Fatty Acid Ethyl Esters in Liver and Adipose Tissues as Postmortem Markers for Ethanol Intake

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Background: Fatty acid ethyl esters (FAEEs) are nonoxidative metabolites of ethanol. FAEEs are found in liver, pancreas, and adipose tissues up to 24 h after consumption of ethanol, and on that basis, they are potentially useful markers for ethanol intake. In this study with rats, we investigated the efficacy of using FAEEs in liver and in adipose tissue as postmortem markers for pre-mortem ethanol ingestion.

Methods: An animal study was conducted in which test rats received injections of ethanol and control rats received injections of normal saline. The rats were killed 2 h after the injections. The bodies of the animals were stored at 4 °C up to 12 h, and samples of liver and adipose tissues were collected at different time intervals and processed for FAEE quantification. In another set of experiments, the rats received injections and were killed as described above, but bodies of animals from both groups were stored at 4, 25, or 37 °C for up to 72 h, and liver samples were collected and processed for FAEE quantification.

Results: FAEEs were detected up to 12 h after death in liver and adipose tissue samples from the bodies of ethanol-treated animals stored at 4 °C; negligible amounts were detected in the bodies of animals that received normal saline. Adipose tissues contained higher amounts of FAEEs than liver, as well as more species: eight FAEE species in adipose tissue and five in liver tissue. Higher concentrations of FAEEs were detected in livers of treated animals stored at 25 °C for up to 48 h than in livers of controls stored under the same conditions.

Conclusions: For at least 12 h after death, FAEEs in liver and adipose tissues are useful postmortem markers of pre-mortem ethanol ingestion.

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after hydrolysis toward triglycerides in both humans and rats (10, 15); (d) ethyl oleate as a representative FAEE within human LDL was degraded in both human and rat blood over a 30-min incubation (10). FAEEs in ethanol-exposed animals were detected in both liver and adipose for at least 12 h after death, and the values were substantially higher than the small amounts found in the liver and adipose of rats not exposed to ethanol. This indicates that FAEEs in liver and adipose are likely to be useful postmortem markers of ethanol intake before death.

Subjects and Methods

Twelve Sprague–Dawley rats weighing 250–300 g each were used. In one set of studies, the experimental group consisted of eight rats that received intraperitoneal injections of pure ethanol (2 g/kg) diluted with normal saline (1:1 by volume). The control group consisted of four rats that received intraperitoneal injections of an equivalent volume of normal saline. The rats were killed by CO₂ asphyxiation 2 h after injection. The bodies of both the experimental and control groups were stored at 4 °C for 12 h. Liver and adipose tissues were removed for analysis from each rat 30, 60, 180, 360, and 720 min after death. The samples were weighed (0.597 ± 0.024 g for liver; 0.275 ± 0.012 g for adipose) and homogenized in phosphate-buffered saline containing 1 mmol/L benzamidine and 20 mg/L phenylmethylsulfonyl fluoride (10 mL of solution per 1 g of sample) using a Fisher PowerGen 125 Homogenizer (Fisher Scientific) equipped with a 10 × 195 mm sawtooth generator. Homogenate (1 mL) was then extracted and analyzed for FAEE content.

In another set of experiments, four Sprague–Dawley rats weighing 250–300 g each were used. The experimental group consisted of two rats that received intraperitoneal injections of pure ethanol (2 g/kg) diluted with normal saline (1:1 by volume). The control group was two rats that received intraperitoneal injections of an equivalent volume of normal saline. The rats were killed by CO₂ asphyxiation 2 h after injection. The bodies of the animals in the experimental and control groups were stored at 4, 25, or 37 °C for up to 72 h. Liver samples were removed for analysis from each rat 48 and 72 h after death. Liver samples were weighed (0.678 ± 0.036 g) and homogenized in phosphate-buffered saline as described above. Homogenate (1 mL) was extracted and analyzed for FAEE content.

FAEE isolation and quantification

FAEEs were isolated and quantified as described by Bernhardt et al. (16) and Kaluzny et al. (17). A 50-μL (1 nmol) internal standard of ethyl heptadecanoate (E17:0) was added to each sample. The samples were extracted using acetone-hexane (2:8 by volume) and then dried under nitrogen to 300 μL. FAEEs were then isolated using solid-phase extraction with a Bond Elute-LRC aminopropyl column (17). FAEEs were quantified by gas chromatography–mass spectrometry (GC-MS) (18) using a Hewlett Packard 5890 Series II gas chromatograph coupled to a Hewlett Packard 5971 mass spectrometer equipped with a Supelcowax 10 capillary column. The oven temperature was maintained at 150 °C for 2 min, increased to 200 °C at 10 °C/min and held for 4 min, increased to 240 °C at 5 °C/min and held for 3 min, and then increased to 270 °C at 10 °C/min and held for 5 min. The injector and mass spectrometer were maintained at 260 and 280 °C, respectively. The carrier gas flow rate was maintained at a constant 0.8 mL/min throughout. Single-ion monitoring was performed, quantifying appropriate base ions for individual FAEE species [i.e., ions m/z 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 m/z 79 and 91 for ethyl arachidonate (E20:4), ethyl eicosapentaenoate (E20:5), and ethyl docosahexaenoate (E22:6)]. FAEEs were quantified by interpolation of the slope generated from individually prepared calibration curves, comparing areas of various concentrations of E16:0–E22:6 to fixed concentrations of the internal standard (E17:0). Mass relationships were obtained for each FAEE using its individual calibration curve. Total FAEE mass was determined by the addition of the masses of the individual FAEEs (E16:0–E22:6).

