



GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

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 intra-pancreatic 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 from 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, Mannheim, 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 chemiluminescence 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

coverslips, and imaged at 37°C on an Olympus IX81 inverted microscope (Olympus America Inc., Center Valley, PA) with a 20 × 0.70 NA objective and a QImaging Retiga EXi CCD camera (QImaging, Surrey, BC, Canada). The FAs and their EEs were added at the final indicated concentrations, and cytosolic calcium levels were determined by alternate excitation at 340 nm and 380 nm, measuring emission at 510 nm. Image acquisition was with the MetaMorph Imaging System (Molecular Devices, Sunnyvale, CA), using the MetaMorph software version 6.3 (Molecular Devices). The 340/380 emission ratio was averaged for 7 to 25 acini per field after background subtraction. Data are presented as means from at least four separate experiments.

Mitochondrial Depolarization Measurement

Acinar cells were loaded with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, 5 µg/mL; Enzo Life Sciences, Farmingdale, NY) at 37°C for 15 minutes. After loading, the cells were washed twice and kept on ice until use. In a quartz cuvette, cells were stimulated with different agonists at the final indicated concentrations, and changes in mitochondrial inner membrane potential (Ψ_m) were determined by excitation at 515 nm and alternate measuring emission at 530 and 590 nm with the use of the F2100 Hitachi Fluorescence Spectrophotometer (Tokyo, Japan). A decrease in the JC-1 aggregate fluorescence at 590 nm and an increase in the fluorescence at 530 nm reflect depolarization. The 530/590 emission ratio was averaged, and data are presented as means from at least four separate experiments.

Biochemical Assays

Serum amylase, lipase, and blood urea nitrogen were measured according to the manufacturer's (Pointe Scientific Inc., Canton, MI) instructions on a ChemWell-T chemistry analyzer (Awareness Technology, Palm City, FL).

FA Measurement

Serum OA (C18:1) level was measured by performing nonesterified FA (NEFA) analysis with the use of gas chromatography, as described previously,^{11,12,35} on samples with adequate volumes (at least 25 µL).

Evaluation of Pancreatic Necrosis and Special Stains

Whole pancreas section slides stained by hematoxylin and eosin were examined by a trained morphologist (K.P.) blinded to the sample as described previously.^{12,35} In brief, all pancreatic parenchymal area was imaged with a 4× objective, and photographs were evaluated for acinar necrosis. Necrotic area and total acinar area were measured in pixels for each pancreas. Percentage of necrosis was reported as a percentage of total area for each

pancreas. The results are depicted as bar graphs, showing means ± SEM for each group. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed on paraffin sections of the lungs and kidneys and was quantified, as described previously.¹²

Immunohistochemical Studies

The horseradish-peroxidase immunohistochemical technique was used to detect myeloperoxidase (MPO) in paraffin-embedded sections of lung tissue. In brief, after deparaffinization and antigen epitope retrieval, tissues were incubated with a primary rabbit polyclonal antibody against MPO (dilution 1:50; Abcam, Cambridge, MA), followed by application of horseradish peroxidase-conjugated (dilution 1:1000; Millipore Corp) secondary antibody. Staining was completed with chromogen incubation with 3-amino-9-ethylcarbazole substrate kit for peroxidase and hematoxylin QS nuclear counterstain (Vector Laboratories, Burlingame, CA). Quantification of MPO positivity was performed as percentage of 3-amino-9-ethylcarbazole-stained nuclear area to total nuclear area in the lung tissue, calculated by color thresholding on Adobe Photoshop CS4 (San Jose, CA).

Cytokine Assays

Cytokine assays were performed on serum samples by fluorescence-based capture sandwich immunoassay (Luminex), using the MILLIPLEX MAP Rat Cytokine-Chemokine Magnetic beads panel (Millipore Corp). These samples were analyzed at the Luminex Core Facility of the University of Pittsburgh Cancer Institute.

Flow Cytometry of Rodent PBMCs

Rodent peripheral blood mononuclear cells (PBMCs) were incubated for 30 minutes at 37°C in serum-free RPMI 1640 media that contained 25 mmol/L HEPES either alone or with 10 µmol/L OA or OAEE. Cytotoxicity was evaluated by labeling cells with annexin V and PI according to the manufacturer's instructions for the fluorescein isothiocyanate-annexin V apoptosis detection kit (BD Pharmingen, San Diego, CA). Flow cytometry of labeled cells was performed on the BD LSR Fortessa Analyzer running the BD FACS Diva software version 8.0.1 (BD Biosciences, San Jose, CA). Acquisition of fluorescence signals was performed after adjusting for forward and side scatter voltages, selecting appropriate fluorochrome channels, and a minimum of 50,000 gated events per experimental condition were analyzed.

