Characterization of enzymes involved in formation of ethyl esters of long-chain fatty acids in humans

Margareta A. Diczfalusy,[1,*] Ingemar Björkhem,[*] Curt Einarsson,[†] Carl-Gustaf Hillebrant,[§] and Stefan E. H. Alexson[*]

Department of Medical Laboratory Sciences and Technology,* Division of Clinical Chemistry, Department of Internal Medicine,† Division of Gastroenterology and Hepatology, and Department of Cardiothoracic Surgery and Anesthesiology,§ Karolinska Institutet, Huddinge University Hospital, SE-141 86 Stockholm, Sweden

Abstract **Elevated fatty acid ethyl ester (FAEE) concentrations have been detected in postmortem organs from alcoholics and patients acutely intoxicated by alcohol, and FAEE have been implicated as mediators of ethanol-induced organ damage. The formation of FAEE is catalyzed by acyl-coenzyme A:ethanol *O*-acyltransferase (AEAT) and by FAEE synthase, which utilize acyl-CoA and free fatty acids, respectively, as substrates. Because little is known about the capacity of various human tissues to synthesize and hydrolyze FAEE, we investigated formation of FAEE by AEAT and FAEE synthase in tissue homogenates from human gastric ventricular and duodenal mucosa, pancreas, liver, heart, lung, and adipose tissue, gallbladder mucosa, and in serum. Liver, duodenal mucosa, and pancreas were found to have the highest capacities to synthesize FAEE, mainly due to AEAT. FAEE hydrolyzing activity was highest in liver and pancreas, but hardly detectable in adipose tissue or heart. Because fatty acids and alcohol are absorbed by the intestinal mucosa, intestine may be a major site of FAEE synthesis, and FAEE may be delivered via the circulation to other organs and taken up by lipoprotein receptor-mediated uptake. A very low rate of FAEE hydrolysis was detected in heart and adipose tissue, which probably accounts for the previously observed accumulation of FAEE in these organs.**—Diczfalusy, M. A., I. Björkhem, C. Einarsson, C-G. Hillebrant, and S. E. H. Alexson. **Characterization of enzymes involved in formation of ethyl esters of long-chain fatty acids in humans.** ***J. Lipid Res.*** **2001.** 42: **1025–1032.**

Supplementary key words alcohol metabolism • fatty acid ethyl esters • AEAT • FAEE synthase

Ingested alcohol is mainly metabolized in the liver by oxidation to acetaldehyde and acetate [for review, see ref. (1)]. Three enzymes are involved in the oxidation of ethanol to acetaldehyde: alcohol dehydrogenase, catalase, and cytochrome P-450 IIE1, the last two enzymes being of minor importance. Further oxidation to acetate is mediated by acetaldehyde dehydrogenase(s). Many of the pathological consequences associated with ethanol abuse have been attributed to high concentrations of acetaldehyde and acetate. However, experiments in male Wistar rats fed ethanol and treated with specific acetaldehyde dehydrogenase inhibitors, which led to sustained elevated levels of acetaldehyde, resulted in prevention of hepatic inflammation and necrosis (2), suggesting that acetaldehyde may not be involved in ethanol-induced organ injury. In addition, several organs with a limited capacity to oxidize ethanol such as heart, pancreas, and brain are also injured in alcoholics, suggesting that factors other than oxidation may be responsible for the ethanol-induced damages in these organs.

More recently, several studies have demonstrated that fatty acid ethyl esters (FAEE), nonoxidative ethanol metabolites, may be involved in organ injury. FAEE have been shown to uncouple oxidative phosphorylation in rabbit heart mitochondria (3), to inhibit protein synthesis and cell proliferation in human hepatoblastoma (HepG2) cells (4), and to increase fragility of pancreatic lysosomes (5). FAEE have been detected in several human tissues at autopsy after acute alcohol intoxication and in chronic alcoholics (6). After acute alcohol intoxication, the highest concentrations were found in adipose tissue, liver, pancreas, and heart, whereas the concentrations in chronic alcoholics were highest in adipose tissue and pancreas. FAEE have also been detected in blood after ingestion of moderate amounts of alcohol (7), and were found to persist in the blood at least 24 h after the ethanol intake, even though ethanol was undetectable at this time (8).

The origin of FAEE in the circulation is not clear. It has been proposed that they may be secreted into the blood after synthesis in liver and pancreas, with subsequent transport to adipose tissue where they may accumulate (6, 7). FAEE-synthesizing activity in leukocytes may also contribute to the occurrence of FAEE in the circulation (9). FAEE can be synthesized by esterification of free fatty acids to ethanol, a reaction catalyzed by FAEE synthases.

Abbreviations: AEAT, acyl-coenzyme A:ethanol *O*-acyltransferase; BNPP, bis-(4-nitrophenyl) phosphate; FAEE, fatty acid ethyl ester; HSA, human serum albumin; p-HMB, p-hydroxymercuri benzoic acid.

[1] To whom correspondence should be addressed
e-mail: margareta.diczfalusy@labtek.ki.se

LPL, an enzyme localized to the lumenal side of the capillary endothelium, has been purified from post-heparin plasma and shown to possess FAEE synthase activity (10). Subsequent studies showed that perfusion of isolated rat heart with chylomicrons and ethanol resulted in the formation of FAEE, suggesting that LPL may contribute to formation of circulating FAEE (11). Thus, several blood components, including the blood vessels, may contribute to synthesize FAEE in subjects drinking alcohol.

A number of intracellular proteins have been isolated from different sources and shown to catalyze esterification of fatty acids to ethanol (12–14). By aminoterminal-sequence analysis, it was shown that two of these purified FAEE synthases are apparently identical to liver microsomal carboxylesterase ES-10, the predominant carboxylesterase in rat liver (15, 16). Other enzymes such as pancreatic cholesterol ester synthase (17) and pancreatic carboxylester synthase (18) also have been shown to possess some FAEE synthesizing activity.

