Red blood cell fatty acid ethyl esters: a significant component of fatty acid ethyl esters in the blood

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Abstract Although alcohol abuse is known to cause an array of ethanol-induced red blood cell (RBC) abnormalities, the underlying molecular mechanisms remain poorly understood. Fatty acid ethyl esters (FAEEs) are toxic, nonoxidative ethanol metabolites that have been found in blood, plasma, and tissues. Because FAEEs have been shown to be incorporated into phospholipid bilayers, we conducted a controlled ethanol intake study to test the hypothesis that FAEEs accumulate and persist within RBCs following ethanol ingestion. We demonstrated that RBC FAEEs account for approximately 5% to 20% of total whole-blood FAEEs, and that the fatty acid composition of FAEEs in RBCs and plasma are different and vary differently over time. These data indicate that a significant percentage of FAEEs in the blood is associated with RBCs and that the metabolism of RBC FAEEs and that of plasma FAEEs (bound to albumin or lipoproteins) are largely independent.—Best, C. A., J. E. Cluette-Brown, M. Teruya, A. Teruya, and M. Laposata. **Red blood cell fatty acid ethyl esters: a significant component of fatty acid ethyl esters in the blood.** *J. Lipid Res.* **2003.** 44: 612–620.

Supplementary key words ethanol • red blood cell membranes • alcohol monitoring • alcoholism • alcohol • addiction

An association between alcoholism and abnormal red blood cell (RBC) size and shape has long been recognized (1). However, the underlying pathophysiologic mechanisms responsible for the morphologic alterations are incompletely understood (2–6). Fatty acid ethyl esters (FAEEs), esterification products of ethanol and fatty acids, have been shown to be incorporated into phospholipid bilayers up to 30 mol % of total membrane fatty acids (7). FAEEs have been implicated as mediators of ethanol-induced organ damage (8–13) and have clinical utility as markers for ethanol intake (14, 15). Serum and plasma FAEE levels closely correlate with blood ethanol levels, but these circulating FAEEs in blood persist for at least 24 h after ethanol ingestion, long after ethanol is no longer detectable (15, 16).

Although the largest reservoir of FAEE synthetic capability appears to reside in the pancreas and the liver (17, 18), enzyme activity that catalyzes FAEE synthesis has been detected in RBCs, white blood cells (WBCs), and platelets. Given the much higher concentration of RBCs in the blood, it has been suggested that RBCs may provide a significant portion of the total FAEE synthase activity in whole blood (19). With this information, the possibility that RBC membranes might synthesize and sequester FAEEs in the blood has been raised.

We conducted a controlled clinical trial to determine if FAEEs accumulate and persist within RBCs following ethanol ingestion. The objectives of this study were to determine: *1*) whether FAEEs appear in RBCs following ethanol ingestion and whether RBC-associated FAEEs correlate with blood ethanol concentration; *2*) how long FAEEs persist in RBCs after ethanol intake is discontinued; *3*) whether the fatty acid composition of RBC FAEEs is distinct from the fatty acid composition of FAEEs in plasma; and *4*) whether RBC and plasma FAEE species undergo a remodeling process with changes in fatty acid composition following discontinuation of ethanol ingestion.

METHODS

The study was approved by the institutional review board of the Massachusetts General Hospital. A written informed consent was obtained from each volunteer prior to subject enrollment.

Abbreviations: AIC, akaike information criteria; E16:0, ethyl palmitate; E18:0, ethyl stearate; E18:1, ethyl oleate; E18:2, ethyl linoleate; FAEE, fatty acid ethyl ester; GC-MS, gas liquid chromatography-mass spectrometry; RBC, red blood cell; SPE, solid-phase extraction; WBC, white blood cell.

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STUDY BEGINS   STUDY ENDS

Ethanol consumption:

No alcohol consumption   Begins   Ends   No alcohol consumption
5 days prior to study   Until 48 hour time point

90 minutes

Blood Sampling: Base line   1.75   3.5 5.5 7.25   24   48
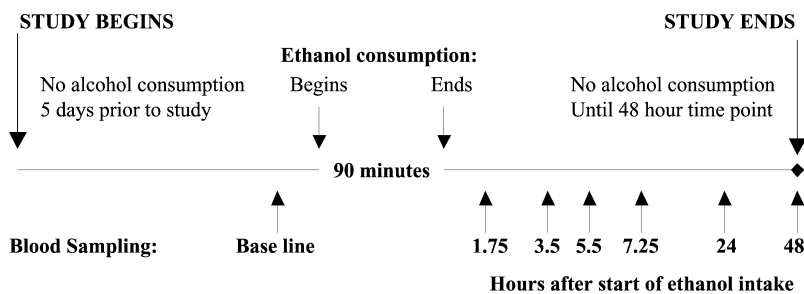
Hours after start of ethanol intake

Fig. 1. Study protocol. Following a 5 day period of alcohol abstinence, study subjects consumed a weight-adjusted amount of alcohol over a 90 min time period and had blood samples collected seven times over a 48 h time period. Each subject's blood was analyzed for ethanol and fatty acid ethyl ester (FAEE) concentrations. Lipids were extracted and FAEEs were isolated and quantitated by gas liquid chromatography-mass spectrometry (GC-MS) as described in Methods.

Study subjects

Study subjects were 21 years or older, were social drinkers (not nondrinkers or alcohol abusers), did not have diagnosed medical conditions, and female subjects were not pregnant. Subjects were excluded from the study if they reported consuming more than 14 drinks per week or more than seven drinks per sitting, or if they had a history of alcohol abuse or dependence.