Statistical Analysis and Graphic Representation

Bar graphs or box plots were used to depict the data, which were collected as independent variables, whereas line graphs were used for continuous variables. All values are reported as means ± SEM. Box plots and whisker plots are used in the

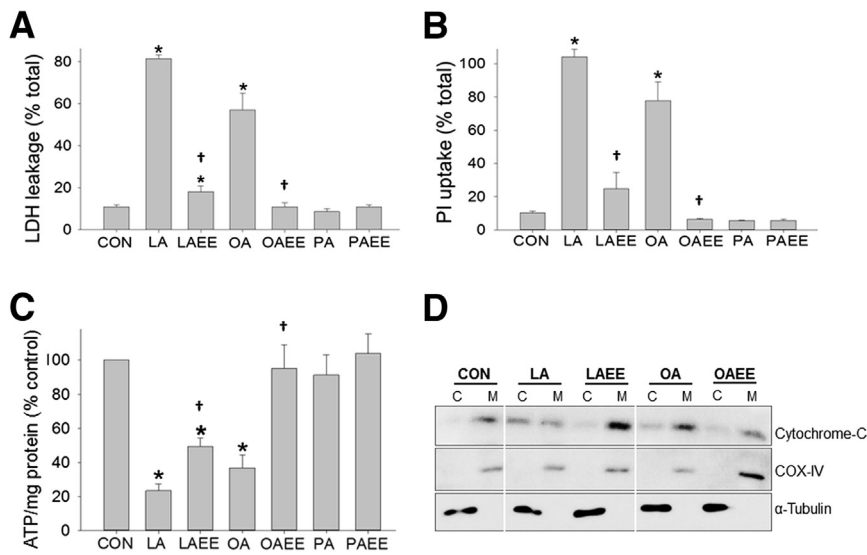


Figure 1 Fatty acids cause more cell death than their EEs. **A** and **B**: LDH leakage (**A**) and PI uptake (**B**) is increased compared with CONs in acini treated with unsaturated fatty acids LA and OA compared with LAEE and OAEE. **C**: LA and OA also cause decrease in ATP levels compared with their EEs. The saturated fatty acid PA does not cause any effect compared with its EE, PAEE. **D**: Western blot analysis for cytochrome *c* shows a leakage of cytochrome *c* from the M compartment to the C compartment in the presence of LA or OA. This intercompartment cytochrome *c* leakage is absent in LAEE and OAEE. Loading controls, α -tubulin for the C compartment and COX-IV for the M compartment, are equally present in all of the groups. Data are expressed as means \pm SEM. * $P < 0.05$ versus CONs; † $P < 0.05$ versus LA/OA. ATP, adenosine triphosphate; C, cytosolic compartment; CON, control; COX, cytochrome *c* oxidase; EE, ethyl ester; LA, linoleic acid; LDH, lactate dehydrogenase; M, mitochondrial compartment; OA, oleic acid; PA, palmitic acid; PI, propidium iodide.

depiction of serum cytokines, in which mean (dotted line), median (solid line), 25th and 75th percentiles (two boxes), and 10th and 90th percentiles (whiskers) are depicted. All significance levels were evaluated at $P < 0.05$ and were compared by the one-way analysis of variance versus controls or two-tailed *t*-test between the triolein (GTO) and OAEE groups. $P < 0.05$ was considered statistically significant.

Results

FAEEs Induce Less Acinar Injury Than the Parent FAs at Equimolar Concentrations

To determine the relative toxicities of FAEEs and the parent NEFAs, 300 $\mu\text{mol/L}$ of the most prevalent NEFAs in the human pancreatic necrosis collections^{11,20,32} were added at relevant concentrations to acinar cells, and LDH leakage was measured over 4 hours (Figure 1A). These NEFA concentrations used are within the range of those noted in human pancreatic necrosis collections.^{11,12,32} Although both UFAs (LA and OA) resulted in $>50\%$ LDH leakage, their respective EEs (LAEE and OAEE) resulted in significantly less acinar injury. Because UFAs are known to eventually induce acinar necrosis,^{11–13} we measured PI uptake and noted a similar pattern (Figure 1B). As previously shown,^{11,13} the saturated FA (SFA) PA did not induce acinar injury, with its EE (PAEE) being similarly nontoxic. UFAs have been shown to result in acinar necrosis via ATP depletion by inhibition of mitochondrial complexes I and V¹¹; we thus compared the ATP levels in acini treated with NEFA or their corresponding EEs. It has been previously shown that ATP levels drop in response to LA over 1 hour.³⁵ Similar to the pattern of LDH leakage, and the previously published data,^{11,35} LA and OA resulted in significantly lower ATP levels, whereas their corresponding

EEs (LAEE and OAEE) had higher levels (Figure 1C). We also noted cytochrome *c* leakage to be more with the FAs than with the corresponding FAEEs (Figure 1D).