FAEE also can be synthesized by transesterification of ethanol and fatty acyl-CoA, a reaction catalyzed by acyl-coenzyme A:ethanol *O*-acyltransferase (AEAT). Less is known about AEAT activity, and only few reports on this enzyme(s) have appeared (19–21). Recently, we characterized AEAT activity in isolated rat liver microsomes (22). In this study, it was shown that microsomal carboxylesterase ES-4 may be important for hydrolysis of FAEE, whereas carboxylesterase ES-10 has much lower FAEE-hydrolyzing activity. In addition, carboxylesterase ES-4 also hydrolyzes acyl-CoAs, and ES-4 was found to compete with AEAT for this substrate. Therefore, accurate assay of AEAT activity requires inhibition of carboxylesterase ES-4 activity in rat liver. In vitro activity measurements on isolated rat liver microsomes suggest that AEAT activity may be considerably more important than FAEE synthase activity for the formation of FAEE. However, there is only limited information available on FAEE synthase and AEAT activities in human tissues. The present study was designed to establish whether human organs are able to synthesize and hydrolyze FAEE, and to define the enzymes involved in these processes. We first optimized reaction conditions for measurement of AEAT, FAEE synthase, and FAEE hydrolase activities in isolated human liver microsomes. These conditions were then used to study the capacity of human tissues to synthesize and degrade FAEE.

MATERIALS AND METHODS

Materials

[1-^{14}C]palmitoyl-CoA (47.8 mCi/mmol), purchased from Du Pont NEN (Sollentuna, Sweden), was diluted with unlabeled palmitoyl-CoA (Sigma, St. Louis, MO) to a final concentration of 2.5 mM and stored at −20°C. [1-^{14}C]palmitic acid (54 mCi/mmol) was from Du Pont NEN. Bis-(4-nitrophenyl) phosphate (BNPP), p-hydroxymercuri benzoic acid (p-HMB), p-nitrophenyl acetate, and human serum albumin (HSA; fraction V, essentially fatty acid free) were from Sigma. Palmitic acid was from Fluka Chemie AG (Buchs, Switzerland). DMSO and silica gel 60 plates for TLC were obtained from Merck (Darmstadt, Germany).

Animals

Sprague-Dawley rats weighing about 200 g were obtained from B&K Universal AB (Sollentuna, Sweden). The rats were euthanized with CO_2 and blood was collected by heart puncture. Serum was prepared by centrifugation of blood samples at 1,200 *g* for 15 min. All animals were maintained at the Animal Care Department at Huddinge University Hospital. All animal experiments were approved by the animal ethics committee.

Patients and tissue sampling

Tissues were obtained from 25 patients undergoing elective surgery. Samples of normal stomach and small intestine were obtained from patients undergoing pancreaticoduodenectomy by Whipple resection because of carcinoma of the pancreatic head or carcinoma of the bile duct. Preoperative liver biopsies were obtained from patients undergoing cholecystectomy and from patients undergoing Whipple resection. Gallbladder samples were obtained from patients operated for uncomplicated gallstone disease. Normal tissue of the pancreatic head and biopsies from adipose tissue were obtained from patients undergoing Whipple resection due to cholangiocarcinoma of the bile duct or carcinoma of the pancreatic head. Normal lung tissue was from patients operated for lung cancer. Heart muscle tissue was obtained from the auricle of the right atrium of patients undergoing coronary by-pass operation. From some of the patients, up to four different tissues were obtained. The tissues were immediately frozen and stored in liquid nitrogen until analysis.

Informed consent was obtained from each patient before the operation, and the ethical aspects of the study were approved by the Ethical Committee at Karolinska Institutet, Stockholm.

Human blood samples were obtained from four healthy subjects. Serum was prepared by centrifugation of blood samples at 1,200 *g* for 15 min.

Preparation of tissue homogenates and isolation of liver microsomes

On the day of preparation, tissues were thawed, minced, and homogenized with a loose-fitting pestle or minced by Polytron in nine volumes of ice-cold 50 mM potassium phosphate buffer containing 0.5 mM EDTA (pH 7.4). The homogenates were filtered through a double layer of gauze and stored at −20°C in aliquots.

Liver microsomes were prepared as described earlier (23). The resulting microsomal pellet was suspended in 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.4), and stored at −20°C in aliquots. Frozen microsomes were used within 2 months. Protein concentration was determined by the method of Bradford, using bovine serum albumin as standard (24).

Western blot analysis

Western blot analysis was performed using a polyclonal antibody raised against purified rat liver microsomal acyl-CoA hydrolase, corresponding to carboxylesterase ES-4 as described earlier (25). Antibodies specific to carboxylesterase ES-4 and ES-10 were raised in rabbits by immunization of peptides that were produced based on the deduced amino acid sequences of the rat enzymes. These antibodies have been described in more detail previously (22).

AEAT assay

AEAT activity was measured as described earlier (22). Briefly, the final assay conditions were as follows: 100 μM [1-^{14}C]palmitoyl-CoA, 0.86 M ethanol, 2 mg/ml HSA, and enzyme (5–10 μg of microsomal protein or 10–72 μg of tissue homogenate protein) in 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.4). The total incubation volume was 0.25 ml. The substrates (ethanol and [1-^{14}C]palmitoyl-CoA) were added after

preincubation for 20 min at 37°C. The reaction was stopped after 15 min by addition of 1.6 ml ice-cold heptane. Ethyl palmitate was extracted with heptane according to Polokoff and Bell (20), with minor modifications (22). Blank incubations, containing all components except for enzyme, were run in parallel. The radioactive product was quantitated in 0.8 ml of the heptane phase by liquid scintillation counting. The AEAT activity, corrected for the blanks, was expressed as nanomoles of [^{14}C]ethyl palmitate formed per milligram of microsomal protein per minute, or per minute and gram of tissue when measured in homogenates.

