ORIGINAL ARTICLE

Basic Study

Distinctive roles of unsaturated and saturated fatty acids in hyperlipidemic pancreatitis

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Abstract

AIM: To investigate how the saturated and unsaturated fatty acid composition influences the susceptibility of developing acute pancreatitis.

METHODS: Primary pancreatic acinar cells were treated with low and high concentrations of different saturated and unsaturated fatty acids, and changes in the cytosolic Ca^{2+} signal and the expression of protein kinase C (PKC) were measured after treatment.

RESULTS: Unsaturated fatty acids at high concentrations, including oleic acid, linoleic acid, palmitoleic acid, docosahexaenoic acid, and arachidonic acid, induced a persistent rise in cytosolic Ca^{2+} concentrations in acinar cells. Unsaturated fatty acids at low concentrations and saturated fatty acids, including palmitic acid, stearic acid, and triglycerides, at low and high concentrations were unable to induce a rise in Ca^{2+} concentrations in acinar cells. Unsaturated fatty acids at high concentrations but not saturated fatty acids induced intra-acinar cell trypsin activation and cell damage and increased PKC expression.

CONCLUSION: At sufficiently high concentrations, unsaturated fatty acids were able to induce acinar cells injury and promote the development of pancreatitis. Unsaturated fatty acids may play a distinctive role in the pathogenesis of pancreatitis through the activation of PKC family members.

Key words: Unsaturated fatty acid; Saturated fatty acid; Hypertriglyceridemia; Acute pancreatitis; Calcium

Core tip: The mechanism by which severe hypertriglyceridemia precipitates acute pancreatitis remains unknown. Abnormal sustained elevated cytosolic Ca^{2+} signals, which cause abnormal intracellular enzyme activation, are crucial in the initiation of acute pancreatitis. Unsaturated fatty acids at high concentrations induced a persistent rise in cytosolic Ca^{2+} concentrations in acinar cells and caused intra-acinar cell trypsin activation and cell damage. Unsaturated fatty acids at low concentrations and saturated fatty acids and triglycerides at low and high concentrations were unable to induce a rise in Ca^{2+} concentrations in acinar cells. Unsaturated fatty acids at high concentrations may play a crucial and distinctive role in the pathogenesis of hypertriglyceridemic pancreatitis.

INTRODUCTION

Hypertriglyceridemia (HTG) is associated with acute pancreatitis (AP) and is observed in 3%-38% of patients with AP[1-6]. HTG is the third-most frequent etiology of AP in Taiwan[7]. Although the mortality rate of hyperlipidemic pancreatitis (HLP) does not differ from that of AP due to other etiologies, the disease severity and complication rates of HLP are higher than those of other forms of AP[8,9].

The mechanism by which severe HTG precipitates AP remains unknown. Havel proposed that pancreatitis results from toxic injury to the capillary endothelium and pancreatic acinar cells through the liberation of free fatty acids (FFA) by pancreatic lipase[10]. This hypothesis was supported by experimental studies by Saharia *et al*[11]; perfusion of the pancreas with FFA induced edema and hemorrhage of the pancreatic preparation. FFA has been reported to induce activation of trypsinogen and to initiate AP[12]. Furthermore, in AP animal models, HTG has been shown to contribute to and accelerate the inflammatory cascade[13]. Previous animal studies revealed that HTG can intensify the course of AP in rats[14] and impair the hemorheology in lipoprotein lipase-deficient mice[15]. However, previous studies are inconclusive with respect to predicting which patients with HTG will develop pancreatitis and why some patients with HTG seldom develop pancreatitis despite the markedly elevated TG level. Further research to understand the pathogenesis of HLP is necessary for the development of specific treatments.

Ca^{2+} signals in the pancreatic acinar cells are generated by the second messengers inositol tris-phosphate (IP3), nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP-ribose (cADPR). Ca^{2+} signals are modulated in a precise spatiotemporal manner, which is necessary for normal acinar cell secretory function[16]. Abnormal, prolonged elevation of cytosolic Ca^{2+} is the critical trigger of pancreatitis[16]. Abnormal, global, and sustained elevation of cytosolic Ca^{2+} signals cause abnormal intracellular enzyme activation, vacuolization and necrosis, which are crucial in the initiation of AP[17,18]. Another pathological signal that appears to be linked to AP is the activation of protein kinase C (PKC) isoforms[19]. PKC isoforms have been linked to NF-κB expression and zymogen activation[20,21]. Although the involvement of PKC isoforms in NF-κB activation by cholecystokinin (CCK) has been addressed, their roles in the pathogenesis of HLP have not been thoroughly studied.

A previous study demonstrated that different serum FFA compositions in patients with AP were related to the severity and complications of AP[22]. Unsaturated fatty acids, mainly linoleic acid, may be involved in the development of AP complications[22]. In addition, palmitoleic acid causes a dose-dependent increase in cytosolic calcium concentrations[23], and oleic acid at high concentrations induces lactate dehydrogenase leakage from acinar cells and causes an increase in cytosolic calcium concentration[24]. We hypothesized that triglyceride composition, *i.e.*, the saturated and unsaturated fatty acid composition, may influence AP susceptibility in HTG patients. To investigate this hypothesis, we used primary pancreatic acinar cell culture to measure the change in cytosolic Ca^{2+} in acinar cells after treatment with different saturated and unsaturated fatty acids. In this study, we also investigated whether saturated or unsaturated fatty acids activate PKC isoforms in isolated pancreatic acinar cells.

