

Novel pathways in fatty-acid induced apoptosis in the pancreatic beta-cell

by

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B.Sc., University of Ottawa, 2004

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Physiology)

University of British Columbia

January 2007

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Abstract

Pancreatic β -cell death is a critical event in the pathogenesis of all forms of diabetes. Type 2 diabetes is caused by the combination of acquired factors such as elevated circulating fatty acids as well as genetic factors. In this study, we show that the free fatty acid palmitate increases markers of endoplasmic stress and apoptosis in pancreatic β -cells. Carboxypeptidase E (CPE), an enzyme involved in the processing of insulin, was identified as the major down-regulated protein spot during palmitate-induced apoptosis using Cy-dye 2D gel proteomics in both the MIN6 β -cell line and human islets. Using MIN6 cells treated with 1.5 mM palmitate complexed to BSA (6:1), a significant decrease in total carboxypeptidase E protein was confirmed through Western blots. The decrease in CPE was seen in the presence of palmitate at both low and high glucose and was not affected in high glucose alone or with the ER-stress inducer, thapsigargin. Palmitate-induced changes in carboxypeptidase E were present after 2 hours, while CHOP, a marker of ER-stress, was not expressed until after 6 hours of incubation, suggesting that the decrease in CPE occurs before ER-stress. This finding, together with experiments using protein synthesis inhibitors and RT-PCR suggested that CPE was likely regulated at the post-translational level. Treatment with the non-metabolizable palmitate, 2-bromopalmitate, did not decrease CPE expression and delayed β -cell death. Addition of the L-type Ca^{2+} channel blocker nifedipine to palmitate-treated MIN6 cells restored CPE protein levels, reduced ER-stress, and rescued β -cells from cell death. The calpain inhibitor E64D also reversed the palmitate-induced decrease in CPE, further implicating Ca^{2+} -dependent proteolysis pathways. This inhibitor however increased ER-stress on its own and did not prevent ER-stress or β -cell death induced by palmitate. Interestingly, islets from CPE mutant mice exhibited increased TUNEL labeling, suggesting elevated apoptosis *in vivo*. Isolated CPE-deficient islets demonstrated increased CHOP and cleaved caspase-3 levels compared to control mice. The effects of palmitate on ER-stress and apoptosis were not additive to those of CPE deficiency. On the other hand, β -cells over-expressing CPE were resistant to palmitate-induced ER-stress and apoptosis. Together, these results show that palmitate markedly lowers CPE protein in the cell in a Ca^{2+} -dependent manner, potentially through Ca^{2+} -dependent proteases, such as the calpains. In addition, a lack of CPE appears to increase the susceptibility of the β -cell to CHOP induction and apoptosis. Conversely, over-expression of CPE protects β -cells from ER-stress and apoptosis induced by palmitate. These findings suggest that CPE represents a novel link between hyperlipidemia and β -cell death in diabetes.

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LIST OF ABBREVIATIONS

ALLM	N-Acetyl-Leu-Leu-Met-CHO
ATF-6	Activating Transcription Factor 6
ATP	Adenosine Triphosphate
BSA	Bovine serum albumin
Ca ²⁺	Calcium
[Ca ²⁺] _i	Intracellular calcium concentration
CHOP	C/EBP-homologous protein
CPE	Carboxypeptidase E
CPT-1	Carnitine palmitoyl transferase 1
DMEM	Dubelcco's modified Eagle medium
E64D	(<i>trans</i> -epoxysuccinyl-L-leucylamido (4-guanidino)-butane
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FFAs	Free fatty acids
GAD	Glutamic acid decarboxylase
GADD 153	Growth-arrest and DNA damage-inducible gene 153
GPR40	G-protein coupled receptor 40
HG	High glucose
IP3	Inositol 1,4,5-trisphosphate
IPGTT	Intraperitoneal glucose tolerance test
Ire-1	Inositol-requiring enzyme 1
Kir6.2	Inwardly rectifying potassium channel 6.2
LADA	Latent automimmune diabetes in adulthood
LC-CoA	Long chain coenzyme A
LG	Low glucose
MEM	Minimal essential medium
MIN6	Mouse insulinoma 6
PBS	Phosphate buffered saline
PC	Prohormone convertase

PI	Propidium iodide
PERK	Pancreatic ER kinase (PKR)-like kinase
PPAR γ	Peroxisome proliferator-activated receptor γ
RIA	Radioimmunoassay
RPMI 1640	Roswell Park Memorial Institute 1640
T2D	Type 2 Diabetes
UPR	Unfolded protein response
XBP-1	X-box binding protein-1

Acknowledgements

The past couple years have flown by in the blink of an eye. And although the time here was short, these two years have shaped me more as an individual than I ever could have imagined. Upon arriving in Vancouver back in September 2004, I met the people who would soon become my family and teach me so much about life and the sciences.

