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Rapid Fatty Acid Ethyl Ester Synthesis by Porcine Myocardium Upon Ethanol Infusion into the Left Anterior Descending Coronary Artery

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Fatty acid ethyl esters (FAEEs), nonoxidative metabolites of ethanol, have been implicated in ethanol-induced heart injury. To assess the in vivo production of FAEEs by myocardial tissue, we used a modified ethanol ablation procedure in pigs. A controlled 60-minute ethanol infusion was administered into the distal left anterior descending coronary artery in seven swine; serial blood sampling of the coronary sinus and peripheral vein before, during, and after infusion allowed measurement of FAEE production and ethanol levels in the coronary sinus and the peripheral circulation. In a single animal, FAEEs were also quantified from nine different sites within the myocardium. FAEEs were produced by the heart within 5 minutes of exposure to ethanol, with very high concentrations of FAEEs detected in coronary sinus blood. Significant variability in amounts of FAEEs was detected in different regions of the heart tissue. A strong correlation was found between coronary sinus FAEEs and ethanol concentration (r = 0.9241, P < 0.00001). FAEE production by the heart after delivery of ethanol into the left anterior descending coronary artery was rapid, reaching levels in the coronary sinus blood 4 to 10 times greater than that found in peripheral blood after ethanol intake. These data demonstrate that FAEEs may be mediators of ethanol-induced cardiotoxicity. (Am J Pathol 2006, 168:1435–1442; DOI: 10.2353/ajpath.2006.050537)

Ethanol has been long known to be a potential cause of cardiac disease and dysfunction.1,2 One outcome of chronic ethanol abuse is the development of alcoholic cardiomyopathy.3,4 Alcoholic cardiomyopathy is a condition that is marked by cardiac hypertrophy, ventricular dilation, fibrosis, and poor cardiac function.5,6 Acute intoxication with ethanol can also produce the “holiday heart syndrome,” aptly named given its increased occurrence in binge drinkers on Mondays and on or after holidays. It is characterized by supraventricular arrhythmias without evident heart disease.7,8 In contrast to all these negative effects of ethanol, in recent years directed infusion has been used for catheter-based treatment of hypertrophic obstructive cardiomyopathy2 as well as for the treatment of ventricular arrhythmia.10

The mechanism by which the controlled myocardial necrosis is induced by ethanol in alcohol septal ablation has not yet been elucidated. Ethanol itself is not considered to play a prominent role as a mediator of cell injury in the heart; or the other organs damaged by ethanol abuse. Attention has instead been directed to metabolites of ethanol as mediators of toxic effects.11–13

The oxidation of ethanol produces acetaldehyde through the action of alcohol dehydrogenase and the cytochrome P-450 system.14 In the liver, where much of the alcohol is metabolized oxidatively, acetaldehyde may play a prominent role in cell injury. However, the enzymatic capacity for acetaldehyde generation is not nearly as significant in the heart as it is in the liver.15 This limited cardiac oxidation of ethanol has led to the hypothesis that nonoxidative ethanol metabolism is responsible for the generation of a toxic mediator responsible for ethanol-induced cardiac dysfunction.12,16 In the early 1980s, it was proposed that fatty acid ethyl esters (FAEEs), esterification products of ethanol and fatty acids, could be the mediators of ethanol-induced cell injury in the heart.16 It was demonstrated that purified FAEEs in emulsions can disrupt oxidative phosphorylation in isolated mitochondria from the heart, and an enzyme known as FAEE synthase was subsequently purified from rabbit heart.17

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We hypothesized that FAEEs are generated rapidly in cardiac tissue in amounts that could produce cytotoxicity and thereby may be the mediators of myocardial necrosis with ethanol-induced ablation. The purpose of this study was to assess the in vivo production of FAEEs by myocardial tissue in a modified ethanol ablation procedure in pigs.

**Materials and Methods**

**Method for Preparation of Animals, Femoral Artery Catheterization, and Septal Ablation**

Experiments were performed on seven pigs (29 to 42 kg) under general anesthesia using a protocol approved by the Massachusetts General Hospital Subcommittee on Research in Animal Care. An open-chest preparation was performed via a thoracotomy and a pericardial cradle was created. Catheters were placed in the femoral artery and coronary sinus for the purpose of repeated coronary sinus sampling. Using angiography, a perfusion balloon coronary catheter was introduced via the carotid artery into the aorta, through the left main artery, and was directed to the left anterior descending (LAD) coronary artery. The balloon was inflated when its location was confirmed by angiography to be in the mid LAD, thus isolating the flow in the distal LAD territory. A flow probe was placed on the LAD distal to the catheter to measure coronary flow downstream from the infusion catheter. Echocardiography was performed using a 3 MHz transducer (Sonos 5500; Philips, Andover, MA).