Subject enrollment

The eligible study subjects had their age, weight, and height recorded, and they completed both a drinking history survey (Khavari Alcohol Test) (20) and a brief questionnaire that revealed their past 24 h of dietary intake.

Study procedures

Over a 90 min time period, the study subjects consumed a weight-adjusted aliquot of alcohol every 10 min as a mixture of 100-proof vodka and juice in a 1:3 ratio in order to attain a blood alcohol level of approximately 100 mg/dl (22 mM). Blood was drawn before the onset of drinking (baseline), and 1.75 h, 3.5 h, 5.5 h, 7.25 h, 24 h, and 48 h after the drinking period was initiated (**Fig. 1**). Not all subjects were available for testing at 24 h and 48 h. Subjects were safely discharged approxi-

mately 6 h after the 90 min drinking period (based on the mean biological elimination rate of ethanol (15–18 mg/dl/h or 3.3–3.9 mmol/l/h) (21).

Prior to the study, all the study subjects ingested a light breakfast. They were instructed to abstain from drinking alcohol for a period of 5 days prior to the study. In addition, subjects were asked to refrain from drinking alcohol for 48 h after ethanol intake in the study was discontinued. The subjects were paid for their participation.

Blood collection and analysis

At all time points, three blood samples were collected. One was collected in a vacutainer tube without anticoagulant (red top), one in a tube containing the anticoagulant sodium citrate (blue top), and one in a tube containing EDTA (purple top). The blue-top tubes were centrifuged immediately at 2,000 g at 5°C for 10 min to separate RBCs from plasma. The RBCs were washed prior to FAEE analysis as described below, and all RBC processing occurred within 2 h of blood collection. Serum and plasma samples from the respective red- and blue-top tubes were stored frozen at −80°C. Serum was used for measurement of ethanol concentration, and citrated plasma was used for FAEE analysis. Whole blood from the purple-top tubes was used to obtain

TABLE 1. Study subject characteristics

Subject Number	1	2	3	4	5	6	7	8
Physical data								
Age (years)	21	29	32	38	46	45	27	22
Sex	F	F	F	M	F	M	M	M
BMI (kg/m^2)	19.1	23.5	35.5	30.5	27.5	23.9	23.9	25.8
Peripheral blood parameters								
RBC × 10^{12}/l	4.2	4.5	4.9	4.6	5.2	4.4	5.1	5.5
WBC × 10^9 /l	5.3	6.6	10.1	7.0	6.8	7.1	6.8	4.6
Platelet count × 10^9/l	308	302	332	202	290	344	205	184
Hemoglobin (g/dl)	12.1	13.4	13.6	13.8	15.9	12.9	15.2	16.5
Mean corpuscular volume (fl)	86.3	83.9	79.6	86.0	87.7	84.6	91.7	84.3
Triglyceride (mg/dl)	90	107	307	128	175	328	71	271
Total cholesterol (mg/dl)	140	172	229	179	186	245	188	209
HDL (mg/dl)	72	45	39	39	57	47	74	36
Albumin (g/dl)	3.9	4.1	4.1	4	4.4	4.1	4.5	4.1
Alcohol intake history								
Beers/month	12	6	1	54	<1	<1	<1	1
Glasses of wine/month	<1	3	<1	12	12	8	20	<1
Drinks of liquor/month	20	<1	<1	<1	0	0	8	1
Total number of drinks/month	32	9	1	66	12	8	28	1
Subjective sense of sobriety	No signs of intoxication	Intoxicated	Intoxicated	No signs of intoxication	Intoxicated	Highly Intoxicated	Lightly intoxicated	Highly Intoxicated

RBC, red blood cell; WBC, white blood cell.

each subject's complete blood count by standard techniques using an ADVIA cell counter (Bayer, Tarrytown, NY).

Lipoprotein analysis and liver function tests

Baseline triglyceride, total cholesterol, LDL, HDL, aspartate aminotransferase, alanine aminotransferase, total protein, and albumin levels were determined by standard laboratory techniques using a Hitachi 917 automated chemistry analyzer (Boehringer Mannheim Diagnostics, Indianapolis, IN). Blood pregnancy tests were conducted on an Elecys 1010 automated chemistry analyzer (Roche Diagnostics, Indianapolis, IN).

Serum ethanol levels

Serum ethanol levels were determined by gas chromatography (22). Serum samples were mixed with an internal standard of 1-propanol, and a 1 μl sample was injected into a Hewlett-Packard 5890 GC (Hewlett-Packard, Palo Alto, CA) containing a 5% Carbowax 20M 60/80 Carbopack B column (Supelco, Bellefonte, PA). The oven temperature was set isothermally at 100°C, and the ethanol peak was identified and quantitated by comparison with a known standard.

