lives than the other participants without any positive TAC readings during 2-week monitoring period.

DISCUSSION

This study quantified the two most abundant of the 48 PEth homologues, PEth 16:0/18:1 and PEth 16:0/18:2 (Gnann et al., 2010, Helander and Zheng, 2009, Nalesso et al., 2011) after controlled alcohol consumption. The results showed: (1) measurable (non-zero) PEth levels

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before alcohol dosing for most participants, despite 7 days of TAC monitoring to promote abstinence; (2) single doses of 0.4 and 0.8 g/kg alcohol produced proportional increases in PEth levels in all but 1 participant; (3) the initial rate of synthesis of both PEth homologues did not differ between the two alcohol doses, but more PEth 16:0/18:2 was synthesized at a greater rate than PEth 16:0/18:1 at both doses; (4) the mean 360 min AUC of both homologues was higher at the 0.8 g/kg dose than at the 0.4 g/kg dose; (5) the mean 360 min AUC of 16:0/18:2 was greater than that of PEth 16:0/18:1 at both alcohol doses; (6) the mean half-life of PEth 16:0/18:1 was longer than that of PEth 16:0/18:2; and (7) there were no sex differences in any of the pharmacokinetic parameters for either of the PEth homologues studied.

All measurements of the rate and amount of PEth formation after single dose alcohol exposure were possible, even though detectable levels of both PEth 16:0/18:1 and 16:0/18:2 were observed prior to any alcohol administration for the majority of participants at baseline. Positive baseline levels were observed even for participants with one week of abstinence objectively confirmed by TAC monitoring. While not a primary hypothesis of this study, baseline PEth levels were significantly related to self-reported % heavy drinking days [$M = 22.1 \pm 14.5$ (*SD*) for men and $M = 12.8 \pm 8.35$ (*SD*) for women] during the month prior to the 7 day abstinence period. This is perhaps not surprising given the long half-lives of PEth 16:0/18:1 [$M = 7.8 \pm 3.3$ (*SD*) days, range 0.79 to 20.8] and PEth 16:0/18:2 [$M = 6.4 \pm 5.0$ (*SD*) days, range 0.73 to 18.1] that we observed during the two-week follow-up period after single dose alcohol exposure. These long half-lives clearly demonstrates that levels of these two homologues can be detected for longer than one week (PEth 16:0/18:2) or longer than 2 weeks (PEth 16:0/18:1). The half-lives for PEth 16:0/18:1 and 16:0/18:2 in whole blood reported here are similar to that reported by others: 3 to 7 days for PEth 16:0/18:1 (e.g., Gnann et al., 2012; Schrock et al., 2017; Zheng et al., 2011) and 4.4 ± 2.2 days (*SD*) for PEth 16:0/18:2 (Schrock et al., 2017). It is important to note that we were the first to monitor alcohol use with TAC, both prior to alcohol consumption as well as during a 14 day follow-up period. Though TAC monitoring did find some low level drinking, both before and after controlled alcohol consumption, we also had confirmed abstinence in most participants and only low level detections in others. These observations were used to show that these few abstinence violations had little effect on our ability to measure formation and half-life over the 2 week follow-up period. Nevertheless, the discovery of a few TAC detectable events highlights the value of using a secondary marker for alcohol consumption in studies where abstinence is requested and expected.

After alcohol consumption, blood levels of both PEth homologues increased in all but 1 participant, indicating that this biomarker has the sensitivity to detect acute consumption of moderate and heavy doses of alcohol (shown here) and low alcohol doses (shown in Javors et al., 2016). The initial rate of synthesis of each PEth homologue did not differ between the 0.4 and 0.8 g/kg alcohol doses, suggesting that the phospholipase D enzyme responsible for PEth synthesis was saturated at the lower dose. Both the mean peak PEth level attained and the mean 360 min AUC for PEth accumulation was greater for the higher alcohol dose, confirming a dose relationship in PEth detection. We also found that the initial rate of synthesis for PEth 16:0/18:2 was greater than for PEth 16:0/18:1 at each alcohol dose.

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Likely because of this higher rate of synthesis, the mean 360 min AUC for PEth 16:0/18:2 was greater than that of PEth 16:0/18:1 at each dose of alcohol.

