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Fatty Acid Ethyl Esters Are Less Toxic Than Their Parent Fatty Acids Generated during Acute Pancreatitis

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Although ethanol causes acute pancreatitis (AP) and lipolytic fatty acid (FA) generation worsens AP, the contribution of ethanol metabolites of FAs, ie, FA ethyl esters (FAEEs), to AP outcomes is unclear. Previously, pancreata of dying alcoholics and pancreatic necrosis in severe AP, respectively, showed high FAEEs and FAs, with oleic acid (OA) and its ethyl esters being the most abundant. We thus compared the toxicities of FAEEs and their parent FAs in severe AP. Pancreatic acini and peripheral blood mononuclear cells were exposed to FAs or FAEEs *in vitro*. The triglyceride of OA (i.e., glyceryl tri-oleate) or OAEE was injected into the pancreatic ducts of rats, and local and systemic severities were studied. Unsaturated FAs at equimolar concentrations to FAEEs induced a larger increase in cytosolic calcium, mitochondrial depolarization, and necro-apoptotic cell death. Glyceryl tri-oleate but not OAEE resulted in 70% mortality with increased serum OA, a severe inflammatory response, worse pancreatic necrosis, and multisystem organ failure. Our data show that FAs are more likely to worsen AP than FAEEs. Our observations correlate well with the high pancreatic FAEE concentrations in alcoholics without pancreatitis and high FA concentrations in pancreatic necrosis. Thus, conversion of FAs to FAEE may ameliorate AP in alcoholics. *(Am J Pathol 2016, 186: 874–884; http://dx.doi.org/10.1016/j.ajpath.2015.11.022)*

Although fat necrosis has been associated with severe cases of pancreatitis for more than a century,[1,2] and alcohol consumption is a well-known risk factor for acute pancreatitis (AP),[3] only recently have we started understanding the mechanistic basis of these observations.[4–7] High amounts of unsaturated fatty acids (UFAs) have been noted in the pancreatic necrosis and sera of severe AP (SAP) patients by multiple groups.[8–12] These high UFAs seem pathogenically relevant because several studies show UFAs can cause pancreatic acinar injury or can worsen AP.[11–14] Ethanol may play a role in AP by distinct mechanisms,[3] including a worse inflammatory response to cholecystokinin,[4] increased zymogen activation,[15] basolateral enzyme release,[16] sensitization to stress,[7] FA ethyl esters (FAEEs),[17] cytosolic calcium,[18] and cell death.[19]

Because the nonoxidative ethanol metabolite of fatty acids (FAs), FAEEs, were first noted to be elevated in the pancreata of dying alcoholics, they have been thought to play a role in AP.[17,19–22] Conclusive proof of the role of FAEEs in AP in comparison with their parent UFAs is lacking. Uncontrolled release of lipases into fat, whether in the pancreas or in the peritoneal cavity, may result in fat necrosis, UFA generation, which has been associated with SAP.[11,12] Pancreatic homogenates were also noted to have an ability to synthesize FAEEs from FAs and ethanol,[20,23] and the putative enzyme for this was thought to be a lipase.[24,25] It has been shown that the FAEE synthase activity of the putative enzyme exceeds its lipolytic capacity by several fold.[25]

Triglyceride (TG) forms >80% of the adipocyte mass,[26–28] oleic acid (OA) being the most enriched FA.[9,29] We recently showed that lipolysis of intrapancreatic TG worsens pancreatitis.[11,12] Therefore, after noting the ability of the pancreas to cause lipolysis of TG into FAs and also to have high FAEE synthase activity

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and FAEE concentrations, we decided to compare the relative ability of FAEEs and their parent FAs to initiate deleterious signaling in pancreatitis and to investigate their impact on the severity of AP.

Materials and Methods

Reagents

Linoleic acid (LA), palmitic acid (PA), glyceryl tri-oleate (GTO), and oleic acid ethyl esters (OAEE; sold as ethyl oleate) were purchased from Sigma-Aldrich (St. Louis, MO). Palmitic acid ethyl esters (PAEE) and Linoleic acid ethyl esters (LAEE) were from Cayman Chemical (Ann Arbor, MI). For *in vitro* studies, these were all dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) as pure solutions, sonicated at 2× final concentration, and used at a final concentration of 0.5% DMSO for the *in vitro* studies. This concentration of DMSO has no effect on any of the markers tested over the duration of the study [eg, control lactate dehydrogenase (LDH) leakage without vehicle over 4 hours was 9.6% ± 0.8% versus 9.4% ± 1.2% in the 0.5% DMSO group]. Ketamine hydrochloride injection was from Fort Dodge Animal Health (Fort Dodge, IA), and xylazine injection (xylazine sterile solution 100 mg/mL) was from Lloyd Laboratories (Shenandoah, IA). The rest of the reagents are detailed in the areas mentioning their use.

