Increased Plasma Fatty Acid Ethyl Ester Levels Following Inhibition of Oxidative Metabolism of Ethanol by 4-Methylpyrazole Treatment in Human Subjects

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Background: Recent experimental evidence suggests that fatty acid ethyl esters (FAEE), nonoxidative metabolites of ethanol, mediate ethanol-induced organ damage. A direct association between pancreas-specific toxicity and increased levels of FAEE following inhibition of the oxidative metabolism of ethanol by 4-methylpyrazole (4-MP) has previously been shown in studies with rats.

Methods: We obtained plasma samples from 32 healthy human volunteers who drank ethanol following 4-MP or placebo ingestion to determine whether in vivo inhibition of oxidative metabolism of ethanol causes a shift to nonoxidative metabolism of ethanol and the subsequent production of increased levels of FAEE. Plasma FAEE were isolated by solid-phase extraction and quantified by gas chromatography–mass spectrometry (GC-MS).

Results: Plasma FAEE levels in subjects receiving 4-MP treatment before ethanol consumption were elevated compared with plasma FAEE concentrations taken from control subjects who received a placebo before ethanol ingestion. Increased FAEE levels in the 4-MP treatment group occurred after peak blood ethanol, and peak FAEE levels were achieved. There was a correlation between the blood ethanol and the plasma FAEE levels, and the correlation persisted in the presence or absence of 4-MP. The peak FAEE values were greater in men than in women, with or without 4-MP treatment.

Conclusions: Our results indicate that the in vivo inhibition of the oxidative metabolism of ethanol using 4-MP results in an increased circulating concentration of FAEE, products of the nonoxidative metabolism of ethanol.

Key Words: Alcohol, Alcoholic Pancreatitis, Ethanol, Fatty Acid Ethyl Esters, 4-Methylpyrazole.

Fatty Acid Ethyl Esters (FAEE) are toxic nonoxidative metabolites of alcohol (ethanol). It was demonstrated in a 1986 report that FAEE were found in highest concentrations in the organs that are most commonly damaged by ethanol abuse, notably the pancreas and the liver, with lower but detectable amounts in the heart and brain (Laposata and Lange, 1986). Subsequent studies in vitro showed that FAEE increased the fragility of pancreatic lysosomes (Haber et al., 1993) and impaired oxidative phosphorylation (Lange and Sobel, 1983) and in intact cells were able to diminish the rate of cell replication and protein synthesis (Szczepiorkowski et al., 1995). More recent studies with lymphocytes and HepG2 cells have shown that FAEE induce apoptosis (K. Alhomsi and M. Laposata, unpublished data, 2005; Aydin et al., 2005). In addition, an in vivo study performed with rats showed that the infusion of FAEE in amounts easily achieved with binge drinking, with a single exposure to ethanol, was associated with pancreatic injury (Werner et al., 1997). Thus, there is growing evidence that FAEE are at least in part responsible for the cellular damage and organ damage associated with ethanol abuse.

Following ingestion, ethanol is largely directed toward oxidative metabolism in the liver by alcohol dehydrogenase (ADH) and at higher concentrations by the cytochrome P450 (CYP2E1) system. The oxidation of ethanol to acetaldehyde by ADH and CYP2E1 is followed by the oxidation of acetaldehyde to acetate (Riveros-Rosas et al., 1997). In addition, a small percentage of ethanol is directed, via a nonoxidative pathway, toward FAEE synthesis (Laposata, 1998). A number of enzymes that have FAEE synthetic activity have been identified, including a family of enzymes specifically named FAEE synthase (Best and Laposata, 2003; Laposata, 1998). The objective of the current study was to evaluate in humans in vivo plasma FAEE concentrations following the inhibition of oxidative ethanol metabolism via the use of 4-methylpyrazole (Fomepizole, 4-MP). Currently, 4-MP is
used clinically as an antidote to ethylene glycol poisoning (Brent et al., 1999) and in the treatment of methanol poisoning (Brent et al., 2001). We obtained plasma samples for FAEE analysis from a clinical trial that evaluated the acute effects of ethanol, and of 4-MP, on ethanol, acetaldehyde, acetate, and lactate in the venous blood as well as breath ethanol and acetaldehyde levels in human subjects (Sarkola et al., 2002). Treatment with 4-MP resulted in a 30% to 40% decrease in the elimination rate of ethanol, compared with placebo controls, with no detectable acetaldehyde formation in the blood (Sarkola et al., 2002). This decrease in ethanol metabolism was approximately the same in magnitude to earlier studies that used similar 4-MP doses (Jacobsen et al., 1996; Salaspuro et al., 1977).