My supervisor, Dr. Jim Johnson, has been not only been an inspirational mentor, but a friend as well. He is an incredibly intelligent individual whose hard work, dedication, and quest for balance have made him successful in all aspects of life. I have learnt so much from him about critical thinking, asking important questions, and looking at problems from all angles. His passion for science is inspirational and has, in turn, driven me to search for my own passions. And while consistently being academically supportive, Jim has always been emotionally available -ready to give advice or a laugh when it was needed most. Plus he put up with my stubbornness, which is not an easy thing to do. Thank you Jim for giving me a chance.

My lab-mates, Tatyana Kalynyak, Grace Li, Emilyn Alejandro, Vardit Dror, Jen Beith, Dan Luciani, and Betty Hu have been an amazing source of information and have been my family for the past couple years. They have always been ready to help me with my experimental follies, as well as provide expertise and advice on the direction of my project. In addition, thanks to my undergraduate lab-mates, Peter Ao, Ting Yang, Matt Frey, Yaryna Bychkivska, May Tee, and Carla Braganini for all their help, support, and particularly their love for parties and food! I would like to say a special thanks to Emilyn for being my first friend in Vancouver and for all her support and guidance both personally and academically. Thank you to Grace for her help not only with my animals, but for being a good friend and great bus-buddy. And thank you to Tatyana for her help in molecular biology and for sharing her life experiences with me. These three women made up my family for the first year of my life in Vancouver and helped me grow not only as a student, but as a person as well. I have had an amazing and invaluable experience working in the Johnson lab and I have all of these individuals to thank for that. All the best to every one of you!

My colleagues and friends have made UBC my second home. Thanks to Tim Kieffer, Chris McIntosh, and Bruce Verchere for their guidance and knowledge. Their ability to see the big picture while still examining small details is something I strive for. Thanks to the members of the Kieffer and the McIntosh labs for their experience and pointers. Thanks to my fellow graduate students/post-docs who have always shared my love for beer, bowling, and baseball. Thanks in particular to Amy Olver for her patience and understanding. Her quiet yet powerful presence has always led to self-reflection.

And finally my parents, sister, grandmother and my husband Dave, whose love and support has always pushed me to believe in myself. Their ability to make me laugh, smile, and relax has made me a more balanced person. Thanks especially to Dave for putting up with the long nights, the sometimes-boring weekends, and willingness and selflessness to move to Vancouver so I could achieve my goals.

1.0 Introduction

Type 2 Diabetes, formerly known as non-insulin dependent diabetes mellitus, is characterized by an inability to control glucose homeostasis by inadequate insulin secretion and/or decreased insulin sensitivity. This disease is rapidly becoming widespread. The prevalence of diabetes is currently approximately 5% of the Canadian population. Alarmingly, it has been predicted that the global prevalence of type 2 diabetes, particularly in developing nations, will roughly double from 171 million in 2000 to 366 million in 2030 (1). Furthermore, type 2 diabetes is becoming more common in children and young adult populations (2). This poses serious health risks for these young individuals, who will spend the majority of their lives battling not only the pathologies of type 2 diabetes, but also the diseases associated with it. While the precise cause of type 2 diabetes remains unknown, it is likely related to a combination of obesity and genetic predisposition. Studies have shown that 45-80% of young individuals diagnosed with type 2 diabetes have at least one diabetic parent and 75-100% of those individuals have a first or second degree relative with diabetes (3, 4). Heredity may increase the risk of developing type 2 diabetes, but environment also plays a key role in determining the development and progression of this disease.

1.1 The role of the environment and diet in the progression of Type 2 Diabetes

Over the past few decades, global diets have been changing toward the "Western" diet. This diet is generally high in saturated fatty acids and low in carbohydrates (5). Accordingly, the increase in these diet patterns has correlated with an increase in diet-related diseases such as type 2 diabetes and many associated conditions including hypertension, stroke, and cardiovascular disease (6). The most common saturated fatty acids found in these diets are palmitate and stearate, which are mainly found in lard, mutton, and beef (7). Epidemiological evidence has suggested that insulin resistance, along with hyperinsulinemia, is linked to the consumption of high amounts of saturated fatty acids (8-12). Conversely, an increase in unsaturated fatty acids has been shown to reverse these effects (13).