As demonstrated in this and previous studies (Gnann et al., 2012; Javors et al., 2016; Schrock et al., 2017; Zheng et al., 2011), substantial between-subject variability in PEth levels, 360 min AUCs, and half-lives of both homologues was apparent (Supplemental Figs. 2, 4). In the study by Gnann et al. (2012), all participants ($n = 11$) drank alcohol to reach a BAC of about 1 g/L, yet whole blood PEth 16:0/18:1 levels varied as much as 3-fold between participants (Gnann et al., 2012). After consuming alcohol in the lab to achieve a BAC of 1g/kg (w/w) in the recent study by Schrock et al., (2017), mean peak levels of PEth 16:0/18:1 = 88.8 ± 47.0 ng/mL (*SD*), with a range of 37.2–208 ng/mL, and peak levels of PEth 16:0/18:2 = 63.5 ± 33.3 ng/mL (*SD*), with a range of 21.0–130 ng/mL. As noted above, there is has also been a lot of variability in the reported half-lives of PEth 16:0/18:1 and 16:0/18:2 (e.g., Gnann et al., 2012; Schrock et al., 2017; Zheng et al., 2011). Factors that might account for this between-subject variability may be differing: (1) phospholipase D levels/activity, (2) phosphatidylcholine homologues (PEth precursors), and (3) PEth elimination rates. Future studies in our laboratory are examining these variables.

Until now, there have been no studies with a large enough sample size to compare the pharmacokinetics of PEth 16:0/18:1 and 16:0/18:2 between men and women. There was a study of overall PEth levels (i.e., all PEth homologues combined) measured over time in alcohol-dependent patients (9 women and 48 men) entering inpatient treatment and no sex differences were found for PEth levels at any time point (Wurst et al., 2010). Under our study conditions, and with equal numbers (n=27 each) of men and women, we did not observe any sex differences in the initial rates of synthesis, 360 min AUC accumulation, or half-lives of PEth 16:0/18:1 or 16:0/18:2. This is important because despite known sex differences in BAC (Baraona et al., 2001; Breslin et al., 1997; Dettling et al., 2007; Fiorentino and Moskowitz, 2013), the extent to which those alcohol levels drive PEth formation seems to be unaffected by sex.

This study was initially designed to assess if there are differences in PEth homologue pharmacokinetics between types of drinkers (light vs. heavy). Unfortunately, our *a priori* plan to recruit light vs heavy groups did not achieve the desired separation of groups which is a definite limitation of the study. In fact, we did not end up with many heavy drinkers or many heavy drinking days. This resulted in too small of a sample size to provide any meaningful interpretation regarding the effects of light vs. heavy drinkers. Nonetheless, we did find that the levels of PEth still detectable after one week of TAC monitored abstinence were significantly related to the % heavy drinking days in the month prior to study participation. We also found that these baseline levels did not alter the rate of formation of PEth or our ability to measure increases due to a single exposure to alcohol. While there have been no *in vivo* studies to our knowledge for comparing PEth pharmacokinetics between light and heavy drinkers, there are two studies that examined the synthesis of PEth *ex vivo* in different types of drinkers. In one study (Mueller et al., 1988), lymphocytes were isolated from 25 alcoholic and 24 nonalcoholic men. The lymphocytes were incubated at 37°C with ethanol (0.5% final concentration) for 180 minutes. PEth synthesis was significantly greater in the lymphocytes from alcoholics than the controls. In another study

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(Varga et al., 2002), blood was drawn from healthy controls ($n = 6$) and alcoholics ($n = 6$) who were inpatients for at least 2 weeks. PEth was not detected in untreated red blood cell, from either controls or alcoholics. When the red blood cells were incubated with 10 and 50 nM ethanol, the *ex vivo* formation of PEth in the red cells from alcoholics was two-fold higher than in control cells. A possible explanation for these findings may be that PLD expression is up-regulated by regular ethanol consumption, leading to higher levels of PEth in heavy drinkers. Furthermore, PEth elimination may differ as a function of variability of red blood cell PLD levels, ethanol absorption, and ethanol history. Gnann and colleagues (2012) suggested that discrepancies observed in the half-life of PEth elimination between studies may be due to individual variability on these parameters. While studies have reported wide ranging differences in elimination among participants (e.g., Gnann et al., 2012; Helander et al., 2012), one study that examined elimination among inpatients, where alcohol abstinence was verified, found less range and variability between individuals (Varga, et al., 2000). Thus, future *in vivo* studies are required to determine if there are differences in the pharmacokinetics of PEth between light and heavy drinkers.