Our analysis of FAEE levels in this study shows that (1) there was an increase in plasma FAEE levels in subjects receiving the 4-MP treatment before ethanol consumption, compared with controls who received a placebo, and that these increases occurred after the peak blood ethanol and peak FAEE were achieved; (2) there was a correlation between the blood ethanol and the plasma FAEE levels and the correlation persisted in the presence or absence of 4-MP; (3) the exposure to 4-MP resulted in the preservation of the original species of FAEE for up to 5 hours, with the control group showing a decrease at the later time points in ethyl oleate; and (4) the peak FAEE values were greater in men than in women with or without 4-MP. Taken together, these studies show that 4-MP is associated with a redirection of ethanol metabolism, toward the non-oxidative pathway and FAEE synthesis. The elevations in FAEE occurred after the peak blood FAEE concentrations were attained.

**MATERIALS AND METHODS**

**Materials**

4-Methylpyrazole was obtained as the free-base liquid from Sigma-Aldrich Inc. [St. Louis, MO (99.1% purity)]. Both the 4-MP solution and the placebo preparation were made/supplied by the University Pharmacy of Helsinki and were stored at 2 to 8°C until use (within 48 hours of preparation). The 4-MP and placebo solutions contained licorice extract, anise extract, and distilled water to disguise the 4-MP treatment solution.

The alcohol drink contained ethanol and lingonberry juice at a final concentration of 10% w/v (final volume 5.0 mL/kg of body weight) and was stored at 2 to 8°C until use (within 4 days of preparation). The placebo drink contained an equal volume of juice only.

**Methods**

The study was carried out according to the provisions of the Declaration of Helsinki. It was approved by an ethical committee and by the Finnish National Agency for Medicine. Signed informed consent was obtained from all participants before study participation.

**Study Subjects**

The study subjects included 10 females who did not take oral contraceptives [age 21 ± 2 years, body mass index (BMI) 21.5 ± 2.8 kg/m²], 10 females who regularly used oral contraceptives (age 25 ± 2 years, BMI 21.9 ± 1.8 kg/m²), and 13 males (age 24 ± 3 years, BMI 23.0 ± 2.7 kg/m²). All subjects were Caucasian, were light to moderate drinkers (they reported drinking less than 14 drinks per week), and had no record of disease. The female subjects’ menstrual cycles were all synchronized, and the females who used oral contraceptives were provided with the same brand of oral contraceptive that contained 30 μg of ethinylestradiol and 75 μg of gestodien.

**Study Design**

The study design was a double-blind, controlled, intervention crossover experiment. Subjects were asked not to drink alcohol for 1 week before the study and to abstain from heavy physical exercise and from eating 4 hours before blood collection. No smoking was allowed during the experimental sessions.

Each subject participated in 4 different experimental sessions (placebo + ethanol, 4-MP + ethanol, 4-MP + placebo, placebo + placebo), in random order. Blood samples were collected from the median cubital vein before the start of the experimental session (baseline), and at 75, 150, 225, and 300 minutes from the start of drinking alcohol or placebo (see Fig. 1). Then, 4-MP (10–15 mg/kg) or placebo was given orally, followed 2 hours later with ethanol (0.5 g/kg, corresponding to 2 to 3 standard drinks) or placebo, given orally as well. The final dose of 4-MP for all subjects was 10 to 15 mg/kg of body weight orally, and the dose per body weight was not significantly different between the study groups (12.9 ± 1.2 vs 13.9 ± 1.7 mg/kg for women and men, respectively, p = 0.12).