RBC isolation

Following the initial centrifugation step to separate the RBCs from plasma, the RBCs were washed three times. Each RBC wash-

Fig. 2. A: Time course for total red blood cell (RBC) FAEE concentration and ethanol concentration over a 24 h period. Data points from 0 to 8 h represent the mean values ± SEM for subjects 1 through 8. The 24 h time point represents the mean value ± SEM for subjects presenting for testing at 24 h (subjects 1, 2, 3, 4, 7, and 8). Ethanol ingestion occurred during the first 1.5 h of the time course. B: Composite time course for total plasma FAEE concentration and ethanol concentration over the 48 h period. Data points from 0 to 8 h represent the mean value ± SEM for subjects 1 through 8. The 24 h and 48 h time points represent the mean value ± SEM for subjects presenting for testing at 24 h (subjects 1, 2, 3, 4, 7, and 8) and 48 h (subjects 1, 3, 7, and 8). Ethanol ingestion occurred during the first 1.5 h of the time course.

ing entailed the addition of five times the volume of 0.9% normal saline, gentle dispersion of RBCs by mixing with a plastic pipette, and centrifugation at 650 g for 10 min at 10°C. The saline wash was aspirated and discarded. The final centrifugation was at 1,500 g for 10 min at 10°C to pack the RBC sample more tightly and to minimize the volume of saline. The final saline wash was discarded, and the cells were resuspended in PBS (pH 7.4). Of the total cells in the sample, the washed RBC fraction contained on average 98.9% RBCs.

FAEEs from the washed RBCs and corresponding plasma samples were isolated by solid-phase extraction (SPE) and were identified and quantitated by gas liquid chromatography-mass spectrometry (GC-MS) as described below (15).

FAEE isolation and quantitation

Extraction of the lipids was initiated by the addition of 2 ml of acetone, followed by the addition of 50 μl (1 nmol) of ethyl heptadecanoate (E17:0) as an internal standard. After vortex mixing for 1 min, the samples were centrifuged at 650 g for 5 min at 4°C. The acetone layer was transferred to a fresh 15 ml conical glass tube, and 6 ml of hexane was then added to the tube, which was vortexed again for 1 min and centrifuged at 100 g for 5 min at 4°C. The supernatant was aspirated and saved in a separate tube. The remaining lower phase was washed with 2 ml of hexane, mixed for 1 min, and centrifuged at 100 g for 5 min at 4°C. The supernatant was removed and pooled with the saved supernatant. The hexane extract was evaporated to dryness under nitrogen, resuspended in 200 μl of hexane, and applied to a conditioned aminopropyl silica column (Bond-Elut LCR, Varian Diagnostics, CA).

SPE

The SPE procedure for FAEE purification was a method modified from that described by Kalunzny et al. (23). The aminopropyl silica columns were placed on a Vac-Elut vacuum apparatus (Analytchem International, Varian Diagnostics) set at 10 kPa. The Bond-Elut column was first conditioned with 4 ml of dichloromethane followed by 4 ml of hexane. Immediately after the solvent reservoir was empty, 200 μl of sample was applied to the column, followed by 4 ml of hexane and an additional 4 ml of dichloromethane. The hexane and dichloromethane fractions were then combined, evaporated under nitrogen, and resuspended in a small amount of hexane for GC-MS analysis.

GC-MS FAEE identification and quantification

GC-MS analysis was performed on a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard 5971 mass spectrometer equipped with a Supelcowax 10 capillary column. The
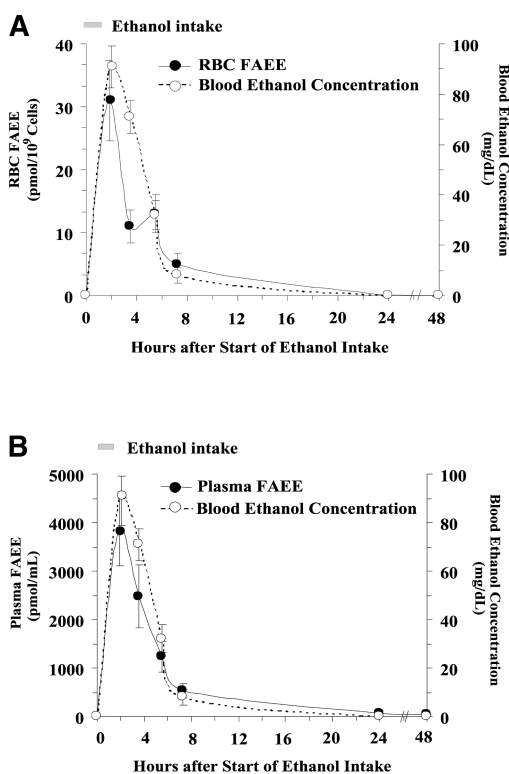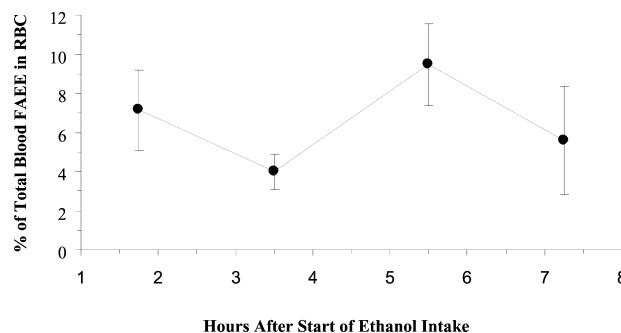