MATERIALS AND METHODS

Reagents

Most of the chemicals, including cerulein, triglyceride, palmitic acid, stearic acid, oleic acid, linoleic acid, palmitoleic acid, docosahexaenoic acid (DHA), arachidonic acid, caffeine, digitonin, rhodamine 100, bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide) dihydrochloride, and U-73122, were obtained from Sigma Co Ltd (St Louis, MO, United States). Xestospongin C was obtained from Calbiochem (EMD Millipore Corporation, Billerica, MA, United States). Fura-2 AM was obtained from Invitrogen (Molecular Probes, Eugene, Oregon, United States). The phospho-PKC antibody sampler kit was obtained from Cell Signaling (Danvers, MA, United States).

Isolation of pancreatic acinar cells

This study was conducted at the animal facility of National Taiwan University College of Medicine after approval by the National Taiwan University College of Medicine and College of Public Health Institutional Animal Care and Use Committee (IACUC) (IACUC Approval No. 20080297). Eight-week-old male BALB/c mice were used for this study. All animals were obtained from an inbred population of BALB/c mice maintained at the animal facility of National Taiwan University. Pancreatic tissue was aseptically removed from the BALB/c mice, which were sacrificed humanely by means of CO_2 asphyxiation after overnight fasting. The pancreatic tissue was dissected free of mesenteric fat, minced, washed with Hanks balanced salts solution (HBSS, pH 7.4) with 0.01% soybean trypsin inhibitor and then incubated with 10 mL 0.05% trypsin and 0.25% EDTA for 5 min at 37 ℃. After centrifugation at 200 g for 5 min, the tissue was rinsed with F-12K Nutrient Mixture (Invitrogen/Life Technologies, California, United States) with 1% antibiotics [100 U/mL penicillin-G, 100 μg/mL streptomycin and 0.25 μg/mL FUNGIZONE (Gemini Bio-Products)], 5 mg/mL bovine serum albumin (BSA) and 0.1 mg/mL soybean trypsin inhibitor. The tissue was centrifuged at 200 g for 5 min, and the pellet was resuspended in 12.5 mL of digestive solution containing 1 mg/ml collagenase V (Sigma Chemical Co.) and 0.2 mg/mL BSA in HBSS for 30 min at 37 ℃. The digested tissue was centrifuged at 200 g for 5 min and resuspended in F-12K medium with 10% fetal bovine serum (FBS) (Invitrogen/Life Technologies) twice. The obtained pancreatic acinar cells were resuspended in F-12K medium with 10% FBS and 100 U/mL penicillin-G, 100 μg/mL streptomycin and 0.25 μg/mL FUNGIZONE (Gemini Bio-Products) at 37 ℃ in an atmosphere of 5% CO_2 and 95% air.

Cytosolic Ca^{2+} concentrations in pancreatic acinar cells treated with unsaturated and saturated fatty acids

Freshly isolated mouse pancreatic acinar cells were loaded with 2.5 μmol/L fura-2/AM in loading buffer containing 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 10 mmol/L HEPES, 0.1% BSA and 0.2% glucose (pH 7.4) for 30 min at room temperature. The cell suspension was washed twice and resuspended in loading buffer. The cells were aliquoted into cuvettes in loading buffer containing 1000 mg/dL triglyceride, 10 nmol/L cerulein, saturated fatty acids (0.1 mmol/L palmitic acid and 1.0 mmol/L stearic acid) or unsaturated fatty acids (0.1 mmol/L or 1.0 mmol/L of the following: oleic acid, linoleic acid, palmitoleic acid, DHA, or arachidonic acid). To dissolve the fatty acids, 0.25% chloroform was added to the buffer. Fluorescence was recorded in a mechanically stirred cuvette using an SLM©AMINCO-8100 spectrophotofluorometer (Spectronic Instruments, Rochester, NY). For measurements of $[Ca^{2+}]$, the excitation wavelength was alternated between 340 and 380 nm every second, and fluorescence intensity was monitored at an emission wavelength of 510 nm. The signal ratio (340:380) was determined after autofluorescence correction, and the free intracellular Ca^{2+} concentration was calculated.

Inhibition analysis

For pharmacological analysis, we added caffeine (20 mmol/L), xestospongin C (10 μmol/L), and U-73122 (10 μmol/L) to the cells for 5 min before adding different fatty acids as inhibitors; this allowed us to delineate the targets of the different fatty acids in the calcium signaling pathway of the acinar cells.