To explore this further, we studied early events in cell death signaling. We compared the effect of agents that induce cell death and their corresponding EEs on two widely published early cell death events, Ψm and Ca_i levels. As previously published, we noted maximal Ca_i increase at 600 $\mu\text{mol/L}$, which is in the range noted in human pancreatic necrosis collections.^{11,32} Consistent with the loss of Ψm being associated with triggering cell death, there was a greater increase in the 530/590 emission ratio of JC-1 induced by LA and OA than by LAEE or OAEE (Figure 2, A and B) at equal concentrations. Similarly, LA and OA (Figure 2, C and D) did cause an increase in Ca_i , but LAEE and OAEE did not. The increase in Ca_i induced by UFAs has been previously shown to be from a thapsigargin-sensitive intracellular store³⁵ and not due to a detergent effect. Thus, the early loss of mitochondrial membrane potential triggers cell death, as supported by cytochrome *c* leakage, which along with inhibition of complexes I and V and associated decrease in ATP levels, sustained increase in Ca_i , eventually resulting in worse necrosis induced by NEFAs than FAEEs.

Intraductal Triolein Results in a Higher Serum Oleate and Mortality Than OAEE

To test the *in vivo* relevance of the *in vitro* findings mentioned in the previous two paragraphs, we went ahead and compared the severity of pancreatitis induced by the TG of OA (which is the most abundant FA found in necrotic collections), ie, triolein (or GTO) or its EE, OAEE. We used the previously described model of pancreatitis involving direct administration of these agents into the pancreatic duct of lean rats.¹² This simulates the lipolysis of visceral fat TG by the pancreatic lipases leaked into fat during pancreatitis. We have previously shown that the lipolysis of the

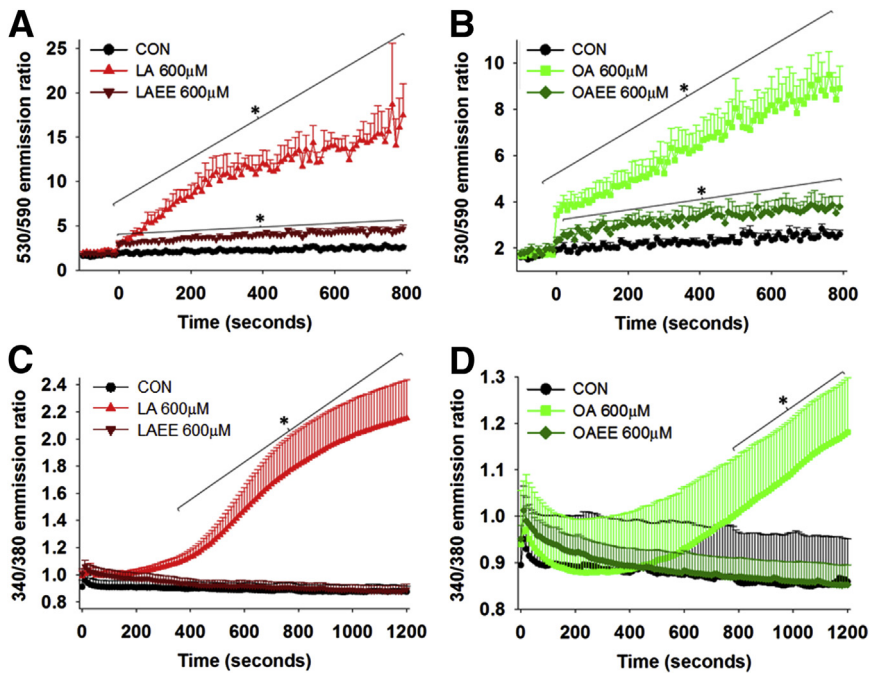


Figure 2 FAEs affect mitochondrial membrane potential and cytosolic calcium levels less than FAs. **A** and **B**: All agents were added at zero second. LA and OA cause a significantly higher loss of mitochondrial membrane potential than their respective FAEs, indicated by the increase in 530/590 emission ratio. **C** and **D**: Cytosolic calcium levels in Fura-2–loaded acini, measured by 340/380 emission ratio, show an increase in resting calcium by FAs, not FAEs. Data are expressed as means \pm SEM. $n = 4$ to 5 experiments. $*P < 0.05$ versus the CON state. CON, control; EE, ethyl ester; FA, fatty acid; LA, linoleic acid; OA, oleic acid.

injected TG and not its mere injection or presence in the pancreas, results in adverse outcomes.¹²