Research has shown that high-fat diets and obesity are positively correlated with an increase in circulating plasma fatty acids levels as well as insulin resistance (14, 15). These findings are in agreement with studies on high-fat fed rats, where an increase in fat consumption led to an increase in fat deposition in the peripheral tissue, particularly the skeletal muscle (16).

This accumulation of fat correlated with an increase insulin insensitivity and hyperinsulinemia (17). While an increase in the circulating levels of fats has been correlated with diabetes in *in vivo* studies, *in vitro* evidence also supports the idea that elevated levels of free fatty acids contribute to the pathogenesis of type 2 diabetes by impairing of insulin action and causing β -cell dysfunction (10, 18-21). Elevated levels of plasma free fatty acids (FFAs) in diabetes (18, 22) are also associated with β -cell apoptosis (23, 24), thereby decreasing the survival and number of insulin producing cells. And while a reduction in insulin secretion, either due to a decrease in β -cell mass or a decrease in β -cell function is detrimental, the deposition of fats into adipose tissue and skeletal muscle makes these target tissues insensitive to the insulin that is already present in the body. A reduction in the ability of these target tissues to respond to insulin, termed insulin resistance, worsens hyperglycemia and perpetuates the progression of diabetes. Together, these studies demonstrate that diets high in fat, likely saturated fats, not only cause obesity but can worsen insulin resistance, lead to a reduction in β -cell mass and promote the pathogenesis of type 2 diabetes.

1.2 Proposed pathogenesis of Type 2 Diabetes

Due to the adverse effects of high-fat diets on obesity and β -cell function, many research groups have examined the role of free fatty acids (FFAs) on β -cell survival *in vitro*. After years of research, it has become clear that while FFAs may lead to increased β -cell apoptosis, not all FFAs have the same effects on β -cell survival. *In vitro* studies, including preliminary studies conducted by J.D. Johnson (Figure 1), have shown that saturated fatty acids, more often than unsaturated fatty acids, lead to β -cell death (13, 25, 26). In fact, addition of unsaturated FFAs to culture medium has been shown to reverse the deleterious effects of saturated FFAs on cell survival (13, 26). Since increased levels of triglycerides derived from saturated fatty acids have been associated with type 2 diabetes (27), an appreciable amount of research has focused on the effect of saturated fatty acids on β -cell death.

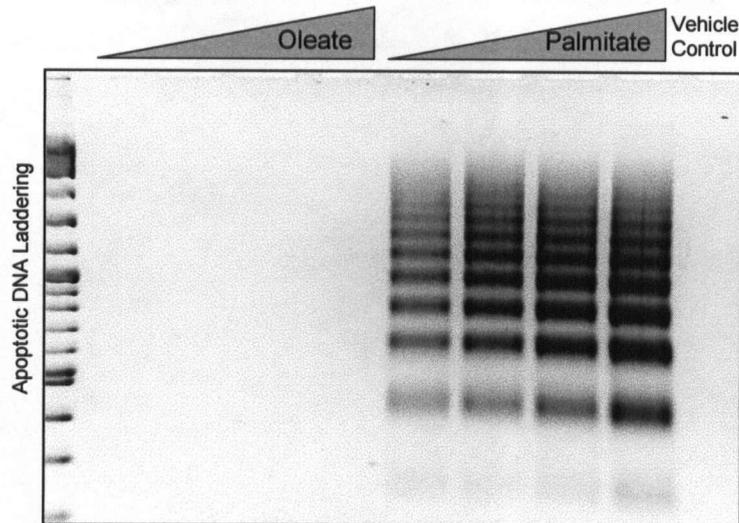


Figure 1. DNA ladders in 24-hour FFA-treated MIN6 cells. MIN6 cells were treated with increasing concentrations of oleate or palmitate, and show evidence of apoptosis through DNA laddering in palmitate-treated cells, but not in oleate-treated cells.

It has been established for years that the loss of functional β -cells is an essential event in the development of type 1 diabetes (28, 29), but the role of β -cell loss in the pathogenesis of type 2 diabetes has been less well defined. In recent years, however, it has become apparent that β -cell apoptosis is a critical event in the progression of type 2 diabetes, potentially exacerbated by dietary saturated fatty acids (30, 31). While in the early stages of the disease an initial compensatory increase in pancreatic β -cell mass and insulin secretion occurs (32), ultimately a decrease in β -cell mass arises in the late stages of type 2 diabetes (3). This leads to an inability to control plasma glucose levels. And though poor diet and genetics may be the underlying *causes* of β -cell death and type 2 diabetes, the precise *mechanism* of this reduction in β -cell mass has remained elusive. One possibility is that β -cell function becomes exhausted and the end result is cell death. This growing inability to control blood glucose levels due to a lack of β -cells may eventually lead to the complications associated with diabetes, such as cardiovascular disease, kidney disease, peripheral vascular diseases, vision impairment and an increased risk of stroke (6, 7).