Echocardiographic contrast (Optison; GE Health Care, Princeton, NJ) was administered to the distal LAD territory and epicardial scanning using a water bath was performed to determine the territory at risk before administration of ethanol.\(^1\) Saline was administered through the central lumen for the first 15 minutes of the experiments on the first two animals, to serve as a control. In all seven animals, alcohol was then administered continuously through the central lumen of the LAD catheter for 60 minutes, to achieve an ethanol level of \(\approx 450\ \text{mg/dl} (100\ \text{mmol/L})\). Because the coronary blood flow varied among animals, the 100% ethanol was diluted based on the coronary blood flow distal to the catheter to approximate an ethanol level of 450 mg/dl. The ethanol was diluted in 100 ml of normal saline and infused at a rate of 100 ml/hour into the distal LAD territory. The balloon was kept inflated during the alcohol perfusion to prevent alcohol spillage into proximal or mid LAD.

Blood was collected in red top tubes containing no anti-coagulant (for alcohol concentration analysis) and in blue top tubes containing the anti-coagulant sodium citrate (for FAEE determination) from the coronary sinus and from the peripheral vein. Imaging and blood collection were performed at baseline and at 15-minute intervals during ethanol infusion and at 30-minute intervals for 2 hours after cessation of ethanol. Additional blood samples were collected at 5 and 10 minutes after ethanol was begun. At the conclusion of the experiment (3 hours total), the animals were euthanized. The heart from a single pig was resected for tissue FAEE analysis (see below) immediately after cessation of ethanol infusion (1 hour total experiment time in this one animal).

There were two technical limitations of note. Because of the coronary anatomy of the pig, we were unable to cannulate the septal perforator artery, so we adapted our model to ablate the distal LAD territory, namely the left ventricular apex. In addition, because of the propensity of the pig to develop ventricular tachycardia/fibrillation in the setting of rapid injection of ethanol, we were unable to give a bolus of 100% ethanol for longer than 1 minute, as is done in human septal ablation procedures. Instead, we attempted to achieve a toxic dose of ethanol \(> 450\ \text{mg/dl}\).

**Blood Preparation and Storage**

The serum or plasma was separated from blood cells by centrifugation \((2000 \times g\) at \(5^\circ C\) for 10 minutes) and was collected for alcohol measurement and FAEE analysis, respectively. All samples were stored frozen at \(-80^\circ C\) until FAEE isolation and quantitation analysis.

**Tissue Specimen Preparation for FAEE Analysis**

The heart from a single pig was sectioned, and corresponding tissue specimens were removed for FAEE analysis immediately after death. The specimens were weighed and homogenized in 1.5 ml of ice-cold phosphate-buffered saline solution using a Fisher PowerGen 125 homogenizer (Fisher Scientific, Pittsburgh, PA) equipped with a \(10 \times 195\)-mm saw tooth generator. After the addition of 2 ml of cold acetone, samples were vortexed and centrifuged at 1117 \(\times g\) for 10 minutes at \(4^\circ C\). The supernatant was transferred to a glass conical tube and analyzed for FAEE content.\(^1\) FAEEs from the plasma samples and the cardiac tissue samples were isolated by solid phase extraction and were identified and quantitated by gas chromatography-mass spectrometry as described previously.\(^2\)–\(^3\)

**Serum Ethanol Determination**

Serum ethanol levels were determined by gas chromatography.\(^4\) Serum samples were mixed with an internal standard of isopropanol, and a 1-\(\mu l\) 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 \(100^\circ C\), and the ethanol peak was identified and quantitated by comparison with a known standard.

**Histopathology Analysis**

The hearts from two pigs were sectioned and tissue specimens were removed for histological assessment immediately after death. Sections were fixed in 10% buffered formalin for at least 24 hours. The sections were...
embedded in paraffin and stained according to standard procedures with hematoxylin and eosin and Sudan Red.

**Statistical Analysis**

Results are expressed as mean ± SEM. Correlation coefficients were determined by regression analysis. Values of $P < 0.05$ were considered statistically significant.