In our opinion, aside from the large variability in half-lives reported herein, one of the most important findings of our study was that PEth 16:0/18:2 is synthesized faster and to higher levels than PEth 16:0/18:1 immediately after alcohol consumption but it has a significantly shorter half-life, confirming our pilot study observations (Javors et al., 2016). This is in contrast to Schrock et al., (2017) who reported that PEth 16:0/18:2 was formed in lower concentrations than PEth 16:0/18:1 in most participants. This difference in conclusion may be due to design differences. Our studies collected blood at 0, 15, 30, 45, 60, 90, 120, and 360 min to more precisely measure rate of formation whereas Schrock and colleagues (2017), only collected blood at 0, 1, 3, 6 and 8 hours after alcohol consumption. Perhaps the differential peak levels of PEth 16:0/18:1 and PEth 16:0/18:2 occurred in between these more sparsely-sampled time points.

Earlier studies (Gnann et al., 2014; Helander and Zheng, 2009) also suggested that PEth 16:0/18:2 is synthesized and eliminated at higher rates compared to PEth 16:0/18:1. For example, as part of a study by Helander and Zheng (2009), 1 outpatient being treated for excessive alcohol use provided several blood samples over 11 weeks, which included a relapse into heavy drinking, to monitor PEth homologue levels. The authors noted that “quantifiable PEth 16:0/18:2 levels in this case was the most important species during the relapse” because 16:0/18:2 was higher than PEth 16:0/18:1 during relapse but it was eliminated faster. In another study, Gnann and colleagues (2014) found that in a group of inpatients ($n = 12$), PEth 16:0/18:2 levels decreased over the course of 20 days while 16:0/18:1, and two other PEth homologues, 18:0/18:2 and 18:0/18:1, remained at nearly constant levels. In that same study, blood collected from social drinkers ($n = 78$) also showed PEth 16:0/18:2 levels to decrease over 20 days, while PEth 16:0/18:1 remained relatively constant and the predominant species throughout. Taken together, most studies (including our own) indicate that PEth 16:0/18:2 is synthesized and eliminated at higher rates compared to PEth 16:0/18:1 in whole blood. With the longer half-life and less rapidly changing levels of PEth 16:0/18:1, it is also possible these data overall support the idea that tracking PEth 16:0/18:2 levels may be the best indicator of recent drinking.

Finally, that PEth 16:0/18:2 may be synthesized and eliminated at higher rates than PEth 16:0/18:1 suggests that phospholipids with palmitic (16:0) and linolenic (18:2) acids at the sn-1 and sn-2 positions provide greater affinity/catalytic capacity for their relative synthetic and catalytic enzymes. This differential synthesis and elimination of these PEth homologues, suggests the possibility that the ratio of PEth 16:0/18:1 to PEth 16:0/18:2 may become useful to estimate the time since the last previous drink or at least whether a person has been abstinent recently. In other words, the longer a person is abstinent after drinking, the greater the ratio of PEth 16:0/18:1 to PEth 16:0/18:2. This topic is being addressed in a current study. Future studies should also examine the synthesis and elimination of the next 3 of the most abundant of the PEth homologues found in human blood [PEth 16:0/20:4 (8 to 13% of total PEth), PEth 18:1/18:1 plus 18:0/18:2 (11 to 12% of total PEth), Gnann et al., 2010, Helander and Zheng, 2009, Nalesso et al., 2011]. Perhaps knowing the levels of a combination of some, or all, of the top 5 most abundant PEth homologues may provide more information regarding the quantity and recentness of alcohol consumption.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BAC	blood alcohol concentration
BrAC	breath alcohol concentration
HPLC/MS/MS	high-pressure liquid chromatography combined with tandem mass spectroscopic detection
PEth	phosphatidylethanol
TAC	transdermal alcohol concentration