Plasma was isolated from whole-blood samples and was stored at −70°C until FAEE analysis. For subjects who used oral contraceptives, the pill was taken 2 hours before the ingestion of ethanol or placebo. The drinking session lasted 15 minutes and the subjects remained seated throughout the experimental sessions. The occurrence of adverse effects was monitored with a questionnaire, and no adverse effects of 4-MP were observed.

**Analytical Procedures**

Blood samples (10 mL) were collected into tubes that contained 22.5 mg of sodium fluoride and 22.5 mg of potassium oxalate as an anticoagulant. The study was carried out according to the provisions of the Declaration of Helsinki. It was approved by an ethical committee and by the Finnish National Agency for Medicine. Signed informed consent was obtained from all participants before study participation.

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anticoagulants. Plasma samples (1 mL) for FAEE measurements were stored at −70 °C until lipid extraction and gas chromatography–mass spectrometry (GC-MS) for isolation and quantification of FAEE.

Ethanol levels were determined from plasma samples by headspace gas chromatography (Perkin-Elmer 8410, Perkin-Elmer Inc., Norwalk, CT). The intraassay and interassay coefficients of variation were 4.0 and 5.1% at the level of 1.5 mmol/L (n = 10), and the detection limit was 0.03 mmol/L.

**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 minute, the samples were centrifuged at 650×g for 5 minutes at 4 °C. The acetone layer (fluid phase above the protein pellet) was transferred to a fresh 15-mL conical glass tube, and then 6 mL of hexane was then added to the tube, which was vortexed again for 1 minute and centrifuged at 100×g for 5 minutes 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 minute, and centrifuged at 100×g for 5 minutes 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 amipropyl silica column (Bond-Elut LCR, Varian Diagnostics, Harbor City, CA; Bernhardt et al., 1996).

**Solid Phase Extraction**

The solid-phase extraction (SPE) procedure for FAEE purification was a method modified from that described by Kalunzny et al. (1985). The amipropyl silica columns were placed on a Vac-Elut vacuum apparatus (Varian) 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 (Bernhardt et al., 1996).

**GC-MS Identification and Quantitation of FAEE**

Gas chromatography–mass spectrometry analysis was performed on a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard 5971 mass spectrometer (Hewlett-Packard, Palo Alto, CA) equipped with a Supelcowax 10 capillary column (30 m × 0.25 mm, 0.2-μm film thickness). The oven temperature was maintained at 150 °C for 2 minutes, ramped at 10 °C/min to 160 °C, and ramped again at 2 °C/min to 180 °C. The temperature was held at 180 °C for 7 minutes and then ramped at 15 °C/min to 230 °C, where it was held for 21 minutes. The injector and mass spectrometer was maintained at 260 and 280 °C, respectively. Carrier gas flow rate was maintained at 0.8 mL/min throughout the GC-MS analysis. Selective ion monitoring was performed, quantifying appropriate base ions for individual FAEE species (i.e., ions 67, 88, and 101 for ethyl palmitate (E16:0), ethyl heptadecanoate (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)). Fatty acid ethyl esters were quantitated by interpolation of the slope generated from individually prepared standard curves, comparing areas of varying concentration of E16:0-E22:6 with fixed concentrations of internal standard (E17:0).

Mass relationships were obtained for each FAEE using an individual standard curve for a given species of FAEE. Total FAEE mass was determined by addition of the masses of the individual FAEE (Best and Laposata, 2003).

**Statistical Analysis**

Results are reported as mean ± SEM. Statistical significance was tested by using paired t test analyses.

**RESULTS**

Figure 1 shows the study protocol. The study subjects proceeded through 4 experimental sessions after a week of abstinence from alcohol. The sessions were placebo + ethanol, 4-MP + ethanol, placebo + 4-MP, and placebo + placebo. These experimental sessions were arranged in random order, and the subjects were blinded to the different treatments within each experimental session. Before each session, a blood sample was taken and then a 15-minute treatment period was commenced. Blood samples were subsequently collected at 5 additional time points over a 300-minute time period. The samples were then analyzed for both ethanol and FAEE levels.