**Results**

The average weight of the swine was 33.4 ± 2.0 kg. The coronary blood flow in the distal LAD ranged from 10 to 22 ml/minute among animals. Figure 1A shows a 3-hour time course of the mean plasma coronary sinus FAEE values (±SEM) for six swine. Ethanol was infused during the first hour of the time course. Coronary sinus FAEE values peaked ~30 minutes into the ethanol infusion. The average peak FAEE value was ~8000 pmol/ml. There was considerable overlap of the curves for coronary sinus FAEE levels (Figure 1A) and coronary sinus blood ethanol concentration (Figure 1B), as the coronary sinus ethanol concentration also peaked at 30 minutes after the initiation of ethanol infusion. The peak coronary sinus ethanol level was ~600 mg/dl ± mg/dl (132 mmol/L). Ethanol was still detectable at the relatively high level of 100 to 200 mg/dl (22 to 44 mmol/L) in the coronary sinus up to 45 minutes after the ethanol infusion was terminated (the 105-minute time point in Figure 1B).

We also measured FAEEs (Figure 2A) and ethanol (Figure 2B) in the peripheral blood in the pigs receiving ethanol by catheter. FAEEs were detected in peripheral blood, but at much lower concentrations than in the coronary sinus. The coronary sinus FAEE values were ~10-fold greater than the peripheral FAEE values throughout the first 90 minutes. Three hours after ethanol infusion was begun and 2 hours after ethanol administration was terminated (the 180-minute time point), the FAEE levels were very similar in the coronary sinus (Figure 1A) and peripheral blood (Figure 2A). There was considerably more variability in FAEEs and ethanol levels of the coronary sinus than in their peripheral blood counterparts (note the smaller error bars in Figure 2, A and B, relative to Figure 1, A and B). The peak FAEE and ethanol concentrations in peripheral blood occurred around 75 minutes, which is 45 minutes after the peak FAEE and ethanol concentrations in the coronary sinus. This was expected because the ethanol originated in the heart and was then systemically distributed.

Figure 3, A–F, shows the correlation between coronary sinus FAEE and ethanol values for each of the six swine. Correlation coefficients (which ranged from $r = 0.5252$ to
Figure 3. The correlation between blood ethanol and FAEEs in the coronary sinus of each pig.
The anatomical locations of nine heart tissue samples taken from an individual pig are shown in Figure 5A, and the total FAEE (nmol/g) tissue detected in each of these areas of the heart are shown in Figure 5B. The highest FAEE levels were detected in the right ventricle free wall and the anterior wall at least 4 cm away from the main LAD at 65 nmol/g tissue and 60 nmol/g tissue, respectively. Figure 5C shows the percent distribution of the individual FAEE species as a percentage of total FAEEs for each area of the heart assayed. Ethyl oleate and ethyl linoleate were the predominant species isolated, representing ~40% and 20% of the total FAEEs, respectively, from all areas sampled except the anterior septum, which had ~30% ethyl palmitate.

**Echocardiography**

In each animal, two-dimensional echocardiography revealed a focal wall motion abnormality that corresponded to the risk area identified using contrast echocardiography. Typically, this involved the apex and distal anterior wall of the left ventricular. No contrast was noted in the right ventricular (RV) free wall or left ventricular posterior wall in any animal. In the six animals that were imaged for the entire 2 hours after the infusion was discontinued, the regional wall motion persisted to 120 minutes after cessation of ethanol. Because the swine were sacrificed 2 hours after procedure, we were unable to assess the reversibility of the wall motion abnormality. In the animal that was sacrificed at the end of ethanol infusion, the echocardiographic contrast pattern was similar to the other animals. There were no wall motion abnormalities noted in the right ventricular free wall or inferior, posterior, or lateral walls during the infusion. The typical left ventricular apical, distal anterior, and septal wall motion abnormalities developed in this animal.

**Discussion**

Myocardial production of FAEEs after delivery of ethanol into the left anterior descending coronary artery was immediate and substantial. The FAEE levels in both the coronary sinus and the peripheral vein correlated with ethanol levels at these locations, and the FAEEs remained detectable 2 hours after ethanol infusion. FAEE synthesis was also detected in various sites of the myocardium. The time course of peak FAEE levels in the peripheral circulation was consistent with cardiac origin and systemic distribution.

The levels of FAEEs achieved in this model were similar to those measured from a single human peripheral venous blood sample taken 30 minutes after a directed infusion of 100% ethanol into a septal perforator during an alcohol septal ablation procedure at our center. That patient had no detectable FAEEs before the ablation and had 272.13 pmol/ml of FAEE in plasma from a peripheral venous sample drawn 30 minutes after ablation. The composition of FAEE was ethyl palmitate, ethyl stearate, ethyl oleate.