Consistent with the lipolysis of injected GTO, there was an increase in serum OA which was significantly higher than in the group receiving OAAE (Figure 3A). This was associated with a significantly higher mortality (70%; $P = 0.003$) in the GTO group within 24 hours of inducing pancreatitis compared with no mortality being noted in the OAAE group over the 5 days these rats were followed (Figure 3B). These findings are consistent with our recent work showing that lipolysis of unsaturated TG worsens AP.¹²

Intraductal OAAE Results in Lesser Pancreatic Injury Than Triolein

Both local and systemic injury may contribute to the severity of pancreatitis. We first compared the effect of GTO and OAAE on local injury. There was gross evidence of hemorrhagic pancreatitis in the pancreata of rats receiving GTO but not OAAE (Figure 4, A–C). Morphometric analysis showed GTO to result in $62.4\% \pm 5.1\%$ necrosis, which was significantly more than that induced by OAAE ($11\% \pm 2.8\%$; $P < 0.001$) (Figure 4, D and E). This was associated with significantly lower serum amylase and lipase in the OAAE group versus the GTO group (Figure 4, F and G). These findings are consistent with the *in vitro* findings that FAEs are less toxic than the parent UFAs.

Intraductal OAAE Results in Lesser Systemic Injury Than Triolein

Because systemic injury occurs during SAP, we compared systemic injury markers in response to GTO and OAAE.

GTO caused a higher serum IL-1 β , IL-6, IL-18, and keratinocyte chemoattractant–growth-regulated oncogene than OAAE (Figure 5, A–D). Similarly, although we noted a low level of MPO positivity in control lungs as has previously been shown by many groups,^{36–40} there was a significantly higher MPO increase induced in the GTO-administered group than in the OAAE group (Figure 5E). GTO-treated but not OAAE-treated rats had a significant increase in TUNEL⁺ cells in the lungs (Figure 6A). The findings in the GTO-treated group are similar to those induced in OA-induced acute respiratory distress syndrome.^{41,42} GTO treated rats also had renal failure evidenced by elevated serum blood urea nitrogen and TUNEL⁺ cells in the kidney tubules at the time of necropsy (Figure 6B). Findings of drop out necrosis in these tubules supported progression to acute tubular necrosis. Flow cytometry of PBMCS showed that treatment with 10 $\mu\text{mol/L}$ OA resulted in significantly

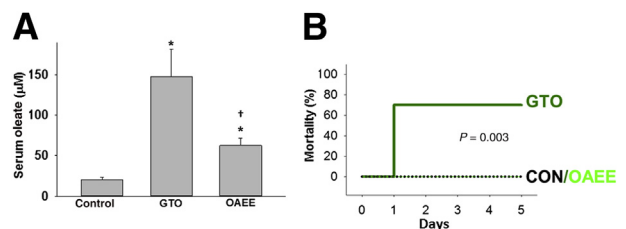


Figure 3 Intraductal injection of triolein causes higher rate of mortality and serum OA concentrations. **A**: Bar graphs of serum oleate level showing a significant increase in its concentrations with intraductal injection of triolein and OAAE. OAAE causes a significantly lesser increase compared with triolein. **B**: Kaplan-Meier mortality curves show 70% mortality with the GTO group versus no mortality in the OAAE group. Data are expressed as means \pm SEM. $*P < 0.05$ versus the CON; $^\dagger P < 0.05$ versus triolein (GTO) group. CON, control; EE, ethyl ester; GTO, glyceryl tri-oleate; OA, oleic acid.