FAEE synthase assay

FAEE synthase activity was determined by quantitation of labeled ethyl palmitate formed from [1-^{14}C]palmitic acid and ethanol. About 50 μg of microsomal protein or tissue homogenate protein in 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.4) was preincubated for 15 min at 37°C, and the reaction was started by addition of 400 μM [1-^{14}C]palmitic acid and 0.86 M ethanol. The total incubation volume was 0.25 ml. The reaction was stopped after 20 min by addition of 1.6 ml of ice-cold heptane. Ethyl palmitate was extracted and quantitated as described above for AEAT determination.

Hydrolysis of [^{14}C]ethyl palmitate

[^{14}C]ethyl palmitate was synthesized from [1-^{14}C]palmitic acid and ethanol as described previously (22). About 2 μg of microsomal protein or 20–60 μg of tissue homogenate protein was preincubated in 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 2 mg/ml HSA (in a total volume of 0.25 ml) for 20 min at 37°C. The reaction was started by addition of 175 μM [^{14}C]ethyl palmitate (dissolved in ethanol) and the samples were incubated for about 15 min. The incubation was stopped by the addition of 2.5 ml chloroform–methanol (2:1, v/v) and 0.25 ml of 0.1 M H_2SO_4 in 0.9% NaCl. The chloroform phase was evaporated under a stream of nitrogen, and radioactive lipids were dissolved in a small volume of chloroform–methanol (2:1, v/v) and chromatographed on Silica Gel 60 plates developed in hexane–diethyl ether–glacial acetic acid (80:20:1, v/v/v). The TLC plates were scanned for radioactivity, and the spots corresponding to [^{14}C]ethyl palmitate and [^{14}C]palmitic acid were scraped off and counted in a liquid scintillation counter.

Effects of amino acid residue-modifying agents

Human liver microsomes were preincubated for 20 min with BNPP or p-HMB as indicated, and subsequently analyzed for FAEE hydrolase activity. p-HMB was dissolved in DMSO. In control incubations, microsomes were incubated with DMSO only.

Carboxylesterase activity

Carboxylesterase activity was measured with 1 mM p-nitrophenyl acetate as substrate in phosphate-buffered saline by a procedure described previously (26). Liberation of p-nitrophenol was