Results

The first analysis involved the quantification of FAEE concentrations per weight of liver or adipose in rats receiving ethanol or saline. The animals received ethanol as an intraperitoneal injection and were killed after 2 h. The bodies were stored at 4 °C for up to 12 h. In Fig. 1, the time after death (with death at time 0) is plotted vs FAEE concentration in nmol/g of liver tissue. The results demonstrate a
substantial difference in FAEE concentrations between the test (Ethanol) and control (Ethanol) groups, indicating that if the bodies are held at 4 °C, FAEE quantification should be effective for up to 12 h in identifying individuals who ingest ethanol within 2 h of death.

The individual species of FAEE in liver tissues, as a percentage of total FAEEs over the same time course, are shown in Fig. 2. The highest percentages were found for E16:0 and E18:0, which accounted for ~60% of the total FAEEs. There were five different species of FAEEs detected in the liver.

The total amount of FAEEs in the adipose tissues stored at 4 °C was ~10-fold higher (per gram of tissue) than was detected in the liver tissues (Fig. 3). This is most likely because FAEEs can be stored in adipose tissue because they have hydrophobicity similar to that of triglycerides. In addition, the FAEE concentrations in the adipose tissues of animals exposed to ethanol were substantially higher than the amounts found in animals not receiving ethanol. The controls had values that approximated 0 nmol/g of adipose tissue, and the animals treated with ethanol all had values on the order of 100 nmol/g adipose tissue or higher. There was a decline in the amount of FAEE per gram of tissue over the first 60 min after death, presumably because of the postmortem hydrolysis of the FAEEs. This decline in FAEE concentrations is not a confounding variable because the concentrations of FAEEs that remained were far greater than those found in animals not exposed to ethanol.

There were eight species of FAEEs identified in adipose tissue (Fig. 4). The minor FAEE species were most likely detectable in adipose because there was a much higher total FAEE concentration in adipose tissue than in liver (13, 19). The major FAEE species in adipose tissues were E18:1, E18:2, and E16:0, accounting for ~80% of the total FAEEs in the tissues. The other five FAEE species in adipose tissues were present at lower concentrations.

In separate experiments, we determined that at 48 and 72 h after death, the amounts of FAEEs per gram of liver tissue in treated animals stored at 4 °C were eight- and fivefold higher than in the control group, respectively, as...
had been found in the initial studies. However, we also found that there was no overlap at 48 h between the controls and those treated with ethanol when the rats were stored at 25 °C (mean ± SE, 161 ± 19 nmol/g with ethanol vs 11 ± 2 nmol/g with no ethanol; P = 0.008; n = 2 per group). Presumably as a result of postmortem synthesis of ethanol by gut bacteria, there was synthesis of FAEE in the bodies of rats that did not receive ethanol intraperitoneally. No statistical differences in FAEE concentrations were detected between test (intraperitoneal ethanol) and control (no ethanol) groups stored at 25 °C for 72 h, at 37 °C for 48 h, and at 37 °C for 72 h.

**Discussion**

This report demonstrates that FAEEs in the liver are readily detectable as markers of ethanol intake up to 48 h in bodies stored at 4 or 25 °C and that the FAEEs in adipose tissues can be used as markers of ethanol for at least 12 h after death in tissues stored at 4 °C. The results clearly differentiated animals exposed to ethanol as an intraperitoneal bolus 2 h before they were killed from those that were not. On the basis of these results, FAEEs in liver and adipose tissue show substantial promise as postmortem markers of premortem ethanol intake.

There is a need for better postmortem markers for ethanol intake. Currently, one of the major limitations is that the blood commonly used to measure blood ethanol is coagulated in subjects at autopsy. If peripheral blood is not available, heart blood may be obtained because it is less likely to be clotted, but the confounding variable of ethanol production by bacteria after death is introduced. Therefore, even if a sample is available for collection, the ethanol value may not reflect premortem ethanol intake. The confounding variable introduced by postmortem production of ethanol by bacteria necessitates additional testing for ethanol measurements in urine or vitreous humor. One of the major advantages for FAEEs in liver tissue as markers is their stability for 48 h after death when the body is stored at 4 or 25 °C. It is not yet known whether the particular FAEE species in liver or adipose tissues also provide diagnostically useful information. We previously demonstrated that E16:0 and E18:1 are the predominant FAEE species in the plasma, and the relative amounts of these two FAEE species are useful in separating chronic alcoholics from episodically excessive (binge) drinkers (19).

It is not clear why the FAEE species differ in liver and adipose tissues. It is presumed that the higher amount in adipose tissue reflects its ability to store large amounts of these hydrophobic molecules and that the liver is less equipped to collect large amounts of fat than is adipose tissue. We suspect that the half-lives for the FAEEs are higher in adipose tissue than in liver tissue, but this has not been studied for FAEEs in the liver. The half-life for FAEEs in adipose tissue is on the order of 16 h (13, 20). This would suggest that FAEEs in the adipose tissues, which we detected 2 h after ethanol intake was discontinued, would still be detectable if the ethanol intake was much longer than 2 h before death. Taken together, these results support the use of FAEEs in liver and in adipose tissue as postmortem markers of premortem ethanol intake.

**References**