Measurement of trypsin activity

The acinar cells were loaded with 10 μmol/L rhodamine 110 bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide) dihydrochloride (BZiPAR) at 37 ℃ for 60 min, which is a specific substrate for the serine protease trypsin that becomes fluorescent after cleavage of the two oligopeptide side chains. Activation can be observed by fluorescence of rhodamine 110 at an excitation wavelength of 485 nm. To ensure that the tryptic activity observed was from intracellular enzymes only and not from trypsin released into the extracellular fluid, the cells for these experiments were prepared in solution containing 5 μmol/L soybean trypsin inhibitor. The localization of the trypsin activity was investigated by confocal microscopy (Zeiss, LSM510, Germany).

Preparation of protein extracts and western blot analysis

After treatment with different saturated or unsaturated fatty acids for 30 min, the pancreatic acinar cells were lysed on ice in TM buffer and a cocktail of protease inhibitors (Biochain Newark, United States). The protein concentration of the extracts was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were fractionated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with Tris-buffered saline supplemented with 5% nonfat dry milk and antibodies against phospho-PKC-α, -δ, -ε, and -ζ (1:1,000 dilution each) for 1 h at room temperature. The membranes were incubated with secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. The blots were developed using an enhanced chemiluminescence detection kit (Pierce, Thermo SCIENTIFIC, Rockford, United States).

RESULTS

Unsaturated fatty acids rather than saturated fatty acids induced sustained elevation of cytosolic Ca^{2+} concentrations in acinar cells

In our experimental acinar cell model, we observed that high concentrations (1 mmol/L) of unsaturated

fatty acids, including oleic acid, linoleic acid, palmitoleic acid, DHA, and arachidonic acid, induced a persistent rise in cytosolic Ca^{2+} concentrations in acinar cells. This rise was similar to that observed with supra-maximal concentrations of CCK in isolated pancreatic acinar cells (Figure 1A-C). However, neither low concentrations (0.1 mmol/L) of unsaturated fatty acids, including oleic acid, linoleic acid, palmitoleic acid, DHA, and arachidonic acid, nor low nor high concentrations of saturated fatty acids, including palmitic acid and stearic acid, were able to induce a rise in Ca^{2+} concentrations in acinar cells (Figure 1B-D). The elevation of cytosolic Ca^{2+} concentrations in acinar cells was dose-dependent on the ratio of unsaturated/saturated fatty acids (Figure 1E-G). High concentrations of triglycerides initially did not cause a rise in cytosolic Ca^{2+} concentrations in acinar cells; however, after long-term incubation with high concentrations of triglycerides, the cytosolic Ca^{2+} concentration in the acinar cells gradually increased (Figure 1H). All experiments were repeated at least three times with the same results.

Inhibition assay

Unsaturated fatty acids at high concentrations induced a persistent rise in cytosolic Ca^{2+} concentrations in acinar cells, similar to that observed with CCK, but this process was not inhibited by the IP₃R antagonists caffeine, xestospongin C or U-73122, which inhibit the hydrolysis of PPI to IP₃ (Figure 2).

Unsaturated fatty acids induced intra-acinar cell trypsin activation

High but not low concentrations of unsaturated fatty acids induced intra-acinar cell trypsin activation, as observed with CCK, whereas neither low nor high concentrations of saturated fatty acids were able to induce this activation and subsequent cell damage and death (Figure 3).