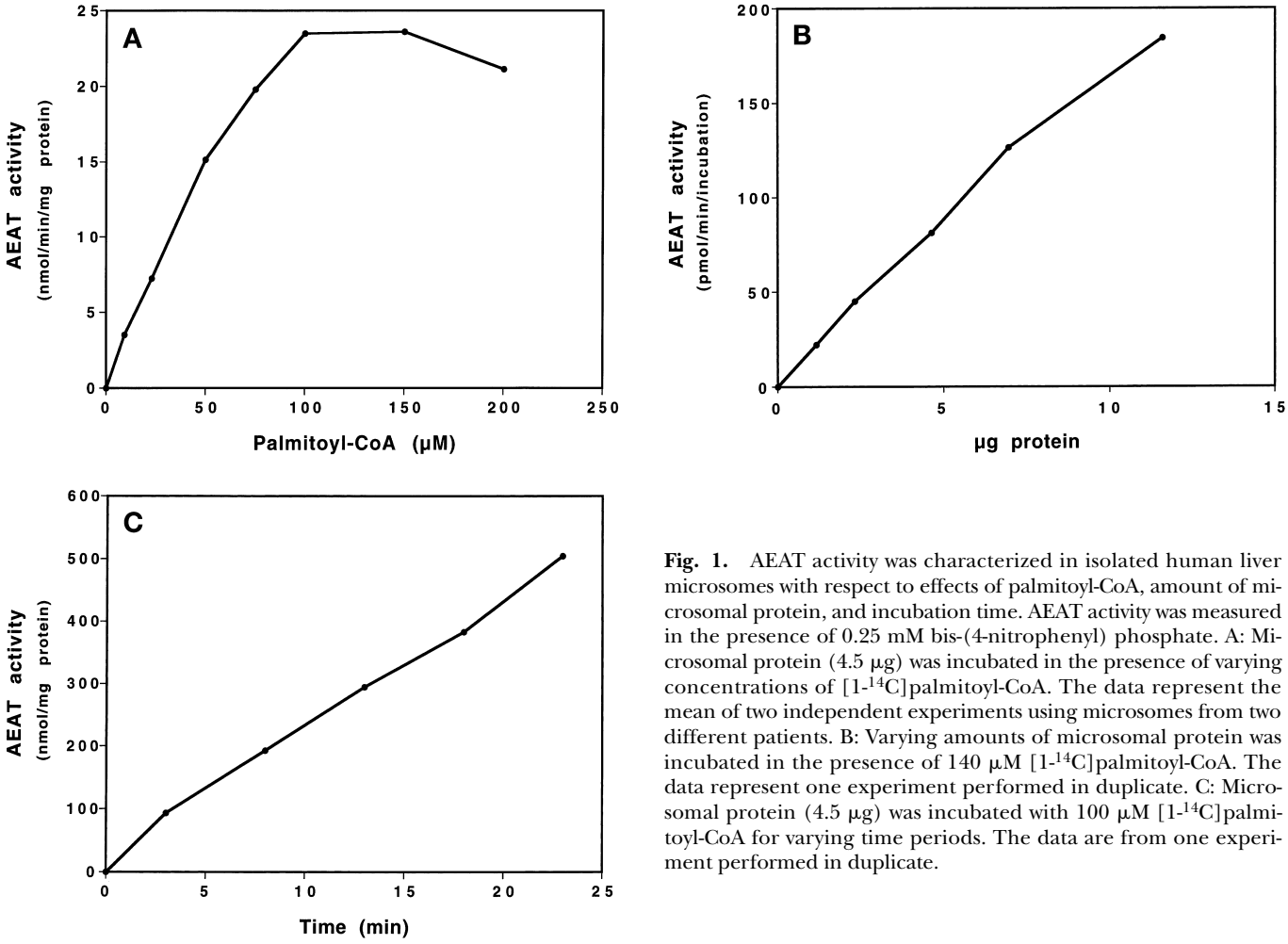

Fig. 1. AEAT activity was characterized in isolated human liver microsomes with respect to effects of palmitoyl-CoA, amount of microsomal protein, and incubation time. AEAT activity was measured in the presence of 0.25 mM bis-(4-nitrophenyl) phosphate. A: Microsomal protein (4.5 μg) was incubated in the presence of varying concentrations of [1-^{14}C]palmitoyl-CoA. The data represent the mean of two independent experiments using microsomes from two different patients. B: Varying amounts of microsomal protein was incubated in the presence of 140 μM [1-^{14}C]palmitoyl-CoA. The data represent one experiment performed in duplicate. C: Microsomal protein (4.5 μg) was incubated with 100 μM [1-^{14}C]palmitoyl-CoA for varying time periods. The data are from one experiment performed in duplicate.

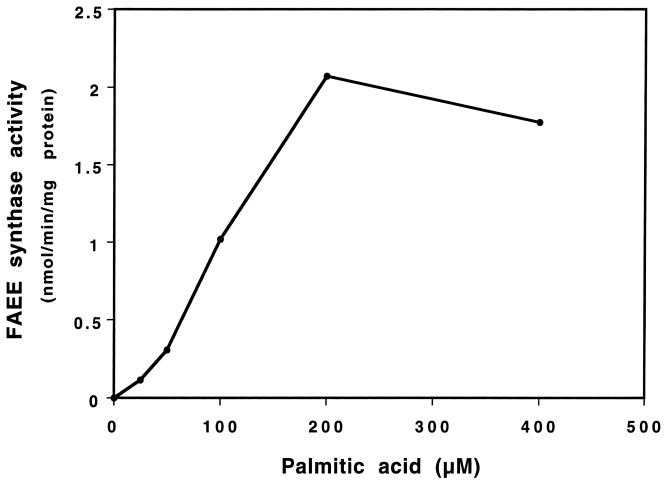

Fig. 2. Synthesis of FAEE by FAEE synthase. Microsomal protein (40 μg) was incubated in the presence of varying concentrations of [1-^{14}C]palmitic acid as described in Materials and Methods. The data are from one patient. Each measurement was performed in duplicate.

monitored spectrophotometrically at 420 nm, and the activity was calculated from an $E_{420} = 3.06\ mM^{-1} \cdot cm^{-1}$.

RESULTS

Characterization of FAEE-synthesizing activities in human liver microsomes

FAEE synthesis. AEAT activity was measured as the formation of ethyl palmitate from radiolabeled palmitoyl-CoA and unlabeled ethanol. First, reaction parameters such as substrate concentration (**Fig. 1A**), amount of added microsomal protein (Fig. 1B), and incubation time (Fig. 1C) were optimized. The final assay conditions were found to be similar to those used for measurement of AEAT activity in rat liver microsomes [see Methods in ref. (22) for details). The AEAT activity measured in human liver microsomes was found to be considerably higher than in rat liver microsomes. The specific activity of AEAT was 17 ± 2 nmol/min/mg protein (mean ± SD, n = 7), which is about 10-fold higher than the specific activity in rat liver microsomes.

The formation of FAEE, catalyzed by FAEE synthase, was measured by incubation of microsomes with [1-^{14}C]palmitic acid and ethanol. The synthesis of FAEE was maximal at 200–400 μM palmitic acid (**Fig. 2**), and 400 μM concentration was chosen for FAEE synthase measurements, which is similar to the concentration used in some previous studies (6, 12, 18). The reaction was linear with microsomal protein up to 90 μg/incubation, the highest protein amount tested (data not shown). The specific activity of FAEE synthase was found to be 2.8 ± 1.6 nmol/min/mg protein (mean ± SD, n = 4), thus being much lower than AEAT activity.

Characterization of FAEE-hydrolyzing activities in human liver microsomes

Turnover of FAEE formed in vivo will also depend on hydrolysis to yield free fatty acids and ethanol. Therefore, we also characterized FAEE-hydrolyzing activity in human liver microsomes. The specific activity was found to be 40 ± 2 nmol/min/mg protein (mean ± SD, n = 7), which is about half of the activity obtained with rat liver microsomes (22). We further tested the effects of BNPP and p-HMB on ethyl palmitate hydrolase activity. BNPP was found to completely inhibit the activity at 250 μM; however, p-HMB (tested at 40 μM) did not affect the hydrolysis of ethyl palmitate. This lack of effect of p-HMB is in contrast to the effect of this inhibitor on ethyl palmitate hydrolase activity in rat liver microsomes, where p-HMB was found to be a strong inhibitor. In a previous study, we identified carboxylesterase ES-4 as the enzyme responsible for most of the ethyl palmitate hydrolyzing activity in rat liver microsomes, and this activity was strongly inhibited by p-HMB (22). The lack of effect by p-HMB on FAEE hydrolysis in human liver microsomes suggests that ES-4 may not be expressed in human liver. Therefore, we ran Western blot analysis on human liver microsomes using specific peptide antibodies to rat liver carboxylesterase ES-4. As expected, no immunoreactive protein was detected, whereas peptide antibodies to rat liver carboxylesterase ES-10 (another member of the same gene family) readily detected a immunoreactive protein of the correct mass (**Fig. 3**). Thus, the enzymes responsible for hydrolysis of ethyl palmitate are apparently different in rat and human liver microsomes.