Fig. 3. The percent of whole-blood FAEE that is RBC associated. The data points from 0 h to 8 h represent the mean value ± SEM for subjects 1–8. The calculations used to obtain the values are presented in Results.
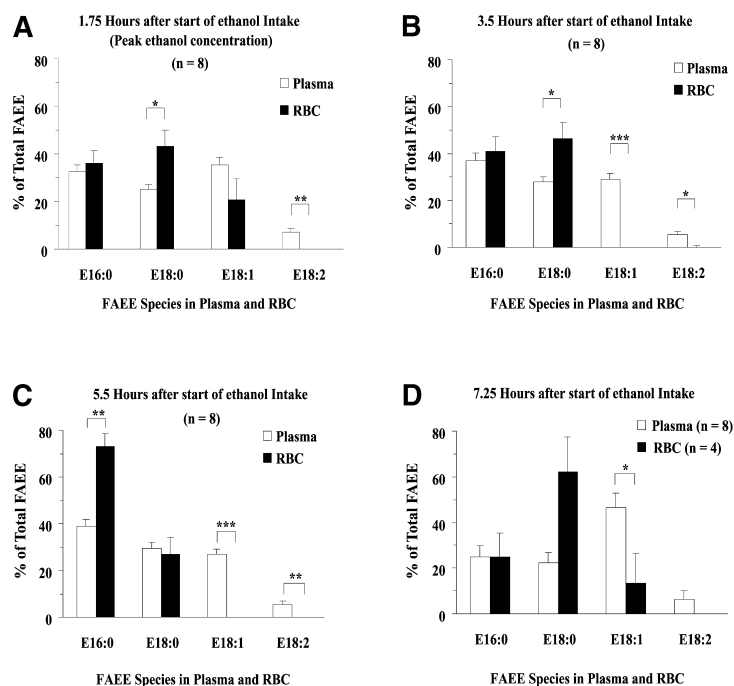
Fig. 4. The fatty acid composition of plasma and RBC FAEEs. FAEEs were isolated and quantitated by GC-MS as described in Methods. The FAEEs are expressed as a percent of total FAEE following ethanol intake. Plasma and RBC FAEE composition at 1.75 h (A), 3.5 h (B), 5.5 h (C), and 7.25 h (D) after the start of ethanol ingestion. E16:0, ethyl palmitate; E18:0, ethyl stearate; E18:1, ethyl oleate; E18:2, ethyl linoleate; $*P \leq 0.05$; $**P \leq 0.01$; $***P < 0.001$.

oven temperature was maintained at 150°C for 2 min, ramped at 10°C/min to 160°C, ramped again at 2°C/min to 180°C and held for 7 min, and then finally ramped at 15°C/min to 230°C, where it was held for 21 min. The injector and mass spectrometer were maintained at 260°C and 280°C, respectively. Carrier gas flow rate was maintained at a constant 0.8 ml/min throughout. Selected ion monitoring was performed, quantifying appropriate base ions for individual FAEE species [i.e., ions 67, 88, and 101 for ethyl palmitate (E16:0), E17:0, ethyl stearate (E18:0), ethyl oleate (E18:1), and ethyl linoleate (E18:2); and ions 79 and 91 for ethyl arachidonate (E20:4), ethyl eicosapentaenoate (E20:5),

and ethyl docosahexaenoate (E22:6)]. FAEE quantification was determined by interpolation of the slope generated from individually prepared standard curves comparing areas of varying concentrations of E16:0–E22:6 to fixed concentrations of E17:0. Mass relationships were obtained for each FAEE using its individual standard curve. Total FAEE mass was determined by addition of the masses of the individual FAEEs (E16:0–E22:6).

Fatty acid isolation

Fatty acids from washed RBCs were methylated according to Moser and Moser (24). Briefly, 250 μl of RBCs was mixed with 1

TABLE 2. Baseline RBC fatty acid composition versus RBC and plasma FAEE fatty acid composition at peak blood ethanol concentration

Fatty Acid	RBC Fatty Acid Composition At Baseline	RBC FAEE Fatty Acid Composition At Peak Blood Ethanol	Plasma FAEE Fatty Acid Composition At Peak Blood Ethanol
		% of total fatty acids	
16:0	27.8 ± 2.7	36.0 ± 6.3	32.6 ± 2.6
18:0	25.5 ± 1.8	43.1 ± 6.8	25.1 ± 1.9
18:1	14.2 ± 0.7	20.9 ± 8.8	35.3 ± 3.4
18:2	9.3 ± 0.8	0.0	7.3 ± 1.4
20:4	9.8 ± 0.8	0.0	Trace[a]
Other	13.4 ± 0.2	0.0	0.0
	Total Concentration	% of Total FAEE in 1 ml Whole Blood	
RBC fatty acid		NA	
nmol/10^9 RBC	311.3 ± 27.2		
nmol/mg Hgb	11.93 ± 0.7		
RBC FAEE		7.2 ± 2.1	
pmol/10^9 RBC	30.5 ± 6.4		
pmol/mg Hgb	1.23 ± 0.22		
Plasma FAEE		92.8 ± 2.1	
pmol/ ml Plasma	3801.5 ± 691.0		
pmol/mg total protein	30.65 ± 6.1		

FAEE, fatty acid ethyl ester. NA, not applicable. Mean ± SEM (n = 8).
[a] Trace indicates a value below reliable limits of quantitation, which is approximately 6–18 pmol/ml.

ml methanol-chloroform [3:1 (v/v)]. After addition of internal standard (∼50 nmol of heptadecanoic acid), 200 µl acetyl chloride was added and the sample was incubated at 75°C for 1 h. After cooling, the reaction solution was neutralized with 4 ml of 7% K$_2$CO$_3$, and the lipids were extracted into hexane. The hexane fraction was washed with acetonitrile and concentrated under nitrogen. The fatty acid methyl ester (FAME) mixture was then resuspended in hexane and analyzed by GC-MS.