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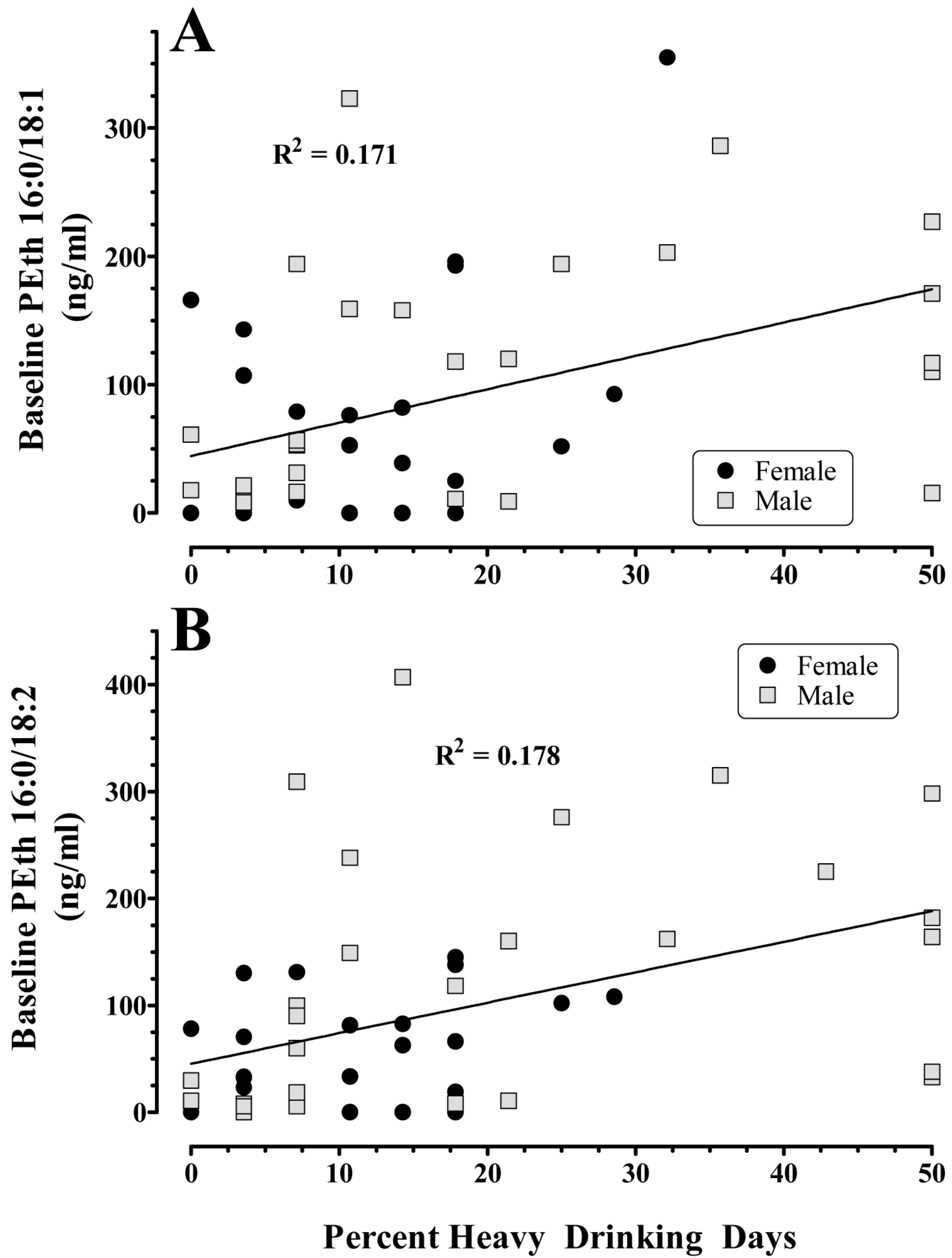

Figure 1.

Correlation of 28 day pre-study self-reported % heavy drinking days with baseline levels of PEth 16.0/18.1 (A) and 16.0/18.2 (B). Baseline levels were observed prior to alcohol administration in men (gray squares) and women (black circles). For each PEth homologue, the PEth level of one male participant was not included because their levels were extreme and outlying values (i.e., 787 ng/ml for 16.0/18.1 and 778 for 16.0/18.2).