Synthesis of FAEE in human tissue homogenates

The results obtained with isolated human liver microsomes above suggest that FAEE can be synthesized in

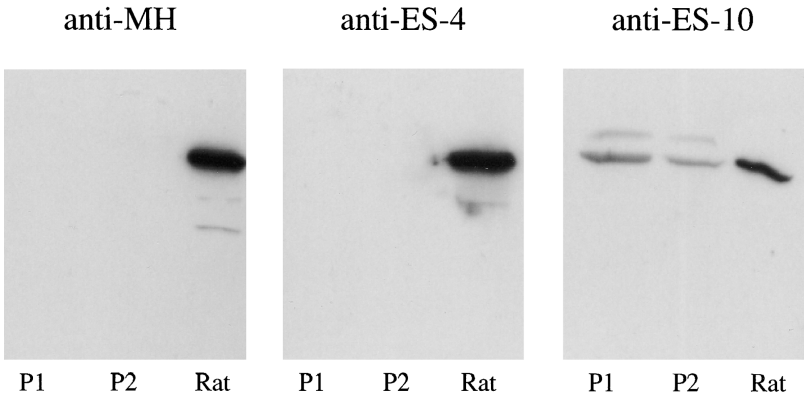

Fig. 3. Western blot analysis. Liver microsomes were isolated from two patients (P1 and P2) and from rat (Rat), and analyzed by Western blotting. Carboxylesterases were detected using a polyclonal antibody raised against purified microsomal rat liver carboxylesterase ES-4 (anti-MH) and peptide antibodies raised against specific epitopes of carboxylesterases ES-4 and ES-10.

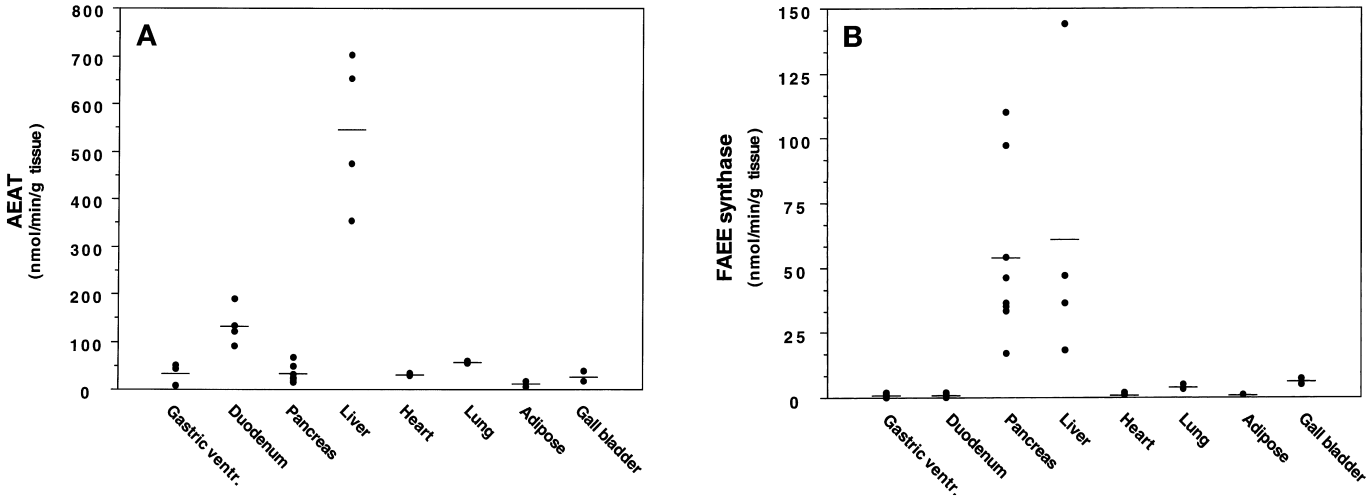

Fig. 4. AEAT and FAEE synthase activities in homogenates of various human tissues. A: AEAT activity. B: FAEE synthase activity. Number of samples used for determination of AEAT and FAEE synthase activities: gastric ventricular mucosa (Gastric ventr.), n = 3; duodenal mucosa (Duodenum), n = 4; pancreas, n = 8; liver, n = 4; heart, n = 2; lung, n = 2; adipose, n = 2; gallbladder, n = 2. The activity of each sample, measured in duplicate, is shown with the horizontal bars indicating the mean activity.

human liver and that AEAT may be quantitatively more important than FAEE synthase for synthesis of FAEE in liver. Synthesized FAEE may undergo hydrolysis, which could potentially be of importance in preventing the accumulation of these esters in some organs. We therefore investigated the capacity of human tissue homogenates to synthesize FAEE by AEAT and FAEE synthase, and their ability to eliminate formed esters by hydrolysis. The enzyme activities were assayed in all tissues obtained.

AEAT activity was measured in tissue homogenates using the standard conditions. As seen in **Fig. 4A**, the highest AEAT activity was found in liver homogenates (mean value 545 nmol/min/g tissue, n = 4). High AEAT activity was found also in homogenates of duodenal mucosa (mean value 132 nmol/min/g tissue, n = 4), whereas the AEAT activity measured in homogenates of other tissues was considerably lower than in liver and intestine.