Figure 2 shows the plasma FAEE concentration relative to the time from ethanol intake, with a comparison between the groups receiving placebo + ethanol versus those receiving 4-MP + ethanol. The figure shows that the subjects receiving 4-MP, the alcohol dehydrogenase inhibitor, showed statistically significantly higher levels of ethanol, 4-MP or Placebo

**Fig. 2.** Time course for total plasma fatty acid ethyl ester (FAEE) and plasma ethanol levels after the intake of ethanol (0.5 g/kg orally) after pretreatment with 4-methylpyrazole (4-MP) (●) or placebo (○). Values are reported as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 (n = 33).
FAEE after the peak FAEE levels were attained at approximately 75 minutes. Thus, the difference between the 2 groups occurred specifically during this period of alcohol metabolism. The increased plasma FAEE concentrations, following 4-MP pretreatment, were coupled with a decrease in the ethanol elimination rate in both men and women (Sarkola et al., 2002). The results, therefore, indicate that inhibition of the oxidative metabolism of ethanol results in increased flux through the nonoxidative pathway of FAEE synthesis.

The addition of 4-MP did not disrupt the linear relationship between blood ethanol concentrations and FAEE levels in the plasma. In subjects receiving placebo along with ethanol, there was a linear association between ethanol and FAEE, with a correlation coefficient of \( r = 0.956 \). When the same subjects were treated with 4-MP, the relationship between ethanol and FAEE was preserved with a correlation coefficient of \( r = 0.959 \). When the same subjects were treated with 4-MP, the relationship between ethanol and FAEE was preserved with a correlation coefficient of \( r = 0.959 \).

Fatty acid ethyl ester species changed over time following ethanol ingestion. The data shown in Fig. 3 represent the fatty acid composition of the FAEE found in the subjects receiving placebo+ethanol (Fig. 3A) and the subjects receiving 4-MP+ethanol (Fig. 3B). The 1 major difference between the 2 groups was that there was a higher concentration of ethyl oleate at the later time periods after ethanol intake in the subjects receiving 4-MP. It is not clear why there is a preferential retention of ethyl oleate in subjects receiving the 4-MP. The ethyl oleate, as a percent of total FAEE, was 32.98 ± 0.9 and 19.65 ± 2.15 at 75 and 300 minutes after ethanol intake in this group. In the subjects receiving ethanol without the alcohol dehydrogenase inhibitor (placebo plus ethanol), the concentration of ethyl oleate declined, over time from approximately 33.97 ± 1.78 to 6.76 ± 2.05, at the 75- and 300-minute time points, respectively. There was a corresponding increase in the concentration of ethyl palmitate.

The subjects were also evaluated by gender for FAEE production in the presence or absence of 4-MP pretreatment. It was demonstrated that in the presence of 4-MP, males achieved a higher peak FAEE concentration following ethanol intake than did the females. The absence of 4-MP pretreatment results showed a slight trend toward higher FAEE values in males, compared with females, but the difference was not statistically significant. No significant differences in plasma FAEE levels were noted in females who did or did not use oral contraceptives.

**DISCUSSION**

This report demonstrates that inhibition of oxidative ethanol metabolism using 4-MP results in a greater flux of ethanol toward the nonoxidative production of FAEE. This important linkage between the 2 pathways is supported by the data in this study involving healthy volunteers who received treatment with 4-MP and ethanol, as well as in previous in vitro and in vivo studies performed with rats (Werner et al., 1997, 2002). In these studies, we were also able to demonstrate that inhibition of oxidative metabolism results in a 2- to 3-fold increase in the production of FAEE in the liver and pancreas. Additionally, we have shown that increased FAEE concentrations in the pancreas in the presence of alcohol dehydrogenase inhibition are associated with pancreatic toxicity, as measured by pancreatic edema, pancreatic trypsinogen-activating peptide levels, and increased vacuolization of acinar cells (Werner et al., 1997). In an earlier study involving the use of 4-MP that investigated ethanol metabolism and FAEE synthesis by the rat pancreas, liver, and lung, a 20-fold increase in pancreatic FAEE concentrations in rats pretreated with 4-MP was reported (Manautou and Carlson, 1991). Male rats received 10% ethanol in their drinking water with or without daily intraperitoneal injections of 4-MP (1 mmol/kg) for approximately 15 days. This current report advances the findings from the in vitro studies and from animal studies to this clinical study involving humans.