We assessed the myocardial synthesis of FAEE in the septal ablation procedure because a number of in vitro and in vivo studies have shown cytotoxic effects of FAEEs and because FAEEs have been detected in the heart tissue of individuals acutely intoxicated at the time of death and in the heart tissue of known alcoholics. We also pursued nonoxidative ethanol metabolites because, although little is known about the mechanism by which ethanol induces myocardial necrosis, it is thought that the oxidative metabolism of ethanol is limited in the heart. In addition, acetaldehyde, the primary oxidative metabolite of ethanol, is cleared from the blood at a rate that is approximately five times that of ethanol (via aldehyde dehydrogenase). In contrast, FAEEs are known to persist in tissues and in the circulation long after ethanol is no longer detectable and may accumulate in pathological conditions.

FAEEs have been shown to damage myocardial cells. Bora and colleagues investigated FAEE-induced cardiac histopathological changes and found that 50 μmol/L ethyl oleate, injected directly into rat myocardium, causes cellular and mitochondrial damage, as evidenced by swollen and irregularly shaped cells. The abnormalities first appeared 4 days after FAEE exposure. The myocardial cell damage caused by FAEE administration worsened throughout time, resulting in pronounced gross morphological changes 30 days after FAEE administration. Additionally in an earlier report, FAEEs were shown to induce dysfunction of mitochondria isolated from the rabbit heart. The above studies raise the possibility that the accumulation of FAEEs after chronic ethanol ingestion may account for the cardiomyopathies associated with alcoholism.

Histological assessment of stained sections of heart tissue in this study did not reveal any morphological changes consistent with either apoptosis or necrosis. This is probably because the animals were given ethanol for 1 hour and were then euthanized 2 hours after the ethanol infusion stopped. In this case observable cell injury would not be expected.

In the current study cardiac dysfunction, as indicated by cardiac wall motion abnormalities, was detected at peak FAEE levels (~8 μmol/L FAEE) after ethanol infusion. The exact mechanism for FAEE-induced cardiac damage is unknown and is being investigated. This study focused on cardiac tissue analysis, even though FAEE synthesis has been shown to be cytotoxic in a number of other organs and cell types, because we were interested in investigating FAEE synthesis after ethanol ablation procedures.

The alcohol ablation procedure is a new nonsurgical method currently used in the treatment of hypertrophic obstructive cardiomyopathy. Hypertrophic obstructive cardiomyopathy is an excessive thickening of the heart muscle and systolic anterior motion of the mitral
Figure 4. The correlation between blood ethanol and FAEEs in the peripheral vein of each pig.
The resultant contact between the thickened upper septum and the mitral valve during systole lead to obstruction of blood flow out of the heart. The alcohol septal ablation procedure is used to induce a focal myocardial infarction and thus cause focal thinning of the left ventricular septum at the site of systolic anterior motion contact and ultimately provide relief from the obstruction. In hypertrophic obstructive cardiomyopathy patients undergoing septal ablation with ethanol, the ethanol is infused into a septal perforator branch of the LAD through an angioplasty catheter, where it causes a controlled localized infarction of the interventricular septum at the site of obstruction, which in turn results in a reduction in the hypertrophy. Little is known about the acute pathological effects of ethanol ablation procedures on the myocardium in humans.

In this study FAEEs were detected throughout the heart tissue (Figure 5). This suggests that in clinical septal ablation procedures, ethanol injected into the septal perforator branch may reach areas of the myocardium other than the septum, including the right ventricle free wall and anterior wall away from the LAD. The ethanol perfusion field, and thus the extent of tissue exposure to FAEEs, is probably directly related to the anatomical characteristics of the coronary artery tree within the heart. The presence of collateral channels between the LAD and right coronary circulations could have led to the high concentration in the right ventricular free wall. The greater variability of FAEE and ethanol concentrations in the coronary sinus, as compared to the peripheral vein (as seen in Figure 1 versus Figure 2), may reflect individual differences in LAD and coronary vein branch anatomy because there is great interindividual variability in branch size, number, distribution, and extent of vascular territory.

In summary, the data in this report demonstrate rapid, high-concentration FAEE production in myocardium and raise the possibility that myocardial necrosis after ethanol-induced ablation is mediated by FAEEs. More work is necessary to understand the molecular mechanisms of ethanol-induced cytotoxicity and resultant myocardial dysfunction.

References