We also measured FAEE synthase activity in tissue homogenates from human organs. FAEE synthase activity was considerably lower than AEAT activity in all tissue homogenates, except for pancreas. Liver contained the highest FAEE synthase activity (about 60 nmol/min/g tissue, Fig. 4B), which was almost one order of magnitude lower than the AEAT activity. In pancreas, the FAEE synthase and AEAT activities were similar (54 and 32 nmol/min/g tissue, respectively). The FAEE synthase activities were 26- and 7-fold higher in liver and pancreas, respectively, compared to previously reported activities (6). These differences are most likely explained by slightly different incubation conditions and different tissue sampling; tissues were obtained by biopsy in the present study, and at au-

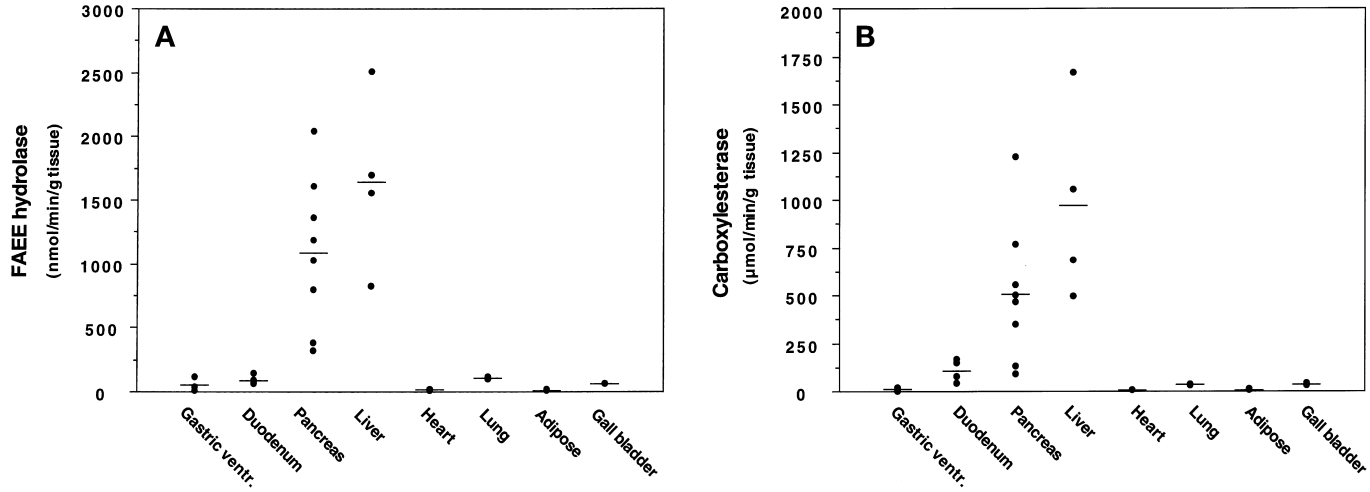

Fig 5. FAEE hydrolase and carboxylesterase activities in homogenates of various human tissues. A: FAEE hydrolase activity. B: Carboxylesterase activity. Same samples as indicated in Fig. 4 were used for measurement of FAEE hydrolase and carboxylesterase activities. The activity of each sample, measured in duplicate, is shown with the horizontal bars indicating the mean activity.

topsy in the previous study. FAEE synthase activity in other tissues was several-fold lower than the AEAT activity, and the most striking difference was found in duodenal mucosa where AEAT activity was more than 100 times higher than FAEE synthase activity.

FAEE hydrolysis and carboxylesterase activity

The hydrolysis of FAEE in tissue homogenates was measured using the same conditions as for liver microsomes. The highest hydrolytic activity was found in liver and pancreas (about 1,600 and 1,100 nmol/min/g tissue, respectively, **Fig. 5A**). The FAEE hydrolyzing activity was much lower in gastric ventricle, duodenal and gallbladder mucosa, and lung than in liver and pancreas (50–100 nmol/min/g tissue). Very low activity was found in heart and adipose tissue (10–15 nmol/min/g tissue).

FAEE synthase has previously been identified as carboxylesterase ES-10 in human and animal tissues by other groups (13, 14). We therefore measured carboxylesterase activity in the various tissue homogenates. Highest carboxylesterase activities were found in liver and pancreas and, to a lesser extent, in duodenum (Fig. 5B). The other organs contained low carboxylesterase activities. As liver and pancreas also contained high FAEE synthase activity, it is possible that the two activities are due to the same protein, that is, carboxylesterase ES-10.

Synthesis and hydrolysis of FAEE in human and rat serum

As discussed above, FAEE can be detected in human blood soon after ethanol ingestion (7). These esters may be formed in the intestine and/or liver, and subsequently secreted into the blood. An alternative possibility is that these esters may be formed in the circulation. We therefore investigated the capacity of serum to synthesize and hydrolyze FAEE. As shown in **Table 1**, human and rat serum contained barely detectable FAEE-synthesizing activities, and FAEE hydrolase activity was very low in human serum. However, ethyl palmitate-hydrolyzing activity was very high in rat serum, in accordance to the previously observed short half-life of FAEE in rat serum (27, 28).

DISCUSSION

The aim of the present study was to measure the capacities of human organs to synthesize and hydrolyze FAEE. In the measurements of enzymatic activities in the human tissue homogenates, we used unphysiological saturating concentrations of the substrates, being about one order of magnitude higher than in vivo concentrations. Still, this approach may be justified from our findings that the K_m values of AEAT, FAEE synthase, and FAEE hydrolase activities were similar, thereby allowing a comparison of the activities ("capacities") at near-saturating substrate concentrations. In the present study, all enzyme activities were measured in tissue homogenates where cell architecture is disrupted. However, because most of the AEAT, FAEE synthase, and FAEE hydrolase activities are found in microsomes (at the lumenal side) upon subcellular fractionation, it is relevant to compare these activities in relation to turnover of FAEE.