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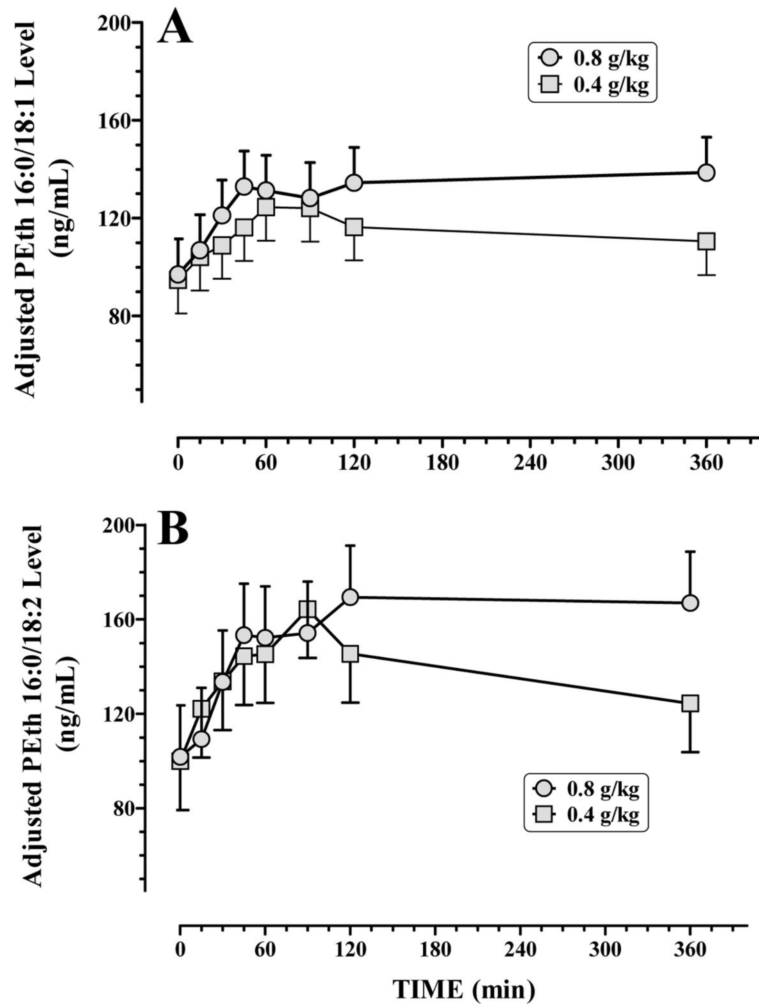

Figure 2.

Adjusted mean PEth concentrations of 16:0/18:1 (A) and 16:0/18:2 (B) at each time point up to 360 min after 0.4 (gray squares) and 0.8 (gray circles) g/kg doses of ethanol consumption. Error bars represent 95% confidence intervals.