GC-MS FAME identification and quantification

FAMEs were quantitated using the same instrumentation and under conditions similar to those discussed above for FAEE quantitation. Peak identification was based upon the retention time of the standard FAME and comparison of spectra with known standards in the database library of the GC-MS device. Total ion monitoring was performed, encompassing mass ranges from 50–550 amus. FAME mass was determined by comparing areas of the unknown FAME to a fixed concentration of 17:0.

Statistical analysis

Results were expressed as mean ± SEM. The unpaired Student's t-test was used to evaluate differences between the means of the groups. The differences were considered statistically significant at $P \leq 0.05$.

To assess potential correlations between RBC FAEE and plasma FAEE levels and ethanol concentration, it was necessary to use a weighted linear regression analysis to appropriately control for the correlation between FAEE levels within individuals. The data were analyzed with SAS PROC MIXED, version 8. The covariance structure of the data was estimated by using restricted maximum likelihood. The optimal model for the covariance was determined by using Akaike Information Criteria (AIC). The appropriate model for the means (FAEE vs. ethanol and time) was determined by performing likelihood ratio tests with the aid of (unrestricted) maximum likelihood.

RESULTS

Table 1 shows the characteristics of the study subjects. A total of eight subjects were included (four women and four men). The age range was 21 to 46 years of age. Subjects 1 and 4 had the most significant alcohol intake history, and during the study they felt no signs of intoxication. This observation implies that these subjects may have developed some level of tolerance to the effect of ethanol.

Figure 2A shows a time course of the mean values for all the subjects for RBC FAEE concentration versus blood ethanol level. The average peak blood ethanol level was 89 mg/dl ±10 and ranged from 48 mg/dl to 137 mg/dl. The legal level of intoxication is 80 or 100 mg/dl in the US, depending upon the state. There was significant overlap of the curves for the RBC FAEE level and the blood alcohol concentration. Figure 2B shows the data for plasma FAEE and blood ethanol concentration. There was an even more significant overlap of the curves in this case, consistent with previous findings (15). It should be noted that after 24 h in this study the plasma FAEEs were still detectable in all samples tested, despite undetectable blood ethanol levels. This also confirms the results of earlier studies (15). RBC FAEEs were detectable at 24 h (Fig. 2A) only in trace amounts in the samples available for testing.
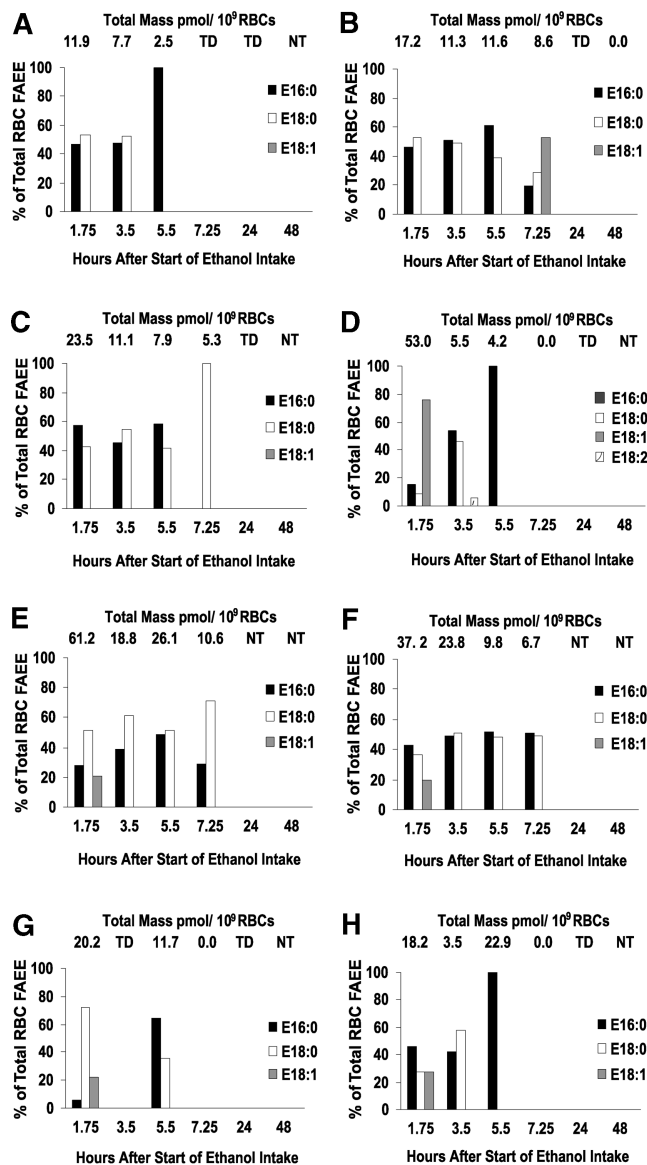

Fig. 5. The RBC FAEE species distribution over time. FAEEs were isolated and quantitated by GC-MS as described in Methods. TD, trace detected indicates a value below the reliable limit of quantitation; NT, not tested. The total mass of FAEE per 10^9 RBC is presented at the top of each panel. A–H: Correspond to subjects 1–8, respectively.

For each time point, the SEM reflects both biological and analytical variability and was unexpectedly low.