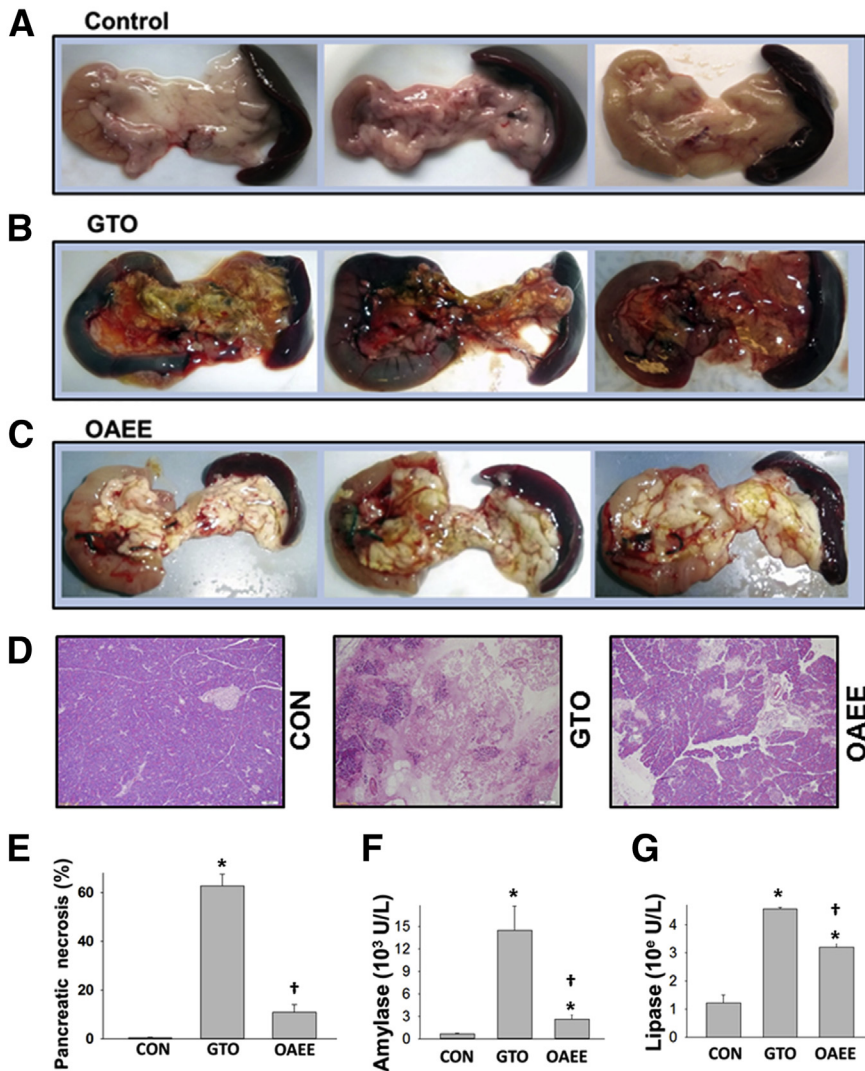


Figure 4 Triolein causes severe hemorrhagic necrotizing pancreatitis. **A–E:** Gross appearance (**A–C**) and hematoxylin and eosin-stained sections (**D**) of the pancreas in CONs (**A** and **D**), intraductal triolein (**B** and **D**), and OAE (**C** and **D**). Intraductal injection of GTO causes significant hemorrhage and necrosis (**B**, **D**, and **E**) compared with the milder necrosis and no hemorrhage in OAE (**C**, **D**, and **E**). **F** and **G:** OAE caused a lesser increase in amylase and lipase than GTO. Data are expressed as means \pm SEM. * $P < 0.05$ compared with CONs; † $P < 0.05$ compared with GTO. Original magnification, $\times 10$ (**D**). CON, control; GTO, glyceryl tri-oleate OAE, oleic acid ethyl ester.

more necro-apoptotic injury than in response to OAE (**Figure 6**, C–F). These results show that OAE is less toxic to PBMCs and renal tubules and does not cause lung injury in contrast to its parent FA, OA.

Discussion

In this study, we compared the ability of FAEEs and their parent FAs to induce cell injury *in vitro* and during AP *in vivo*. Adipocytes are increased in obesity, which many studies show is a risk factor for SAP.^{43–50} Stored TG forms >80% of the adipocyte mass.^{26–28} Because FAEEs are metabolites of ethanol, which is a common cause of AP, this study helps understand the role of FAEEs in AP compared with FAs generated from visceral fat lipolysis. With the use of the TG form of the most abundant FA found in pancreatic necrosis collections, ie, OA or its EE (OAE), we find that the FAEE causes milder acinar injury and pancreatitis than the parent UFA; and, with the use of FAs *in vitro* at concentrations within the range of pancreatic necrosis

collections, we note them to be more potent than their EEs in inducing an increase in Ca_i , loss of Ψ_m , decrease in ATP, cytochrome *c* leakage, and necrotic cell death. These findings shed light on the role FAEEs may play in AP.

The historic first report that measured FAEEs in humans analyzed tissues from acutely intoxicated alcoholics (blood ethanol concentration, >80 mg/dL) dying from an accident, homicide, a nonalcohol-related natural cause, or alcoholic liver disease.²⁰ Although adipose tissue and pancreas of these individuals had the highest levels of FAEEs, none of them had pancreatitis. Ethyl oleate was reported to be the most abundant FAEE in pancreata of humans and rodents exposed to ethanol.^{20,23} When supplied OA and ethanol, the pancreas synthesizes FAEEs at the highest rate compared with other tissue.²⁰ This activity is noted in pancreatic homogenates in the presence of ethanol and after ethanol infusion^{51,52} and may exceed the lipolytic capacity of the putative enzyme by >60-fold.²⁵ This suggests that in the presence of sufficient ethanol, FAs may be converted to FAEEs *in vivo*.