1.3 Fatty acid metabolism and its toxic effects

Although excessive fatty acids may have detrimental consequences, they are essential cellular building blocks. Fatty acids form the foundation for cellular membranes and are required for sterol and hormone synthesis. In addition, they provide an important source of energy for basic cellular functions. In the body, fatty acids circulate through the blood bound to various proteins, including serum albumin. Once the fatty acids have reached their target destination, they dissociate from their carriers to cross the plasma membrane of various cell types, most commonly adipocytes and myocytes (7, 14, 21, 33-35). In addition to simple diffusion across the plasma membrane, fatty acids can also gain access to the cell with the help of fatty acid binding proteins and transporters. However, it remains controversial which pathways take precedence.

Under basal conditions, long chain free fatty acids, such as palmitate, are converted to long chain-CoA (LC-CoA) by acyl CoA synthase found in the endoplasmic reticulum. These complexes are then transported to the mitochondria by carnitine-palmitoyl-transferase-1 (CPT-1) where β -oxidation takes place to produce acetyl-CoA (36), which is also a product of glucose metabolism through the pyruvate dehydrogenase reaction. Essentially, acetyl-CoA is the hub where fatty acid and glucose metabolism meet to enter the citric acid cycle. Under hyperglycemia, however, it has been proposed that an increase in citrate from mitochondrial glucose metabolism causes the accumulation of malonyl CoA in the cytosol via ATP citrate lyase and acetyl CoA carboxylase (37). The increase in malonyl CoA inhibits the function of CPT-1, leading to an accumulation of LC-CoA in the cytosol (23). These findings align with other studies on obese and type 2 diabetic subjects, where increases in muscle lipid have correlated with increases in malonyl-CoA concentrations and decreases in CPT-1 activity (37, 38). The implications for the rise in LC-CoA are not well understood, but have been associated with the toxic effects of FFA, widely known as lipotoxicity.

While some research groups have focused on the role of fatty acid metabolism in inducing apoptosis, other groups (39-43) have focused on understanding the signalling pathways involving the recently discovered free fatty acid G-protein coupled receptor, GPR40 (42). GPR40 is activated by medium and long-chain fatty acids and is preferentially expressed in the plasma membrane of the β -cell. Furthermore, it has also been proposed to be necessary for insulin secretion (41, 44, 45). Upon activation via FFA, GPR40 leads to the phospholipase C-mediated production of IP₃ and diacylglycerol. IP₃ binds to its receptor on the ER, promoting the release and rise of Ca²⁺ in the cytosol, allowing for the secretion of insulin (40, 44). Interestingly,

it has been shown previously that high-fat fed mice lacking β -cell GPR40 are protected from the glucose intolerance that is developed in wild-type mice, while over-expression of GPR40 led to decreased β -cell function and diabetes (40). While there is evidence supporting the interaction of fatty acids with GPR40 to induce insulin secretion, paradoxically, it was recently shown that islets from GPR40 knock-out mice are not protected from lipotoxicity (43). This suggests that GPR40 is not involved in lipid associated cell death. And although our understanding of β -cell apoptosis is growing, it is clear that the molecular pathways leading to diabetes-related β -cell death remain elusive.

Another comparatively new focus in the field of FFA research is the role of FFAs on the endoplasmic reticulum. A mounting body of research has shown that β -cell apoptosis in the presence of the FFA, palmitate, is accompanied by an increase in endoplasmic reticulum stress (ER-stress) (23, 46-49). Preliminary studies by J.D. Johnson have confirmed these findings. Human islets exposed to 250 μ M palmitate for 24 hours showed an increase in the protein expression of C/EBP-homologous protein (CHOP), a marker of severe ER stress (Figure 2). In MIN6 cells, ER-stress was induced as expected by thapsigargin and cyclopiazonic acid (Figure 3), which inhibit the ER sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase pump. Together these finding imply that palmitate-induced apoptosis is mediated in part by ER stress.