Comparison of the activities of FAEE-synthesizing enzymes show that AEAT activity was several-fold higher than FAEE synthase activity in all tissues tested, except for pancreas where the two activities were comparable. Because fatty acids are rapidly esterified to CoA when entering cells, the high in vitro capacity of AEAT suggests that it may be of major importance in the formation of FAEE. Interestingly, duodenal mucosa was found to contain rather high AEAT activity (and very low FAEE synthase activity), being about one-fourth of the activity in liver. This is in accordance with a previous finding showing that isolated rat intestinal microsomes contain high AEAT activity (20). Although the total mass of the intestinal mucosa in humans is difficult to estimate, it is reasonable to assume that the intestinal AEAT activity contributes significantly to total FAEE synthesis. Fatty acids released from ingested fat in the intestinal lumen are taken up in the intestinal mucosal cells where they are activated to the corresponding CoA esters prior to esterification into triacylglycerols, phospholipids, and cholesteryl esters. Because ingested alcohol is also taken up by the intestinal mucosa, it is likely that a significant amount of alcohol is esterfied to fatty acids in the mucosal cells. FAEE are very hydrophobic and may therefore dissolve in the triacylglycerol synthesized in the mucosal cells and subsequently be co-transported in chylomicrons to peripheral tissues. Elevated levels of FAEE can be detected in serum soon after alcohol intake, and levels remain elevated for about 24 h after the intake (8), being in accordance with the present finding of very low ethyl ester-hydrolyzing capacity of human serum. Previous analysis of serum samples from human subjects with elevated levels of FAEE showed that about 30% of the FAEE were associated with lipoproteins and that the remaining FAEE were found in the serum fraction containing albumin (7). Fatty acid esters are prone to constant hydrolysis and re-esterification during transport across membranes. It is therefore possible that LPL plays a significant role in the synthesis of FAEE during hydrolysis of triacylglycerols in the presence of circulating ethanol (11), and thereby contributes to the serum fraction of FAEE. The remaining FAEE that are associated with the lipoprotein fraction may originate from the intestine, and may be taken up by the LDL and VLDL receptors. Uptake of FAEE in various tissues is thus likely to vary extensively, due to extent of lipoprotein degradation by lipases, receptor density, and so forth.

Table 1. AEAT, FAEE synthase, and FAEE hydrolase activities in human and rat serum

Activity	Human Serum	Rat Serum
AEAT	0.2 ± 0.21	ND
FAEE synthase	ND	2.6 ± 1.6
FAEE hydrolase	0.34 ± 0.29	195 ± 31

Activities are expressed as nmol/min/ml. Values given as means ± SD, n = 4. Each sample was determined in duplicate. AEAT, acylcoenzyme A:ethanol *O*-acyltransferase; FAEE, fatty acid ethyl ester; ND, not detectable.

Accumulation of FAEE in various organs depends on uptake, synthesis, and hydrolysis of FAEE. Our results show that liver contains the highest FAEE-synthesizing activity among the tissues tested. The high synthesizing capacity in liver homogenates is mainly due to AEAT activity, which is many-fold higher than FAEE synthase activity. The liver (together with pancreas) also contains the highest FAEE-hydrolyzing activity, suggesting that FAEE formed in the liver may have a rapid turnover. This is supported by the relatively low degree of accumulation of FAEE in liver during alcohol intake and in alcoholics (6). The FAEE-synthesizing activities in the other tissues investigated (adipose tissue, gallbladder, lung, and heart) were all rather low, suggesting that intraorgan synthesis is unlikely to be of critical importance. FAEE appear to accumulate to a much higher degree in adipose tissue than in any other organ investigated so far. The explanation is probably the low turnover of fatty acids and a very low FAEE hydrolyzing activity in this tissue.

A possible involvement of FAEE in ethanol-dependent organ injury is still unclear. Tissues reported to be prone to organ injury by ethanol intake include heart, which we found to contain very low FAEE-synthesizing and -hydrolyzing activities, and pancreas, which contains high FAEE-synthesizing and -hydrolyzing activities. A rapid turnover of FAEE in pancreas is consistent also with a previous demonstration that FAEE concentrations were about 5-fold higher in pancreas in acutely ethanol-intoxicated subjects compared with alcoholics (6). It is possible that toxic effects caused by FAEE may remain after normalization of FAEE levels in pancreas, and can be explained by an increased lysosomal fragility (5), with subsequent release of lipases and proteases that deteriorate subcellular structures.

In the present study, we found that AEAT activity is much higher than FAEE synthase activity in most tissues investigated. The identity of the AEAT enzyme is still unknown. AEAT activity is not due to ACAT because we have observed that FAEE formation is unaffected in incubations when ACAT activity is inhibited (data not shown). Other enzymes utilizing acyl-CoAs for transesterifications are enzymes involved in triacylglycerol synthesis. Recently, diacylglycerol acyltransferase (DGAT) was cloned and expressed as a recombinant protein in insect cells (29). However, characterization of the recombinant protein showed that DGAT does not transesterify fatty acyl-CoA to ethanol. It therefore appears that the identification of AEAT has to await the purification of the responsible enzyme and the further characterization of the protein.

We are grateful to Lisbet Benthin and Dr. Johan Permert for help with collecting human tissues. This study was supported by the Swedish Natural Science Research Council, the Swedish Research Council for Engineering Sciences, the Swedish Medical Research Council, Svenska Sällskapet för Medicinsk Forskning, Åke Wibergs Stiftelse, and Magn. Bergvalls Stiftelse.

Manuscript received 6 October 2000 and in revised form 13 February 2001.