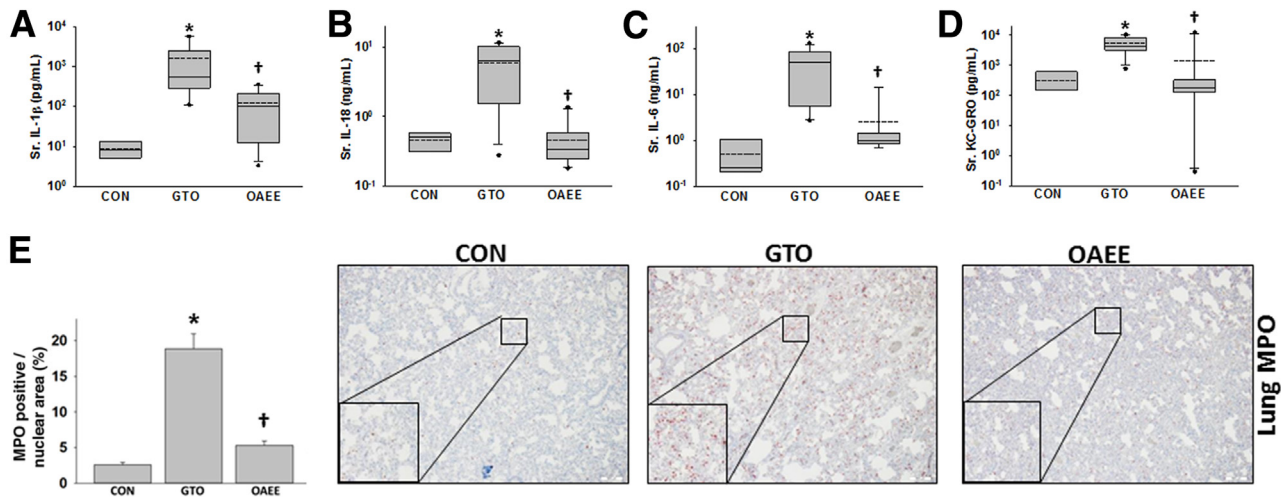


Figure 5 Triolein administration causes a worse cytokine response and inflammation compared with OAEE. **A–D:** Box plots depicting cytokines IL-1 β (**A**), IL-18 (**B**), IL-6 (**C**), and KC-GRO (**D**) show significant increase in the group administered triolein intraductally compared with CONs. Administration of OAEE significantly reduces these cytokines. **E:** MPO⁺ cells are significantly increased in the GTO group versus the OAEE group. * $P < 0.05$ compared with CONs; † $P < 0.05$ compared with GTO. Original magnification: $\times 20$ (**E**); $\times 60$ (**insets**). CON, control; KC-GRO, keratinocyte chemoattractant–growth-regulated oncogene; GTO, glyceryl tri-oleate; MPO, myeloperoxidase; OAEE, oleic acid ethyl ester; Sr., serum.

Our current study clearly shows that FAEs are less toxic than their parent UFAs at equivalent concentrations. The concentrations chosen mirror those in human pancreatic necrosis collections.^{11,12,32} This partly explains the high levels of FAEs noted in the tissues of alcoholics without pancreatitis and why only a minority of alcoholics may develop pancreatitis^{53,54} despite high FAEs in the pancreas. In contrast, lipolytic FA generation universally exacerbates pancreatitis to a lethal disease in the mechanistically distinct IL-12/IL-18,¹¹ biliary,¹² rat,³² and mouse⁵⁵ caerulein models.^{11,12}

Ethanol has diverse effects on AP outcomes in rodents. In some studies ethanol administration did not worsen pancreatitis,⁵⁶ but it enhanced exocrine secretion along with increasing plasma cholecystokinin levels.⁵⁷ Ethanol may cause AP when co-administered with caerulein at doses that are one-tenth the highest caerulein dose used to induce pancreatitis,⁴ but which is still in the supra-physiologic range. When co-administered with a high-fat diet, chronic ethanol administration increased the fragility of lysosomal and zymogen membranes^{58,59} and resulted in chronic pancreatitis in $<30\%$ animals. Interestingly, although most studies show FAEs to be more toxic than in ethanol alone,¹⁷ separate studies show that infusion of FAEs causes relatively mild changes (pancreatic edema, trypsinogen activation, vacuolization)¹⁹ compared with UFAs (eg, OA), which when infused cause acute respiratory distress syndrome^{41,42,60–62} and renal injury,^{63,64} components of multisystem organ failure in SAP.