Unsaturated fatty acids induced the expression of PKC isoforms in acinar cells

Treatment of acinar cells with high concentrations of fatty acids, induced the expression of PKC-α, -δ, and -ε. This effect was strongest upon treatment with oleic acid, linoleic acid, palmitoleic acid, DHA, and arachidonic acid and weaker with palmitic acid and stearic acid (Figure 4). PKC-ζ expression was only induced by DHA and arachidonic acid (Figure 4).
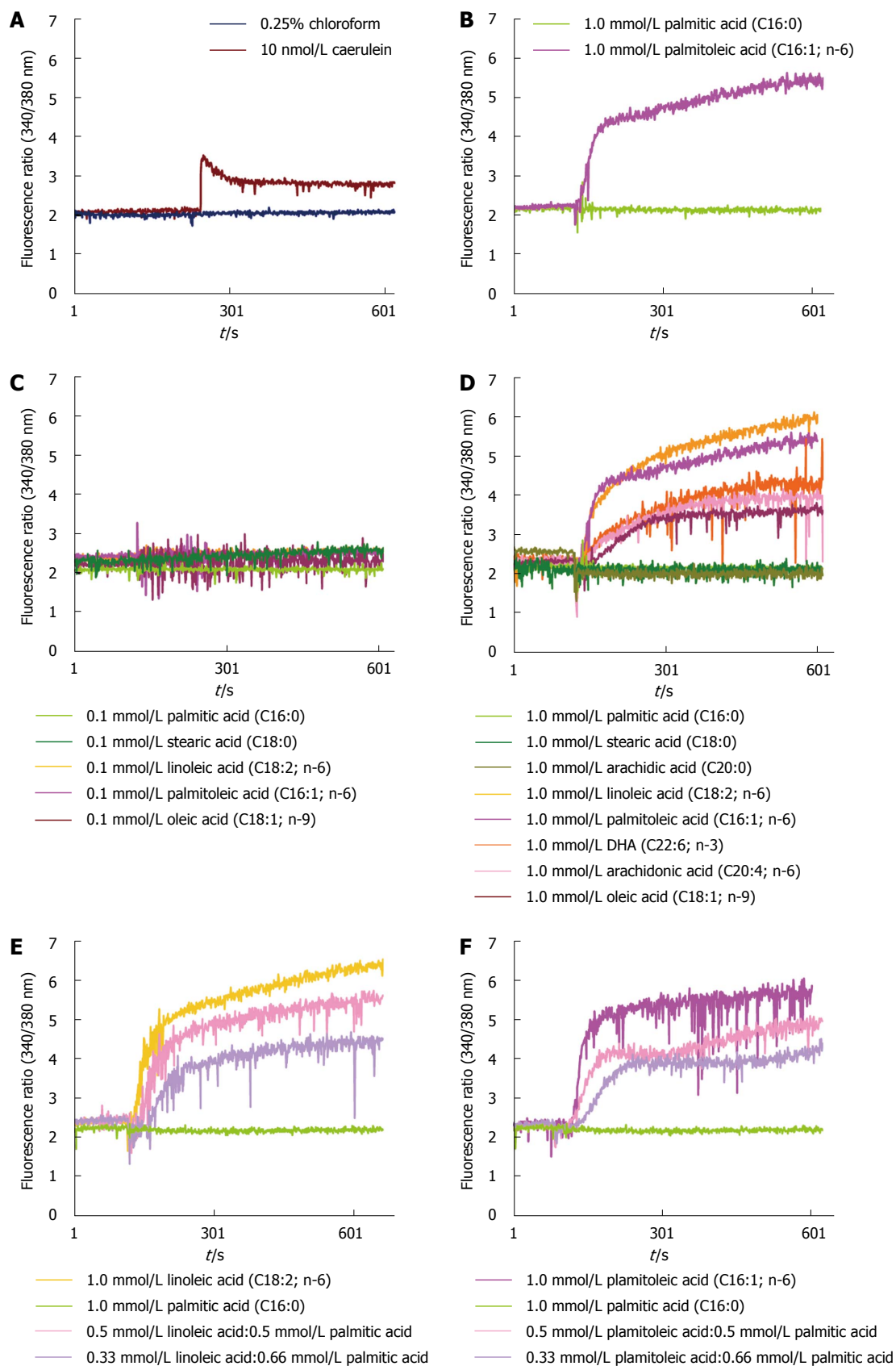
DISCUSSION

The mechanism by which HTG leads to pancreatitis is not well understood, and it is difficult to predict which hyperlipidemic patients are likely to develop an episode of pancreatitis. Hydrolysis of triglycerides by pancreatic lipase with localized release of large quantities of FFAs has been proposed to be the pathogenic mechanism by which HTG causes pancreatitis. An early feature of AP is the premature intracellular activation of trypsinogen within pancreatic acinar cells. This activation requires a rise in cytosolic Ca^{2+} from intracellular compartments[16]. A previous study reported that different serum FFA compositions in patients with AP were related to the severity and complications of AP[22,25]. Unsaturated fatty acids, mainly linoleic acid, may be involved in the development of complications of AP[22]. In addition, palmitoleic acid causes a dose-dependent increase in cytosolic calcium concentrations[23], and high concentrations of oleic acid induce lactate dehydrogenase leakage from acinar cells as well as an increase in cytosolic calcium concentrations[24]. Our data demonstrate for the first time that high but not low concentrations of unsaturated fatty acids and neither high nor low concentrations of saturated fatty acids induced an elevation of cytosolic Ca^{2+} concentrations in acinar cells as well as intra-acinar cell trypsin activation, cell damage and death. This elevation of cytosolic Ca^{2+} concentrations in acinar cells is dose-dependently related to the ratio of unsaturated/saturated fatty acids. Triglycerides are unable to induce a rise in cytosolic Ca^{2+} concentrations in acinar cells. These results indicate that triglycerides cannot induce an attack of AP. Only when triglycerides are hydrolyzed by lipase into FFAs and the concentration of unsaturated fatty acids is sufficiently high do acinar cells become injured, thereby resulting in pancreatitis. These results provide a potential explanation for why only a portion of HTG patients develop HLP clinically. High levels of unsaturated fatty acids may play a crucial role in the pathogenesis of HLP.

In pancreatic acinar cells, Ca^{2+} release from the endoplasmic reticulum (ER) is mediated by IP₃, cADPR and NAADP and primarily occurs through the activation of inositol trisphosphate receptors (IP₃Rs) and ryanodine receptors (RyRs)[16]. Caffeine and xestospongin C exhibit inhibitory effects on IP₃Rs on the ER membranes of calcium stores, and U-73122 inhibits the hydrolysis of PPI to IP₃. However, pretreatment of acinar cells with these inhibitors failed to block the persistent elevation of cytosolic Ca^{2+} observed in acinar cells exposed to high concentrations of unsaturated fatty acids. These results imply that the elevation of cytosolic Ca^{2+} concentrations in acinar cells induced by high concentrations of unsaturated fatty acids does not occur through activation of IP₃Rs. Criddle *et al*[26] reported that the unsaturated fatty acid palmitoleic acid can elicit prolonged elevation of the global cytosolic Ca^{2+} concentrations and that this elevation cannot be inhibited by IP3 receptor blockade, largely due to the inhibition of mitochondrial ATP production. The mechanism by which unsaturated fatty acids induce pathological Ca^{2+} release in acinar cells from the ER or other acidic stores requires further investigation.