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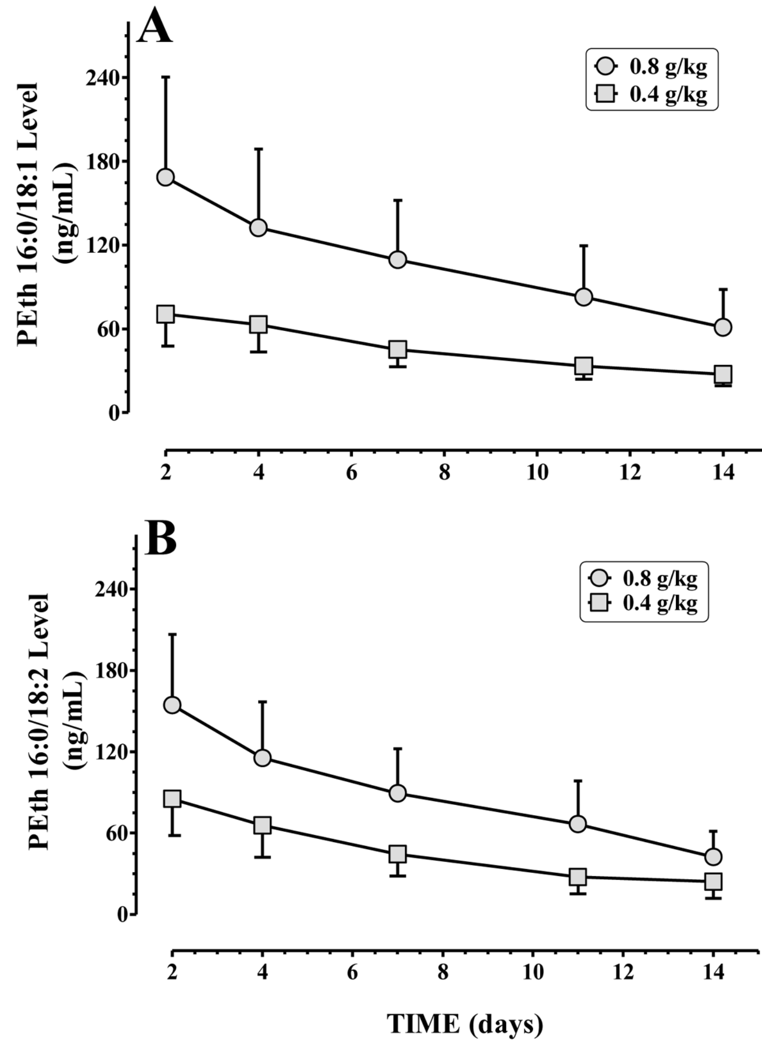

Figure 3.
(A) Mean PEth 16:0/18:1 and (B) mean PEth 16:0/18:2 concentrations up to 14 days after 0.4 (gray squares) and 0.8 (gray circles) g/kg doses of ethanol consumption. Error bars represent 95% confidence intervals.

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Table 1

Demographics

Characteristic	0.4 g/kg dose		0.8 g/kg dose		*P value*
	Men (n = 14)	**Women (n = 14)**	**Men (n = 13)**	**Women (n = 13)**	
	M(SD)	***M(SD)***	***M(SD)***	***M(SD)***	
Age (Years)	25.57 (3.55)	28.21 (8.79)	25.92 (4.34)	30.92 (6.32)	.38
Weight (kg)	81.82 (9.63)	63.37 (9.13)	80.25 (10.61)	63.28 (7.49)	.82
# Drinking Days (out of 28 days prior to study entry)	11.71 (4.73)	8.57 (3.08)	14.77 (4.59)	8.00 (3.27)	.34
Standard Drinks/Drinking Day	5.94 (4.00)	4.03 (1.55)	4.65 (1.86	4.17 (2.54)	.44
Standard Drinks/Week	17.85 (12.09)	8.57 (4.28)	17.95 (9.81)	7.75 (4.09)	.94
% Heavy Drinking Days	21 (18)	13 (8)	23 (17)	12 (9)	.89
Race (C/AA/O)*	8/1/5	9/0/5	8/3/2	10/1/2	.15
Ethnicity† (H/N)	11/3	8/6	7/6	7/6	.29

Note.

*Race is represented as the frequency of individuals in each group identifying as African-American (AA), Caucasian (C), or Other (O).

†Ethnicity is represented as the frequency of individuals in each group identifying as Hispanic (H) or Non-Hispanic (N).

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Table 2

Adjusted Mean Areas under the PEth Curves (AUC), Initial Rates of PEth Synthesis, and Half-Lives

0.4 g/kg (*n* = 28)	**PEth 16:0/18:1** ***M (SD)***	**PEth 16:0/18:2** ***M (SD)***
AUC (ng-min/mL)	8204 (8533)	15853 (13900)
Initial Rate of Synthesis (ng/ml-min)	0.624 (0.5561)	1.224 (1.224)
Half-Life (days)	9.24 (4.18)	6.72 (5.55)
0.8 g/kg (*n* = 26)		
AUC (ng-min/mL)	14426 (10687)	22918 (12249)
Initial Rate of Synthesis (ng/ml-min)	0.880 (2.67)	1.55 (1.340)
Half-Life (days)	8.47 (7.4 – 9.5)	6.59 (5.39)