Studies by Criddle et al¹⁷ over the past decade have contributed significantly to our current understanding of the role of FAEs in pancreatitis. Their seminal study in 2004 showed palmitoleic acid (POA) and its EE (POAEE) to cause a sustained increase in cytosolic calcium and to

induce calcium-dependent cell death within the first hour of exposure. The role on calcium in early cell death induced by FAs was subsequently confirmed by us.¹¹ However, we also noted that the amount of cell death within the first hour was small compared with the $>80\%$ necrosis that occurred over the subsequent 4 hours and was insensitive to calcium chelation.¹¹ In 2006 Criddle et al²¹ showed that FAEs induced necrosis via conversion to FAs by an esterase sensitive to bis-(4-nitrophenyl) phosphate. However, the esterase so far has not been characterized, and the influence of bis-(4-nitrophenyl) phosphate on its activity was not measured in the study. Their study also showed that in most cases POA was toxic at one-half the dose of POAEE.²¹

The putative enzyme responsible for FAE generation has remained elusive. The enzyme was mostly referred to as carboxylesterase²⁵ or cholesterol esterase in the 1990s.²⁵ It was later proposed that formation of FAEs is catalyzed by both an acyl-CoA:ethanol O-acyltransferase and a FAE synthase which use FAs and acyl-CoA as substrates, respectively.⁶⁵ Subsequently, this enzyme that has its first 15 amino acids identical to pancreatic cholesterol esterase⁶⁶ was noted to be sensitive to inhibitors of serine proteases.⁶⁷ In 2005 polymorphisms in the carboxyl ester lipase gene were noted to be associated with the risk of developing alcoholic pancreatitis.

In 2014,²² Criddle et al¹⁷ in contrast to their 2004 study noted that POA in most cases did not induce a sustained increase in cytosolic calcium. In addition, in contrast to their 2006 study which showed FAE cytotoxicity depends on their conversion to FAs²¹; their 2014 study²² showed this to depend on the conversion of POA to POAEE. This increase in cytosolic calcium and severity of pancreatitis were significantly reduced with the agent 3-benzyl-6-chloro-2-pyrone (3-BCP), a generic serine protease inhibitor,^{68,69} which they proposed