Animal Work

Male Wistar rats of 250 to 300 g, purchased form Charles Rivers Laboratories (Wilmington, MA), were acclimatized for at least 2 days before use. These rats were housed with a 12-hour light-dark cycle, fed standard laboratory chow, and allowed to drink *ad libitum* until the night before the surgery, when they were deprived of food. After adequate anesthesia with 90 mg ketamine/kg and 8 mg xylazine/kg, intraperitoneally, under sterile precautions, either the TG of oleic acid (GTO) also known as triolein, or the ethyl esters (EE) of OA, ie, OAEE, also known as ethyl oleate (both dosed as 50 μL/100 g of body weight) were injected into the biliopancreatic duct in rats. The biliopancreatic duct was ligated after the injection. Postoperatively, animals were intramuscularly administered 100 mg cefazolin/kg (WG Critical Care, Paramus, NJ) for infection prevention, buprenorphine SR (ZooPharm, Windsor, CO; at 0.6 mg/kg, i.p.) for pain management, and normal saline subcutaneously daily. These animals were followed up to 5 days for survival or were sacrificed at the time near mortality (moribund state). In addition, a separate group of animals in the OAEE group were sacrificed electively after 24 hours of AP induction to compare the severity with GTO, because this was the time when nearly all mortality was observed in the GTO group. On necropsy, serum and pancreata were harvested to study for various markers of pancreatitis initiation, histologic assessment, cytokine levels, lipotoxic mediators, and markers of end-organ injury, the details of which are described in *Materials and Methods*. There were 10 to 12 animals in each group. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and the Mayo Clinic (Scottsdale, AZ).

Acinar Harvest and *in Vitro* Assays

Mice pancreatic acini were harvested, as described previously.[30,31] Fresh acini were used for all assays. Viability, confirmed by Trypan blue exclusion, was >95%. Acini were exposed to FAs (LA, OA, PA) and their corresponding FAEEs (ie, LAEE, OAEE, PAEE) at concentrations relevant to human pancreatic debridement fluids. The human necrotic fluid collections contained 1235 ± 412 μmol/L OA, 613 ± 214 μmol/L PA, and 586 ± 185 μmol/L LA.[32] Necrotic cell injury was assessed by LDH leakage, propidium iodide (PI) uptake (both as percentage of total), and adenosine triphosphate (ATP) levels of the pellets at the end of the incubation (4 hours). These were performed with colorimetric, fluorescence, or luminescence assay as described previously.[33] LDH activity in the medium and PI uptake into the cells were measured and expressed as a percentage of total generated by lysis of cells with the use of 1% Triton X-100. ATP measured was normalized to untreated controls as 100%. In addition, cell pellets were collected at 90 minutes and fractionated into cytosolic and mitochondrial fractions. These lysates were then probed for cytochrome *c* oxidase IV, α-tubulin, and cytochrome *c* by Western blot analysis, as described further.

Western Blot Analysis

Cytosolic and mitochondrial cell fractions were homogenized in lysis buffer that contained various protease inhibitors (Complete, EDTA Free; Roche, Mannhein, Germany) and then normalized for 1 μg/μL protein after protein estimation with a Pierce protein assay kit (Thermo Fisher Scientific, Rockford, IL). Lysates were boiled (5 minutes) in Laemmli buffer that contained SDS and β-mercaptoethanol, loaded (10 μg/lane) on 12% polyacrylamide gels and underwent electrophoresis denaturing conditions (SDS-PAGE). Western blot analysis was performed by incubation with primary antibody cytochrome *c* oxidase IV (dilution 1:2000; Invitrogen, Grand Island, NY), cytochrome *c* (dilution 1:750; Cell Signaling Technology, Danvers, MA), and α-tubulin (dilution 1:1000; Developmental Studies Hybridoma Bank, Iowa City, IA) and then probed with horseradish peroxidase-labeled corresponding secondary antibodies (dilution 1:10,000; Millipore Corp, Billerica, MA). Band intensity was visualized by chemiluminesence by using electrochemiluminescence plus Western blot detection kit (Amersham GE Healthcare, Buckinghamshire, UK).

Calcium Imaging

Calcium imaging was performed as described previously[34,35] in acinar cells loaded with fura-2AM, adhered to glass