The mean RBC FAEEs accounted for approximately 7% of total whole-blood FAEEs (**Fig. 3**). The RBC FAEE amount varied from 4 ± 1% to 10 ± 2% (mean ± SEM) of total whole-blood FAEEs over the 7 h after the start of ethanol intake. To determine the mean percent of RBC FAEEs in 1 ml of whole blood (noted above), we performed the following calculations: *1*) the total RBC FAEEs in pmol/ml whole blood was determined by multiplying the RBC FAEEs pmol/10^9 RBCs by the number of RBCs × 10^9 in 1 ml of whole blood; *2*) the total plasma FAEEs in pmol/ml whole blood was determined by multiplying the total FAEE pmol in 1 ml of plasma by (1-hematocrit); *3*) to determine the percent of total whole-blood FAEEs that
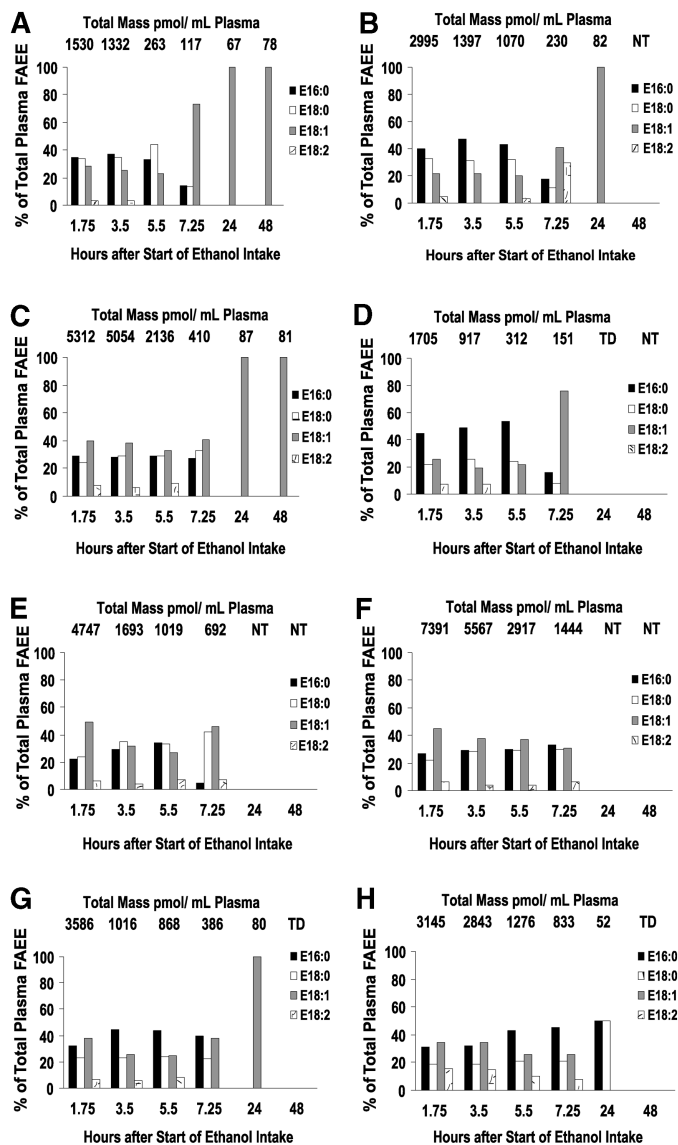
Fig. 6. The plasma FAEE species distribution over time. FAEEs were isolated and quantitated by GC-MS as described in Methods. TD, trace detected indicates a value below the reliable limit of quantitation, which is approximately 6 to 18 pmol/ml. The total mass of FAEE per ml plasma is presented at the top of each panel. A–H: Correspond to subjects 1–8, respectively.

was RBC associated, we divided the total RBC FAEEs in pmol/ml of whole blood by the total whole-blood FAEEs (RBC FAEEs plus the total plasma FAEEs).

In WBCs, we have detected widely variable amounts of FAEEs after ethanol ingestion. We performed a controlled ethanol intake study, in which blood ethanol levels reached 61 ± 6 mg/dl (mean $\pm$ SEM, n = 4). The peak FAEE concentration in lymphocytes, which have the highest FAEE synthetic activity among WBCs, was 10.0 ± 1.3 pmol/10^6 lymphocytes (mean $\pm$ SEM, n = 4). However, we found no FAEEs in lymphocytes isolated from ethanol-positive blood samples obtained from 18 patients admitted to our hospital's emergency department. In these cases, the WBCs were not available immediately upon blood collection, whereas in our controlled intake study they were processed within minutes. Using isolated populations of RBCs and WBCs in an in vitro study, we have shown that FAEEs are hydrolyzed at a rate approximately 1,000-fold greater in WBCs than in RBCs (25). Our working hypothesis from all of these findings is that WBC-associated FAEEs represent at most 1% to

2% of FAEEs in whole blood at peak blood ethanol concentration, and unlike RBC-associated FAEEs, are nearly completely degraded by 2 h after formation or uptake.