Several studies have demonstrated that PKC isoforms modulate pathological secretion during AP
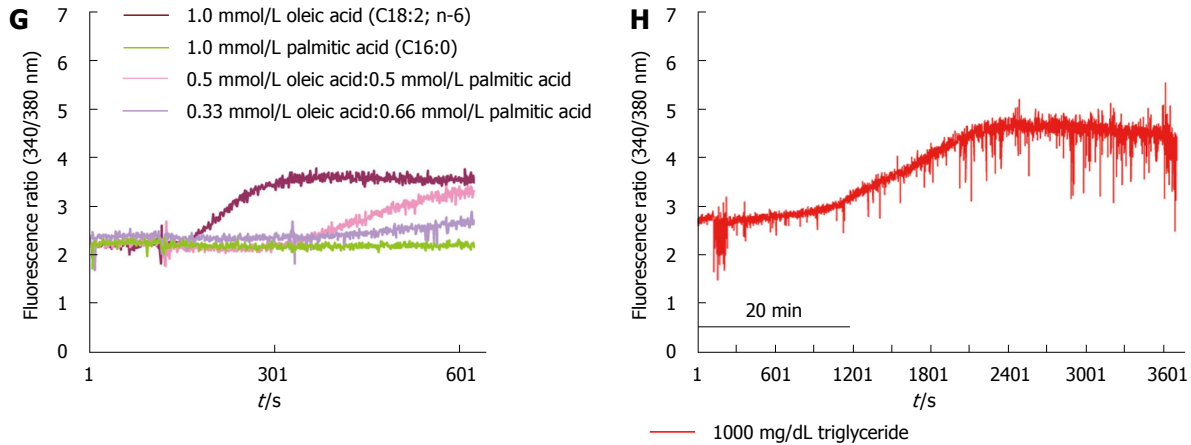
Figure 1 Effects of unsaturated and saturated fatty acids on cytosolic Ca²⁺ concentrations in acinar cells. A: A 0.25% chloroform and 10 nmol/L cerulein solution was used as the control; B: A high concentration (1.0 mmol/L) of the unsaturated fatty acid palmitoleic acid, but not of the saturated fatty acid palmitic acid, induced a sustained cytosolic Ca²⁺ elevation; C: Various unsaturated fatty acids and saturated fatty acids at low concentrations (0.1 mmol/L) did not induce sustained