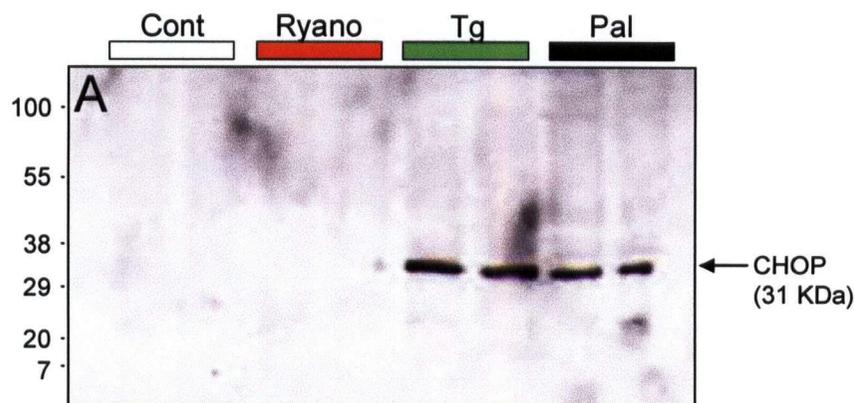


Figure 2. ER-stress in human islet cells. Twenty-four hour incubation of human islet cells in 250 μ M palmitate (Pal) or 1 μ M thapsigargin (Tg), a known inducer of ER-stress increased expression of the ER-stress marker, CHOP.

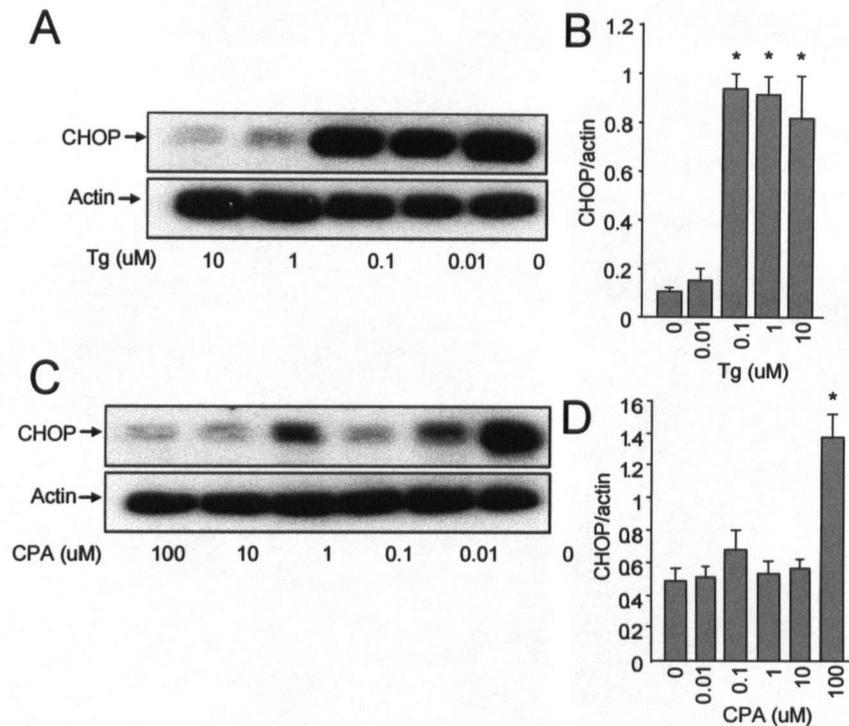


Figure 3. The effects of thapsigargin and cyclopiazonic acid on ER-stress in MIN6 cells. MIN6 cells (n=3) were treated for 24 hours with increasing concentrations of (A) Thapsigargin (Tg) or (C) Cyclopiazonic acid (CPA). (B, D) Densitometric analysis of (A) and (C). * denotes a significant difference from the control ($P < 0.05$).