Figure 4 is a 4-panel figure that shows the fatty acid composition of the FAEEs in both plasma and RBCs over the first 7.25 h following the onset of ethanol intake. The two conclusions from these experiments are that the fatty acid composition of RBC FAEEs and plasma FAEEs are different and that their respective fatty acid composition profiles change differently over time. When compared with plasma, RBCs contain more saturated FAEEs as a percent of total FAEEs. Compared with plasma FAEEs, RBC FAEEs have significantly more E18:0 (as % of total FAEEs) at both 1.75 h and 3.5 h after the start of ethanol consumption ($P = 0.03$, $P = 0.03$, respectively). In addition, RBC FAEEs have significantly more E16:0 at the 5.5 h time point than do the plasma FAEEs ($P = 0.003$). In plasma, E18:2 consistently represented approximately 6% of FAEEs, but E18:2 was not detected in RBCs ($P < 0.001$). The presence of E18:1 in the RBCs diminished signifi-
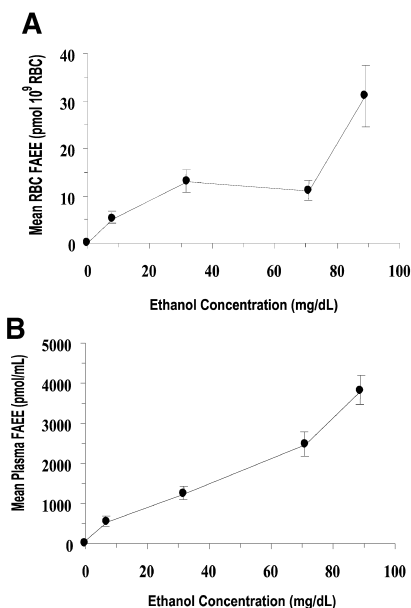
Fig. 7. A: RBC FAEE concentration (pmol/10^9 cells) correlation with serum alcohol concentration (mg/dl). B: FAEE concentration (pmol/ml) correlation with serum alcohol concentration (mg/dl). FAEEs were isolated and quantitated by GC-MS, and serum ethanol concentration was measured as described in Methods.

cantly from $21 \pm 9\%$ at peak ethanol concentration (1.75 h time point) to 0% at 3.5 h and 5.5 h post ethanol consumption ($P \leq 0.001$). In contrast, E18:1 in plasma increased as a percent of total FAEEs over time [$29 \pm 3\%$ at 3.5 h post ethanol ingestion to $47 \pm 7\%$ at 7.25 h post ethanol ingestion ($P = 0.035$)]. Over time, the percent of E16:0 in RBCs increased from $36 \pm 6\%$ at 1.75 h post ethanol ingestion to $73 \pm 6\%$ at 5.5 post ethanol ingestion ($P = 0.003$), and then the E16:0 declined substantially to $12 \pm 7\%$ at the 7.25 h time point. Taken together, these data show differences in FAEE composition and metabolism in RBCs and plasma.

Table 2 shows the total RBC fatty acid composition relative to the fatty acid composition of the RBC FAEEs and plasma FAEEs at peak blood ethanol concentration. The fatty acid composition of RBCs was determined upon initial exposure to ethanol (at baseline), and FAEEs were detected only after ethanol intake. The fatty acid composition of the RBC FAEEs did not reflect the total fatty acid composition (primarily phospholipid-associated fatty acids) of the RBCs. For example, compared with the percentage of RBC E18:0 (25.5%), RBC FAEEs showed an approximately 2-fold higher value for E18:0 (43.1%) as a percent of total RBC FAEEs. The relative amount of 18:1 in plasma FAEEs was greater than in total RBC fatty acids and RBC FAEEs. Thus, the fatty acid composition of RBC FAEEs and plasma FAEEs does not directly reflect the total fatty acid composition of RBCs.

Figure 5 is an eight-panel figure that shows the RBC FAEE fatty acid composition after the start of ethanol intake for each of the eight subjects. The individual panels show the marked variability in the distribution of FAEEs among the different subjects. In addition, all of the eight

subjects show significant remodeling of the fatty acids in the FAEEs over the time course, with different patterns of remodeling among the subjects. In subjects 2, 3, 5, and 6, FAEEs persisted in RBCs 7.25 h following ethanol ingestion, unlike in subjects 1, 4, 7, and 8, who showed RBC FAEEs only until the 5.5 h time point. Additionally, subjects 1, 4, and 8 all had 100% of their FAEEs as E16:0 at 5.5 h post ethanol consumption, and then had no detectable FAEEs at 7.25 h after the start of ethanol intake. Subjects 5 and 6 did not return for the 24 h blood collection, and subjects 4, 5, and 6 did not return for the 48 h post ethanol ingestion blood collection.

Figure 6 presents the data for plasma FAEEs for each of the eight subjects, and variations in remodeling patterns for fatty acids within plasma FAEEs were observed. There was a tendency toward a predominance of E18:1 with increasing time after ethanol ingestion. Subjects 1 and 4, with the highest ethanol intake history, showed the most rapid and most significant increase in E18:1 over time among the eight subjects. Of all the subjects tested except subject 8, at 24 h and 48 h post ethanol consumption, 100% of the FAEEs were E18:1. Subjects 1 and 3 had approximately 80 pmol FAEEs/ml plasma at 48 h post ethanol consumption, and subjects 7 and 8 had trace amounts of ethyl esters at 48 h post ethanol consumption. This extension to 48 h after the start of ethanol intake (and 46.5 h after discontinuation of intake) is a new finding with regard to how long FAEEs may be detectable in the plasma.