cytosolic Ca²⁺ elevations; D: Various unsaturated fatty acids at high concentrations (1.0 mmol/L), but not saturated fatty acids, induced sustained cytosolic Ca²⁺ elevations. The effects of different ratios of unsaturated fatty acids and saturated fatty acids on the elevation of cytosolic Ca²⁺ concentrations: linoleic acid and palmitic acid (E), palmitoleic acid and palmitic acid (F), and oleic acid and palmitic acid (G); H: High concentrations (1000 mg/dL) of triglycerides did not induce the elevation of cytosolic Ca²⁺ concentrations.
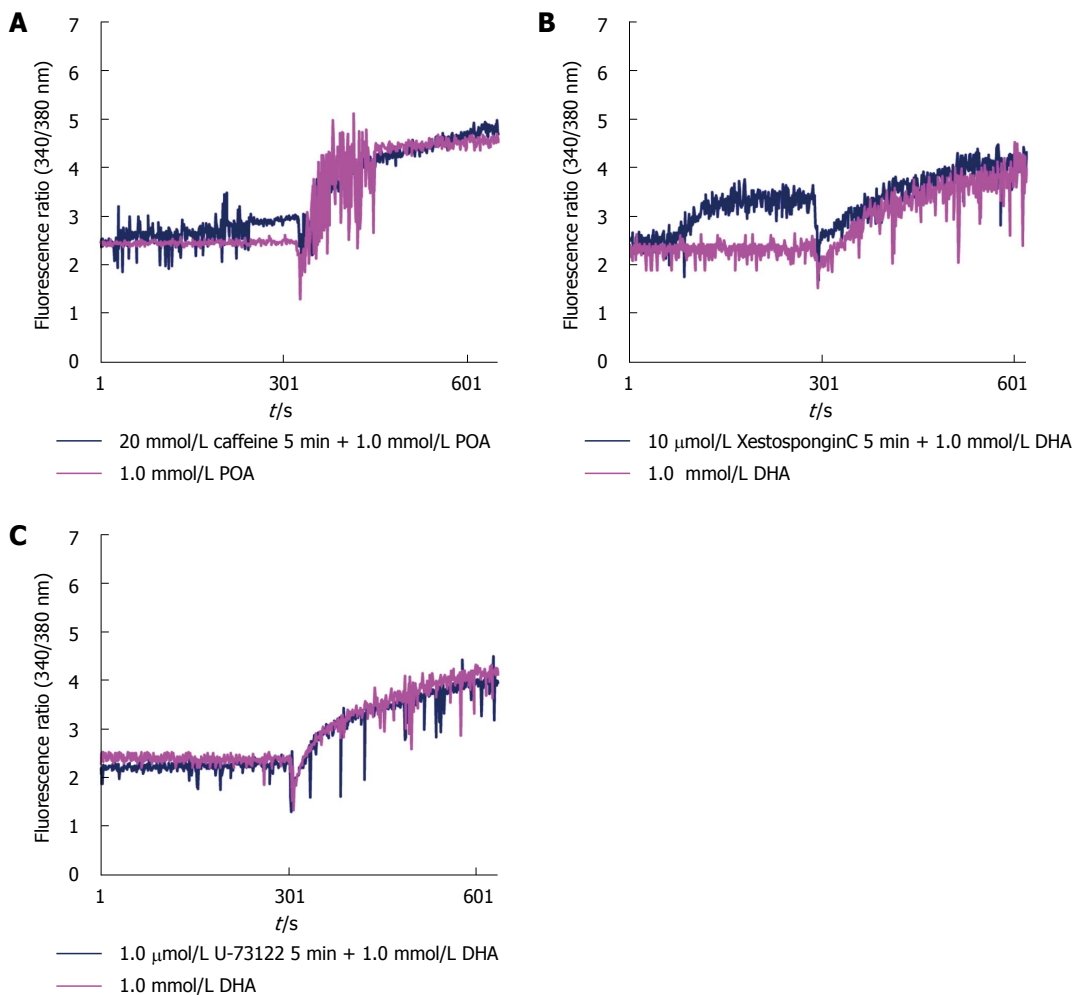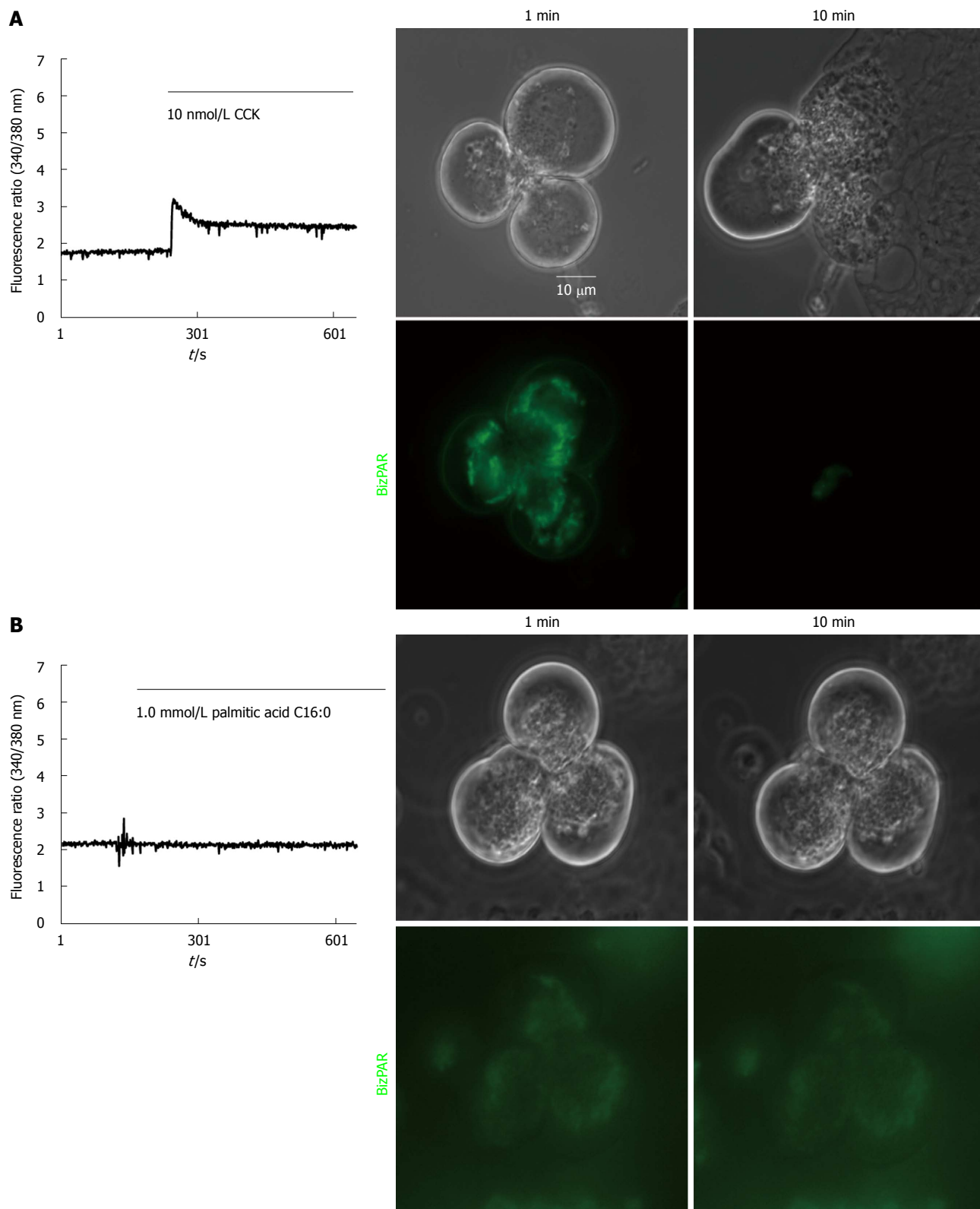