REFERENCES

1. Lieber, C. S. 1985. Alcohol and the liver: metabolism of ethanol, metabolic effects and pathogenesis of injury. *Acta Med. Scand. Suppl.* **703:** 11–55.
2. Lindros, K. O., K. Jokelainen, and A. A. Nanji. 1999. Acetaldehyde prevents nuclear factor-kappa B activation and hepatic inflammation in ethanol-fed rats. *Lab. Invest.* **79:** 799–806.
3. Lange, L. G., and B. Sobel. 1983. Mitochondrial dysfunction induced by fatty acid ethyl esters, myocardial metabolites of ethanol. *J. Clin. Invest.* **72:** 724–731.
4. Szczepiorkowski, Z. M., G. R. Dickersin, and M. Laposata. 1995. Fatty acid ethyl esters decrease human hepatoblastoma cell proliferation and protein synthesis. *Gastroenterology.* **108:** 515–522.
5. Haber, P. S., J. S. Wilson, M. V. Apte, and R. C. Pirola. 1993. Fatty acid ethyl esters increase rat pancreatic lysosomal fragility. *J. Lab. Clin. Med.* **121:** 759–764.
6. Laposata, E. A., and L. G. Lange. 1986. Presence of nonoxidative ethanol metabolism in human organs commonly damaged by ethanol abuse. *Science.* **231:** 497–499.
7. Doyle, K. M., D. A. Bird, S. Al-Salihi, J. Hallaq, J. E. Cluette-Brown, K. A. Goss, and M. Laposata. 1994. Fatty acid ethyl esters are present in human serum after ethanol ingestion. *J. Lipid Res.* **35:** 428–437.
8. Doyle, K. M., J. E. Cluette-Brown, D. M. Dube, T. G. Bernhard, C. R. Morse, and M. Laposata. 1996. Fatty acid ethyl esters in the blood as markers for ethanol intake. *J. Am. Med. Assoc.* **276:** 1152–1156.
9. Wright, M., K. J. Bieser, P. M. Kinnunen, and L. G. Lange. 1987. Nonoxidative ethanol metabolism in human leukocytes: detection of fatty acid ethyl ester synthase activity. *Biochem. Biophys. Res. Commun.* **142:** 979–985.
10. Tsujita, T., and H. Okuda. 1994. Fatty acid ethyl ester-synthesizing activity of lipoprotein lipase from rat postheparin plasma. *J. Biol. Chem.* **269:** 5884–5889.
11. Chang, W., C. Waltenbaugh, and J. Borensztajn. 1997. Fatty acid ethyl ester synthesis by isolated perfused rat heart. *Metabolism.* **46:** 926–929.
12. Mogelson, S., and L. G. Lange. 1984. Nonoxidative ethanol metabolism in rabbit myocardium: purification to homogeneity of fatty acid ethyl ester synthase. *Biochemistry.* **23:** 4075–4081.
13. Tsujita, T., and H. Okuda. 1992. Fatty acid ethyl ester synthase in rat adipose tissue and its relationship to carboxylesterase. *J. Biol. Chem.* **267:** 23489–23494.
14. Bora, P. S., D. G. Guruge, D. D. Miller, B. R. Chaitman, and M. S. Ruyle. 1996. Purification and characterization of human heart fatty acid ethyl ester synthase/carboxylesterase. *J. Mol. Cell Cardiol.* **28:** 2027–2032.
15. Robbi, M., and H. Beaufay. 1983. Purification and characterization of various esterases from rat liver. *Eur. J. Biochem.* **137:** 293–301.
16. Mentlein, R., A. Ronai, M. Robbi, E. Heymann, and O. von Deimling. 1987. Genetic identification of rat liver carboxylesterases isolated in different laboratories. *Biochim. Biophys. Acta.* **913:** 27–38.
17. Riley, D. J. S., E. M. Kyger, C. A. Spilburg, and L. G. Lange. 1990. Pancreatic cholesterol esterases. 2. Purification and characterization of human pancreatic fatty acid ethyl ester synthase. *Biochemistry.* **29:** 3848–3852.
18. Tsujita, T., and H. Okuda. 1994. The synthesis of fatty acid ethyl ester by carboxylester lipase. *Eur. J. Biochem.* **224:** 57–62.
19. Grigor, M. R., and I. C. Bell, Jr. 1973. Synthesis of fatty acid esters of short-chain alcohols by an acyltransferase in rat liver microsomes. *Biochim. Biophys. Acta.* **306:** 26–30.
20. Polokoff, M. A., and R. M. Bell. 1978. Limited palmitoyl-CoA penetration into microsomal vesicles as evidenced by a high latent ethanol acyltransferase activity. *J. Biol. Chem.* **253:** 7173–7178.
21. Treloar, T., L. J. Madden, J. S. Winter, J. L. Smith, and J. de Jersey. 1996. Fatty ester ethyl ester synthesis by human liver microsomes. *Biochim. Biophys. Acta.* **1299:** 160–166.
22. Diczfalusy, M. A., I. Björkhem, C. Einarsson, and S. E. H. Alexson. 1999. Formation of fatty acid ethyl esters in rat liver microsomes. Evidence for a key role of acyl-CoA:ethanol *O*-acyltransferase. *Eur. J. Biochem.* **259:** 404–411.
23. Einarsson, K., L. Benthin, S. Ewerth, G. Hellers, D. Ståhlberg, and B. Angelin. 1989. Studies on acyl-coenzyme A:cholesterol acyltransferase activity in human liver microsomes. *J. Lipid Res.* **30:** 739–746.

24. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein/dye binding. *Anal. Biochem.* **72:** 248–254.
25. Alexson, S. E. H., R. Mentlein, C. Wernstedt, and U. Hellman. 1993. Isolation and characterization of microsomal acyl-CoA thioesterase: a member of the rat liver microsomal carboxylesterase multi-gene family. *Eur. J. Biochem.* **214:** 719–727.
26. Beaufay, H. A., A. Amar-Costesec, H. Feytmans, D. Thines-Sempoux, M. Wibo, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. *J. Cell Biol.* **61:** 188–200.
27. Hungund, B. L., Z. Zheng, and A. I. Barkai. 1995. Turnover of ethyl-linoleate in rat plasma and its distribution in various organs. *Alcohol. Clin. Exp. Res.* **19:** 374–377.
28. Saghir, M., J. Werner, and M. Laposata. 1997. Rapid in vivo hydrolysis of fatty acid ethyl esters, toxic nonoxidative ethanol metabolites. *Am. J. Physiol.* **273:** G184–G190.
29. Cases, S., S. J. Smith, Y-W. Zheng, H. M. Myers, S. R. Lear, E. Sande, S. Novak, C. Collins, C. B. Welch, A. J. Lusis, and S. K. Erickson. 1998. Identification of a gene encoding an acyl CoA: diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. *Proc. Natl. Acad. Sci. USA.* **95:** 13018–13023.