Similar to previous observations, we noted changes in the FAEE species distribution over time following ethanol ingestion and we observed that 4-MP influences the distribution of FAEE species (Fig. 3). The major FAEE species detected in this study were as follows: ethyl palmitate, E16:0; ethyl oleate, E18:1; ethyl stearate, E18:0; and ethyl linoleate, E18:2. The data support the concept that there is fatty acid remodeling within the FAEE pool over time as shown in a number of other studies (Best et al., 2003; Doyle et al., 1996; Soderberg, 1999). In a previous clinical trial involving 8 healthy volunteers, we compared red blood cell (RBC) and plasma FAEE species, and we
assessed the variability in the distribution of FAEE among the different subjects following ethanol ingestion over a 48-hour time course. The percent distribution of FAEE species in RBC and in plasma both changed over time, but not in an identical fashion. The subjects who reported the highest ethanol intake by history (32 and 66 drinks per month) showed a significant increase in ethyl 18:1 in the plasma over time, as a percent of total FAEE, compared with the subjects who were light drinkers (who reported drinking less than 12 drinks per month; Best et al., 2003).

The variability in distribution of specific FAEE species among individuals may be clinically relevant as differences in the fatty acid composition of FAEE may be useful in the differentiation between chronic alcoholism and binge drinking. Soderberg et al. (2003) reported that chronic alcoholic individuals have a higher concentration of ethyl 18:1 in the serum compared with individuals who report low to moderate alcohol intake. Why the 4-MP treatment in this case resulted in the preferential preservation of ethyl oleate over time (Fig. 3) is not clear, especially given that these subjects were not alcoholic individuals.

Furthermore, these findings may be clinically important because variations in FAEE production may explain why only 5% to 10% of heavy drinkers develop pancreatitis. (Bisceglie and Segal, 1984; Mergener and Baille, 1997). There are a number of factors that may explain the individual variability in the pathological consequences of ethanol abuse or varied susceptibility to organ damage. These include variations in FAEE species distribution, tissue-specific accumulation of FAEE or high circulating FAEE levels. Elevated concentrations of FAEE may result from a reduction in the oxidative metabolism of ethanol because of variations in the activity of enzymes, which oxidize ethanol to acetaldehyde; such variability may be a result of genetic alterations, liver disease, or pharmacological interventions. (Day et al., 1993; Frenzer et al., 2002; Maruyama et al., 1999; Matsumoto et al., 1996; Verlaan, 2004; Zavras et al., 2002).

In this study, we also demonstrated a difference between male and female FAEE levels during the 4-MP pretreatment experimental sessions. The males had higher concentrations of FAEE at peak concentration, despite the absence of a difference in blood ethanol levels between the males and females exposed to 4-MP (Sarkola et al., 2002). The similarity in blood ethanol concentration was expected because the males and females ingested a similar weight-based dose of ethanol to achieve a targeted blood ethanol level. We previously observed that despite similar blood ethanol levels, men had higher FAEE values than women (Soderberg, 1999). The finding in the current study that men have a higher FAEE level than women, but only with 4-MP exposure, also reflects greater FAEE production in men. There was no difference, however, between men and women in this study in the absence of 4-MP pretreatment (Fig. 4).

Taken together, the current study demonstrates in human subjects ingesting ethanol that there is a linkage between the oxidative and nonoxidative pathways of ethanol metabolism and that inhibition of the oxidative pathway results in increased flux of ethanol toward the nonoxidative metabolite, FAEE. It is possible that increased FAEE production, owing to a shift from oxidative to nonoxidative metabolism of ethanol, might result in enhanced cytotoxicity and damage to organs such as the pancreas and liver.

**REFERENCES**