Figure 2 Inhibition of IP₃R did not prevent the elevation of cytosolic Ca²⁺ concentrations in acinar cells induced by unsaturated fatty acids: caffeine (A), xestospongin C (B), and U-73122 (C).
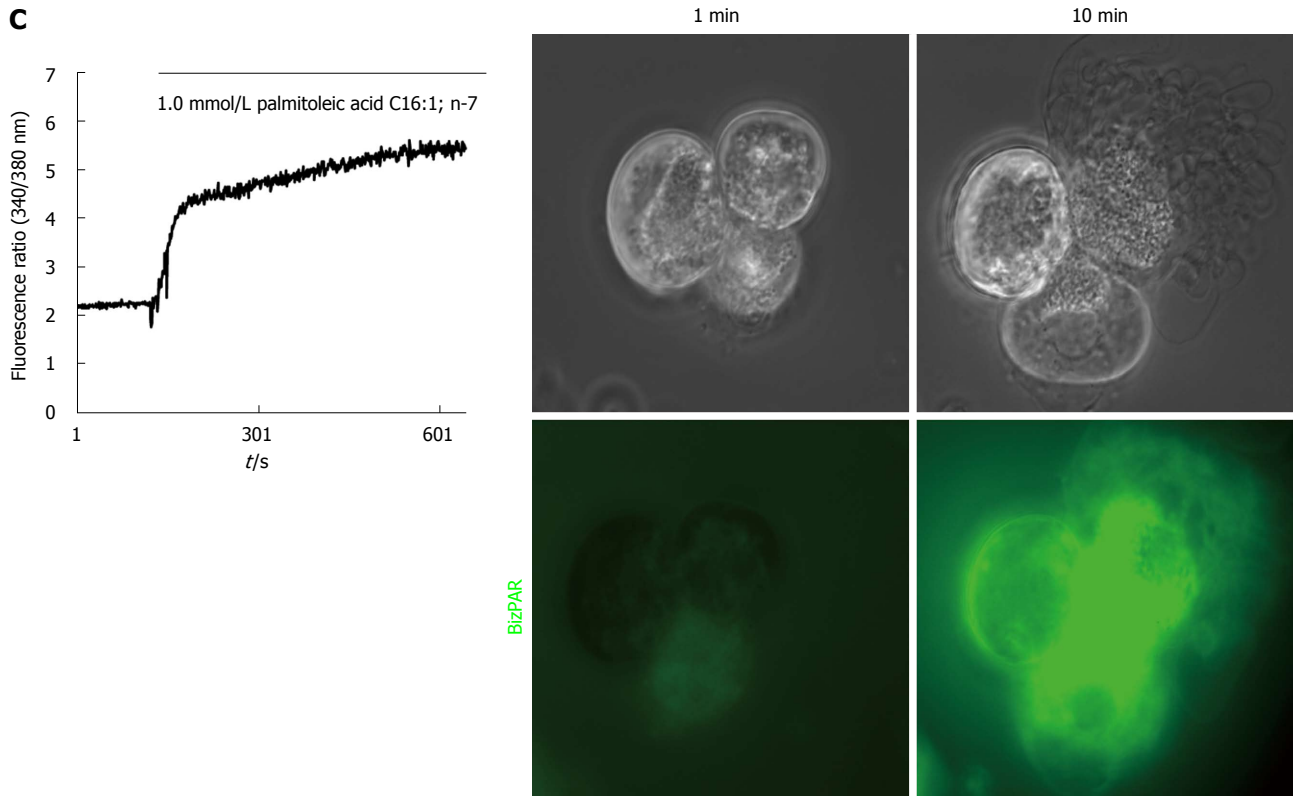
C