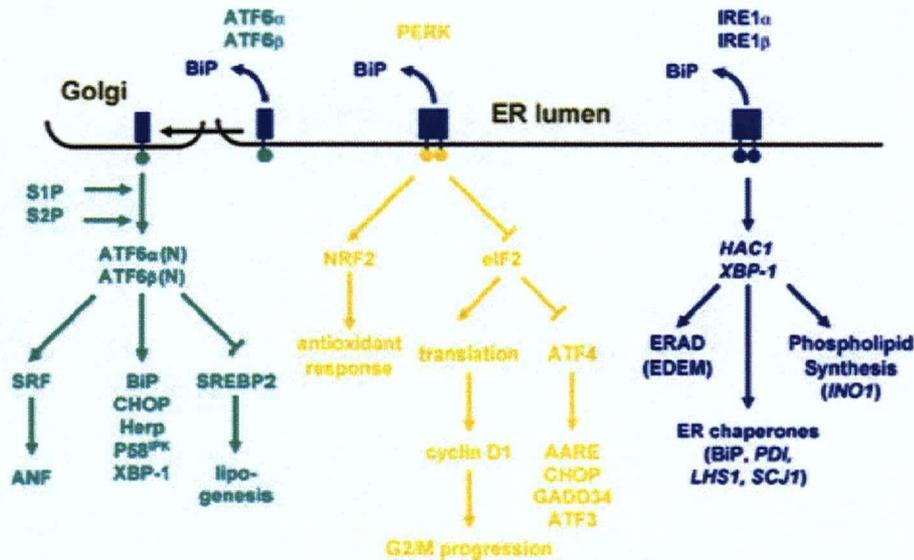
1.4 ER-stress in β -cell apoptosis

Protein synthesis, as well as protein folding and assembly, requires the involvement of the endoplasmic reticulum (ER). Protein translation occurs in the ribosomes on the rough endoplasmic reticulum, while in the lumen of the smooth ER, translated proteins undergo post-translational protein modifications such as *N*-glycosylation and the formation of disulfide bonds. Furthermore, the lumen is also the site where proteins are folded into their native three-dimensional conformation by chaperone proteins. Optimal ATP and Ca^{2+} levels are necessary for protein folding, while oxidizing capabilities are necessary for disulfide-bond formation (50). Due to the vast array of duties performed in the ER, the ER is very sensitive to disturbances in its environment. Therefore, to ensure the production of functional proteins is occurring, there are several checkpoints that a secreted protein must reach before it is ready to be released from the ER. In the first step of quality control, chaperone proteins such as calreticulin, calnexin, ERp57, and BiP examine the newly synthesized protein for exposed hydrophobic, unpaired cysteine residues, unformed disulfide bonds and the formation of aggregates to ensure the protein is

folded into its native conformation (50). Misfolded proteins are retained in the ER until properly folded or until they are forced to the cytosol where they are tagged with ubiquitins for degradation by the proteasome (51). The second step in protein quality control is cell specific and pertains to the retention of the folded protein in the ER and the timing of its release (50). Premature release of a protein may lead to a cascade of events ranging from poorly formed membranes to apoptosis, thus proper cellular function is highly dependent on the functionality of its proteins.

The concentration of total protein in the ER is estimated at approximately 100 g/l (52), a concentration that exceeds that of the ER chaperones. This high concentration of protein can lead to an unmanageable workload for the chaperones that modify and fold new proteins into their native state. In order to circumvent exhaustion of the chaperones, the ER evolved an unfolded protein response (UPR) that balances the concentration of newly synthesized protein in the ER with the folding capabilities of the chaperones. Under normal conditions, several proteins including the activating transcription factor 6 (ATF6), pancreatic ER kinase (PKR)-like kinase (PERK), and inositol-requiring enzyme 1 (IRE1) remain situated in the ER membrane anchored by chaperone protein BiP/Grp78. During cellular perturbations, such as low glucose, changes in cytosolic or luminal Ca^{2+} , or overproduction of proteins, the environment of the ER lumen becomes compromised. This situation is referred to as ER-stress and the ER reverts to the unfolded protein response (UPR) as a protective mechanism (53-56). Initially during the UPR, chaperones such as BiP/Grp78 sense an accumulation of unfolded proteins in the ER and release each of the UPR proteins from the membrane. Upon release, activated PERK phosphorylates elongation initiation factor 2 α (eIF2- α) halting the translation of non-essential proteins. ATF-6 cleavage produces cytosolic bZIP-containing fragments that migrate to the nucleus to up-regulate genes encoding proteins involved in the UPR (51). While the goal of the UPR is to decrease the translation of general cellular proteins, the up-regulation of UPR proteins aids in the folding of misfolded proteins in the ER. Upon activation via phosphorylation, IRE1 reduces the cell's potential to further translate proteins by recognizing specific mRNAs by their location and sequences and degrading them. While involved in mRNA degradation, IRE1 also splices XBP-1, a UPR protein, targeting it to the nucleus (55). The nuclear targeting of the XBP-1 transcription factor leads to the increase in UPR related gene transcription, including up-regulation of its own expression.

There are three arms of pathways involved in the unfolded protein response. The initiator proteins ATF6, PERK, and IRE1 lead each of these pathways (Figure 4).



Schroder M. and Kaufman R.J. 2005. *Mutation Research*. 569: 29-63

Figure 4. Overview of ER-stress pathways. There are three arms in the pathways involved in the unfolded protein response. The initiator proteins ATF6, PERK, and IRE1 lead these pathways and remain situated in the ER membrane anchored by chaperone protein BiP/Grp78 during normal conditions. During cellular perturbations each of the UPR proteins is released from the membrane. Upon release, activated PERK phosphorylates eukaryotic initiation factor 2 α (eIF2- α) halting the translation of non-essential proteins. ATF-6 cleavage produces cytosolic bZIP-containing fragments that migrate to the nucleus to upregulate genes encoding proteins involved in regulating the UPR. And finally, upon activation via phosphorylation, IRE1 reduces the cell's potential to further translate proteins by recognizing specific mRNAs by their location and sequences and degrading them.