Figure 7 shows the relationship between the serum ethanol concentration and FAEE levels in RBCs and plasma over the first 7.25 h following the start of ethanol intake. There is a statistically significant positive relationship (that is approximately linear) between RBC FAEEs and blood ethanol concentrations (Fig. 7A). The estimate of the mean increase in RBC FAEEs associated with a 1 mg/dl increase in blood ethanol level is 0.123 pmol/10^9 RBCs $\pm$ 0.02 pmol/10^9 RBCs, $P = 0.0007$. There is a similar relationship between plasma FAEEs and blood ethanol concentrations, which is consistent with previous findings (15) (Fig. 7B). The estimate of the mean increase in plasma FAEEs associated with 1 mg/dl increase in blood ethanol level is 36.0 pmol/ml $\pm$ 3.4 pmol/ml, $P < 0.0001$. All mean models were fitted with the covariance model, with the lowest AIC for FAEE versus ethanol concentration. The time after drinking was initiated did not affect the correlation.

DISCUSSION

This report presents several novel observations regarding the presence of FAEEs and their metabolism in whole blood. Following ethanol consumption, approximately 7% of whole-blood FAEE was found in the RBCs, with the remaining 93% in plasma. The fatty acid composition of RBC FAEEs and that of plasma FAEEs were different. In addition, there were different changes in the fatty acid composition of RBC FAEEs versus plasma FAEEs over time. These changes in composition indicate that there

are independent RBC FAEE and plasma FAEE remodeling processes following ethanol ingestion.

The presence of certain fatty acids in FAEEs may be dependent on relative fatty acid substrate availability, selective fatty acid incorporation, and/or selective degradation of certain FAEE species. Existing literature also provides evidence suggesting that there are differences in FAEE metabolism among individuals and differences in the metabolism of particular FAEE species (26).

Chronic ethanol consumption is associated with abnormal RBC morphology and an increased susceptibility to hemolysis (27, 28). The underlying molecular mechanisms responsible for the effect of alcohol on RBC morphology are poorly understood (2–6, 29). Ethanol consumption may alter membrane cholesterol content, phospholipid class distribution, and fatty acid composition (30, 31). Membrane abnormalities have been suggested to occur because of an increased cholesterol-phospholipid ratio. However, cholesterol levels do not always increase after ethanol intake (4, 32).

Electron spin resonance (33), fluorescence (32, 34), and NMR techniques (28) have revealed a membrane-disordering effect of ethanol exposure. Ethanol has been shown to "fluidize" membranes, and chronic ethanol exposure limits this increase in membrane fluidity. The RBC membranes of alcoholics are more ordered (less fluid, more rigid) than are those of controls (35).

The incorporation of FAEEs into membranes may result in more rigid membrane structures with increased molecular order. Bird et al. demonstrated that the carbonyls of E18:1 and E16:0 are exposed to the aqueous interface of phospholipids, and they suggested that FAEEs align parallel to the fatty acid moieties (7). Partitioning of the FAEEs into the lipid bilayer may alter the biochemical properties of the cell membrane, the membrane shape, organization, and permeability, possibly by preventing lateral diffusion or rotation of phospholipids. Additionally, the presence of neutral hydrophobic FAEEs could reduce membrane fluidity, impair the deformability of RBCs, and consequently affect blood rheology.

We found in the present studies that RBCs contained more E16:0 and E18:0 (saturated FAEE) as a percent of total FAEE than does plasma FAEE (Fig. 4). Plasma FAEE consistently had higher amounts of E18:1 and E18:2. The increase in E16:0 as a percent of total FAEE in RBCs following ethanol intake may be because ethanol preferentially increases the rate of uptake of palmitic acid (relative to oleic acid) into the RBC membranes (36).

Following ethanol ingestion, E18:1 becomes the predominant FAEE species in plasma (Fig. 4). The subjects in our study with the highest ethanol intake history (1 and 4) showed a significant remodeling of the fatty acid within FAEEs to E18:1 at 7.25 h after ethanol ingestion. These findings are consistent with the observation that alcoholics have more plasma E18:1 as a percent of total FAEE than do binge drinkers (25). Alcoholics also tend to increase their fatty acid composition in plasma and cell membranes in general toward oleate (38–40).

In this report, RBC FAEEs comprise approximately 0.01 mol % of total RBC fatty acids, and we did not detect any RBC morphological abnormalities. The FAEE mol % required to alter RBC morphology is unknown, but it may be quite low. Calculations based on molecular dimensions of phospholipids indicate that the differential expansion or contraction of one lipid monolayer by 0.4% to 1.3% of its area is sufficient to produce an abnormal RBC morphology (37).

Further investigation is necessary to determine if the observed FAEE changes in RBCs are related to the RBC membrane disorders associated with chronic ethanol ingestion. Recently in an in vitro study, Tyulina et al. showed that FAEEs cause elevated RBC hemolysis and significant elevations in phosphatidylserine externalization to the outer leaflet of the membrane bilayer (41). In this study, the observed changes were not due to ethanol or acetaldehyde, a product of oxidative ethanol metabolism. They also revealed that the effects of FAEE on RBC instability and structure were more pronounced when albumin was absent (41). This finding may explain why altered RBC morphology occurs with alcoholic liver disease, given that injured livers often synthesize decreased amounts of albumin.

Summarily, these data show that RBC FAEEs account for approximately 7% of total whole-blood FAEE, that the fatty acid composition of RBC FAEEs changes over time following ethanol ingestion, and that RBC FAEE fatty acid composition is significantly different from plasma FAEE fatty acid composition. These results may be clinically significant because FAEE-induced alterations in membrane structure may lead to pathologic changes in RBC membrane function. In addition, RBC FAEEs may be useful in monitoring ethanol intake, along with other markers of ethanol intake.▨

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