1 min   10 min

Figure 3 Unsaturated fatty acids at high concentrations (1.0 mmol/L), but not saturated fatty acids, induced intra-acinar cell trypsin activation and cell damage observed by confocal microscopy: CCK (A), palmitic acid (B), and palmitoleic acid (C).
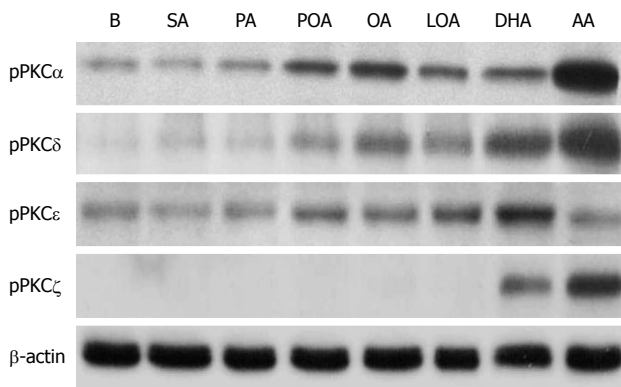

Figure 4 Effects of unsaturated fatty acids at high concentrations (1.0 mmol/L) on protein kinase C isoform expression in acinar cells as detected by western blot. B: Blank; SA: Stearic acid; PA: Palmitic acid; POA: Palmitoleic acid; OA: Oleic acid; LA: Linoleic acid; DHA: Docosahexaenoic acid; AA: Arachidonic acid.

and also regulate the expression of inflammatory mediators[19,20,27]. In pancreatic acinar cells, the conventional PKC-α, the novel PKC-δ and -ε and the atypical PKC-ζ isoforms have been identified[28]. PKC-δ has been shown to participate in premature zymogen activation within pancreatic acinar cells and in activation of the transcription factor NF-κB during experimental pancreatitis[19,29]. PKC-δ is activated and translocated to the plasma membrane and participates in amylase secretion[30], regulates protease activation[31] and modulates inflammatory molecule

expression in pancreatic acinar cells[32]. The initiation of AP requires both zymogen activation and the retention of active enzymes in acinar cells. Fatty acids have been shown to increase PKC activity in HTG and AP animal models[33]. In our study, we found that unsaturated fatty acids at high concentrations upregulated the expression of the PKC isoforms PKC-α, PKC-δ, PKC-ε and atypical PKC-ζ in mouse pancreatic acinar cells. Previous studies reported that supraphysiological concentrations of CCK cause activation of the novel PKC isoforms δ and ε and the atypical PKC isoform ζ as well as the activation of NF-κB[34]. Unsaturated fatty acids at high concentrations have a similar effect to that of CCK at supraphysiological concentrations with respect to the activation of PKC-α, -δ and -ζ, implying that HLP may be triggered by unsaturated fatty acids and through the activation of specific isoforms of PKC. In a recent study, Cui *et al*[35] reported that FFAs induced ER stress by enhancing Ca^{2+} influx in pancreatic β cells, which contributed to β cell dysfunction and cell apoptosis. In addition, in an arginine model of experimental AP, ER stress sensing and signaling mechanisms were reported to be activated in acinar cells early in the development of AP[36]. Furthermore, HTG was reported to aggravate ER stress[37]. Further studies of the roles and effects of unsaturated fatty acids at high concentrations in key pathogenic cellular events, such as calcium release from acidic stores, mitochondrial dysfunction, ER stress, autophagy, impaired trafficking, and lysosomal

and secretory responses in AP are needed.

In conclusion, our *in vitro* results provide an explanation for the clinical observation that only a portion of HTG patients develop AP and that some patients with HTG seldom develop pancreatitis despite marked elevation of triglyceride level. Triglycerides were unable to induce an attack of AP. Only when triglycerides are hydrolyzed by lipase into FFAs and the concentration of unsaturated fatty acids is sufficiently high do acinar cells become injured, thereby resulting in pancreatitis. Unsaturated fatty acids may play a distinctive role in the pathogenesis of HLP through the activation of PKC family members. Further analysis of the composition of unsaturated/saturated fatty acids in acute phase sera of patients with HLP are needed. In addition, consumption of food containing different types of fat might be a strategy to reduce the risk of the development of AP in HTG patients.

COMMENTS

Background
Hypertriglyceridemia (HTG) is the third-most frequent etiology of acute pancreatitis (AP) in Taiwan. The detailed mechanism by which severe HTG precipitates AP remains unknown. Clinically, it is difficult to predict which patients with HTG will develop pancreatitis and why some patients with HTG seldom develop pancreatitis despite the markedly elevated TG level.

Research frontiers
Different serum free fatty acid (FFA) compositions in patients with AP were related to the severity and complications of AP. Unsaturated fatty acids, mainly linoleic acid, may be involved in the development of AP complications.

Innovations and breakthroughs
When triglycerides are hydrolyzed by lipase into FFAs and the concentration of unsaturated fatty acids is sufficiently high do acinar cells become injured, thereby resulting in pancreatitis. Unsaturated fatty acids may play a distinctive role in the pathogenesis of HLP.

Applications
Consumption of food containing different types of fat might be a strategy to reduce the risk of the development of AP in HTG patients.

Terminology
HTG is defined by fasting serum triglyceride level of > 150 mg/dL. HTG is considered a risk for pancreatitis when levels are > 1000 mg/dL.

Peer-review
This is a well conducted study that has resulted in a well written manuscript that helps answer a question about the aetiology of hypertryglyceride induced induced acute pancreatitis. The manuscript is worthy of publication but the introduction and discussion could both be reduced in length without any adverse effect on the paper. The authors might also wish to consider how they can demonstrate similar processes are occurring in a human population.

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