The activation of these proteins leads to the induction of three separate pathways, all involved in coping with ER-stress; and the purpose of the UPR is to protect the cell from the deleterious effects of misfolded proteins. However, in situations of prolonged ER-stress, the compensatory mechanisms allowing the cell to survive become exhausted. As a result, the ER-stress pathways that once served to protect the cell activate proteins involved in inducing apoptosis. Chronic ER-stress leads to the activation of the transcription factor, C/EBP homologous protein (CHOP), also known as growth-arrest and DNA damage-inducible gene 153 (GADD153). CHOP is a small nuclear protein that heterodimerizes with transcription factors from the C/EBP family, which are involved in the regulation of genes involved in many physiological functions including cell differentiation and proliferation (56). Under normal conditions, CHOP is ubiquitously expressed at very low levels in the cytosol. During ER-stress, CHOP translocates to the nucleus, where upon binding to the C/EBP transcription factors, CHOP serves as a dominant negative inhibitor

to regulate growth and cell division (57, 58). While originally thought to be expressed during genotoxic events, it has become clear that the induction of CHOP is dependent on ER-stress, not DNA damage (57, 58). During ER-stress, CHOP expression occurs in all three signaling pathways of the UPR and is currently implicated in playing a role in apoptosis as its absence has been shown to prevent apoptosis. Embryonic fibroblasts from CHOP^{-/-} mice also demonstrated reduced apoptosis compared to wildtype fibroblasts when treated with tunicamycin, thapsigargin, and A23187, drugs known to induce ER-stress (60). Furthermore, disruption of the CHOP gene in the heterozygous Akita mice delayed the onset of diabetes by 8-10 weeks (61). CHOP knock-out mice subjected to bilateral common carotid artery occlusion exhibited decreased ischemia-associated apoptotic loss of neurons compared to wild-type mice (59). Together, these results suggest an important role for CHOP in ER-stress-mediated cell death under a variety of conditions.

Interestingly, aberrant ER-stress has been associated with many disease states. It has also been implicated in the pathogenesis of several rare forms of human diabetes including Wallcott-Rallison syndrome and Wolfram syndrome. In Wolcott-Rallison syndrome, affected individuals have inactivating mutations in the PERK mutation and display osteoporosis and skeletal mineral deficiencies (62, 63). Individuals with Wolfram syndrome are deficient in WFS-1 protein, which leads to ER-stress, impairments in cell cycle progression, and the development of diabetes (64, 65). In animals, the Akita mouse is a model of pancreatic β -cell specific ER-stress and insulin dependent diabetes. Its genetic mutation in the insulin 2 gene leads to the lack of a disulphide bond between the insulin A and B chains and results in insulin misfolding (65, 66). This change in insulin conformation leads to the induction of proteins involved in the UPR, including CHOP. The Akita mouse is consequently hyperglycemic, exhibits reduced β -cell mass and at approximately 3 weeks of age, develops diabetes. Thus, the maintenance of proper ER function is therefore critical not only in the survival of specific cell types, but also in the maintenance of overall health and the prevention of disease.

1.5 Ca²⁺ in FFA-induced ER-stress and β -cell apoptosis

While diseases have been linked to the ER-stress response, it has only recently been discovered that saturated FFAs such as palmitate initiate an ER-stress response. Although associations have been made, it remains unclear *how* these FFAs instigate ER-stress. Furthermore, exposure of cells to saturated FFAs leads to chronic ER-stress and eventually to

apoptosis. The link, however, between FFA-induced ER-stress and apoptosis has yet to be clarified.

Due to the importance of Ca^{2+} , not only as a second messenger, but also as a regulator of proper protein folding in the ER, much attention has been directed at Ca^{2+} and its role in ER-stress. Ca^{2+} is intricately involved in many cellular functions, and the timing and concentration of Ca^{2+} introduction play important roles in the specificity of Ca^{2+} signaling. Changes in cytosolic Ca^{2+} are manifested as oscillations, which vary in frequency, amplitude and sub-cellular localization. Ca^{2+} oscillations are supported by the entry of Ca^{2+} from the extracellular space and its mobilization from intracellular Ca^{2+} stores, primarily the ER. Drastic alterations in ER Ca^{2+} can be detrimental to the cell, resulting in ER-stress and potentially apoptosis, as observed in cells treated with thapsigargin and cyclopiazonic acid, ER-calcium channel inhibitors (Figure 3).