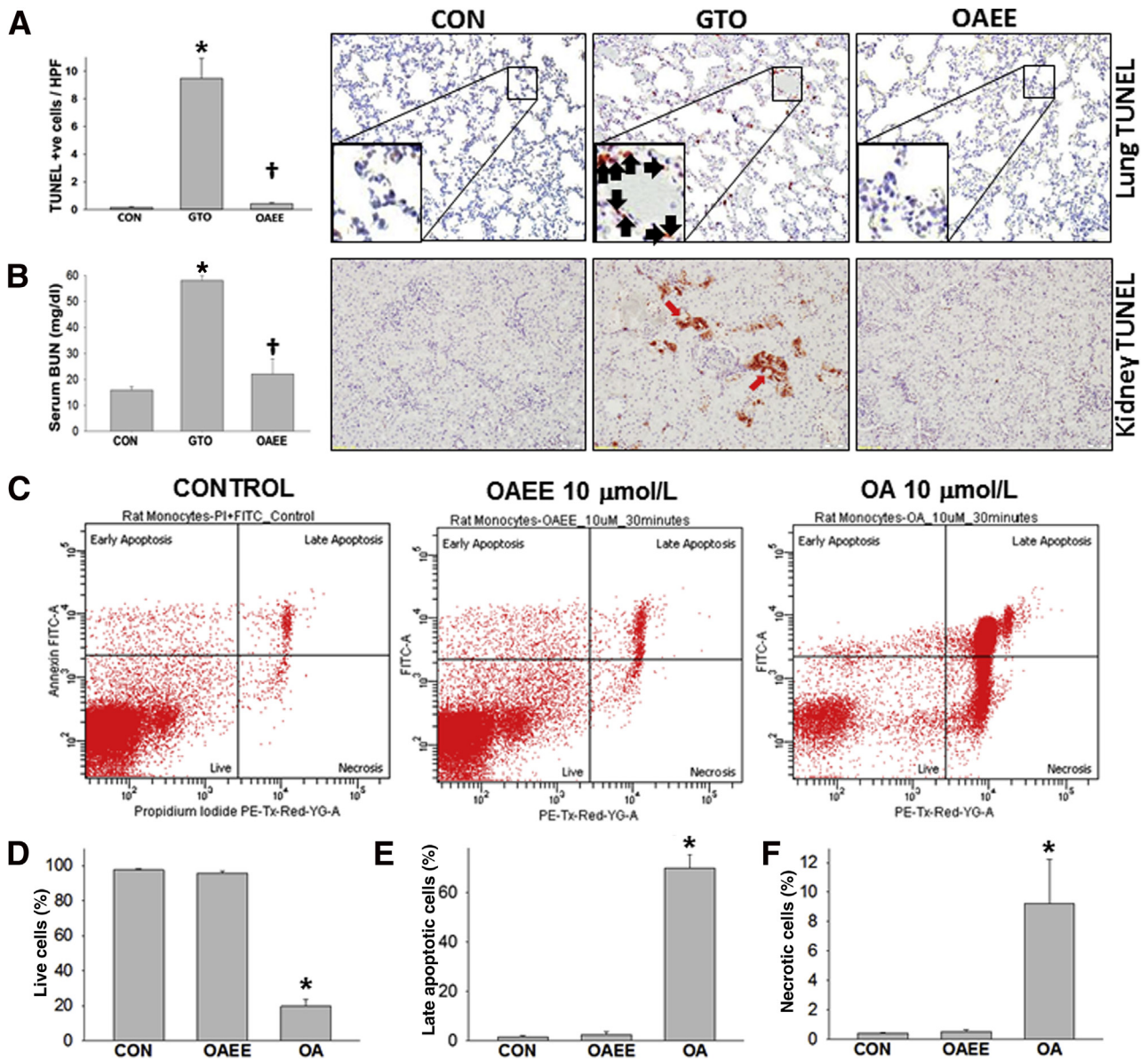


Figure 6 Triolein causes worse systemic injury compared with OAEE. **A:** Presence of TUNEL⁺ cells (inset, black arrows) in lungs are significantly higher in the GTO group than the OAEE group. **B:** Intraductal GTO also causes a significant increase in BUN and TUNEL⁺ cells in the kidney tubules. Representative TUNEL-stained image for each condition is shown to the right of the bar graphs (**A** and **B**). **C–F:** Peripheral blood mononuclear cells treated with OA show a significant decrease in live cells (**C** and **D**) and an increase in late apoptotic cells (**C** and **E**) and necrotic cells (**C** and **F**) compared with OAEE at equimolar amounts (10 μmol/L). **P* < 0.05 compared with CONs; †*P* < 0.05 compared with GTO. Original magnification: ×40 (**A**); ×20 (**B**); ×100 (insets). BUN, blood urea nitrogen; CON, control; EE, ethyl ester; FITC, fluorescein isothiocyanate; GTO, glyceryl tri-oleate; HPF, high power field; OA, oleic acid; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

to be a specific FAEE synthase/carboxyl ester lipase inhibitor.²²

These recent findings published by Criddle et al²² partly differ from our current work and their earlier studies.^{17,21} In our current work, similar to their 2006 study, we note FAEEs to be less toxic than the parent FAs. In addition, the following points help resolve differences. i) Previous work⁶⁸ has shown 3-BCP to also be an inhibitor of serine esterases such as chymotrypsin,⁶⁹ α-lytic protease, pig liver elastase, and cholinesterase, some of which have been implicated in initiating or worsening pancreatitis. Thus, this broad

inhibitory effect of 3-BCP may contribute to its protection. ii) Carboxyl ester lipase is a pancreatic lipase, which is involved in the lipotoxic AP exacerbation in the mechanistically distinct biliary¹² and IL-12 + IL-18 models.¹¹ Inhibition of lipases by 3-BCP thus may contribute to the amelioration noted in their study. iii) As we note, FAEEs do cause cell death and pancreatitis, but it is substantially milder than from the parent FAs. iv) The pattern of necrosis noted by intraperitoneal administration of FAEEs or FAs + ethanol²² shows this to be localized to the edges of the lobules, with relative sparing of acinar cells in the center,

suggesting a local effect from the relatively large amounts of FAs/ethanol/FAEEs which were delivered into the peritoneal cavity.

Interestingly, a literature review of the roles of FAEEs also reveals their benign aspects. These include i) their levels being elevated in the pancreas of alcoholics without AP,²⁰ and ii) FAEEs are used in treating hypertriglyceridemia. These include the Food and Drug Administration-approved EEs of eicosapentaenoic acid and docosahexaenoic acid,⁷⁰ the parent FAs of which are as toxic to acinar cells as LA/OA (unpublished data). iii) Chronic OAEE administration to rats (3 months)⁷¹ and humans⁷² does not cause toxicity.

The above-mentioned studies, along with evidence that i) high FA levels are noted in the sera^{8,10} and pancreatic necrosis debridement^{9,11} of patients with SAP; ii) previous studies show the pancreatic toxicity of FAs^{11,13}; iii) the ability of pancreatic tissue to rapidly form FAEEs, when supplied ethanol and FAs²⁰; iv) high pancreatic FAEE concentrations in alcoholics without pancreatitis; and v) this study showing that FAEEs are less toxic than their parent FAs, suggests that the formation of FAEEs is a protective mechanism to detoxify FAs. In summary, the conversion of FAs to FAEEs in alcoholics may play a protective role in AP.

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