The effects of FFAs on cellular Ca^{2+} levels have been examined by several groups. Previous work on palmitate-induced apoptosis in cardiomyocytes has shown that alterations in intracellular Ca^{2+} flux enhanced apoptosis (67). Furthermore, L-type Ca^{2+} channels have also been implicated in palmitate-induced Ca^{2+} signals in both mouse pancreatic β -cells (68) and in the β -cell-line, the INS-1 cells (69, 70). Therefore, due to the important role of Ca^{2+} in protein folding and hormone secretion, it is plausible that disruptions in Ca^{2+} homeostasis mediated by free fatty acids could lead to ER-stress events and the subsequent apoptosis in exposed β -cells.

1.6 Calpain-10 in FFA-induced β -cell death

It is thought that increased levels of circulating FFAs affect the progression of type 2 diabetes by exacerbating β -cell death, but poor diet and obesity do not necessarily lead to type 2 diabetes in every individual. Thus, genetic susceptibility is thought to determine the likelihood that an individual might develop diabetes independent of obesity and elevated circulating free fatty acids. Genome-wide scans have implicated a number of candidate genes potentially involved in T2D susceptibility, including peroxisome proliferator-activated receptor (PPAR γ) (71-74), Kir6.2 (75-78), and CD38 (79-82).

Given the dependence of β -cell function and insulin secretion on intracellular Ca^{2+} , it is particularly interesting that one of the first type 2 diabetes susceptibility genes identified by positional cloning was calpain-10. Calpain-10 is a gene belonging to the calpain family of Ca^{2+} -dependent proteases and was first implicated as a diabetes susceptibility gene in 2000 (83). Upon

examination of various human populations, Horikawa *et al.* (83) discovered that certain groups of people, including Germans, British, and Mexican-Americans possessed variations within the calpain-10 gene introns that resulted in an increased vulnerability to type 2 diabetes. Additional studies on Pima Indians, a population with an increased susceptibility to type 2 diabetes, showed that while the calpain-10 gene was not associated with an increase in diabetes, individuals in this population had reduced calpain-10 mRNA in their skeletal muscle (84). Furthermore, a decrease in post-absorptive glucose and glucose turnover was also associated with decreased glucose oxidation in these subjects. Although, the precise consequences of genetic variation within the calpain-10 gene remain unclear, genetic variants in the calpain-10 gene have been found to be associated with elevated free fatty acids and insulin resistance (85). Calpain inhibitors have also been found to reduce insulin-stimulated glucose uptake into fat and skeletal muscle, as well as reduce glycogen synthesis in myocytes (86). Interestingly, previous work has implicated calpain-10 in palmitate-induced cell death in mouse pancreatic islets (87). Islets of calpain-10 knock-out mice treated with palmitate showed reduced apoptosis, as evidenced by DNA ladders, compared to the control (87). Furthermore, palmitate-induced apoptosis was increased in islets over-expressing calpain-10 (87). These findings not only implicate calpain-10 as a potential candidate in T2D susceptibility, but also imply a role for calpain-10 in pancreatic β -cells. Thus, calpains may play a role in the complex apoptotic pathways involved in the pathogenesis of type 2 diabetes and potentially link the genetic susceptibility with acquired stresses, such as FFAs.

1.7 An unbiased proteomic screen for palmitate targets in β -cells

As the amount of saturated fatty acids is increasing in the diets of the general population, research into the detrimental effects of these fats on β -cell survival is important and necessary. In order to further define the protein targets of saturated FFAs in the pancreatic β -cell, a proteomics screen was conducted at the Proteomics Core at Washington University (St. Louis, MO). In collaboration with this facility, the β -cell proteome was screened for proteins significantly altered by the saturated FFA, palmitate, during a 24-hour incubation period. Mass spectroscopy revealed that, in both human islets and MIN6 cells, carboxypeptidase E (CPE) was the most reduced protein spot on the gel, indicating a possible reduction in CPE protein expression in the presence of palmitate (Figure 5). CPE was reduced at several adjacent protein spots, suggesting the possibility of post-translational modifications. However, the spots that were decreased and identified as CPE had an apparent molecular weight corresponding to the approximate size of

full-length CPE, suggesting the total levels of the protein were likely altered. The potential effect of palmitate in reducing CPE protein prompted further investigation into CPE as a potential target of saturated FFAs and as a possible player in the pathogenesis of type 2 diabetes.

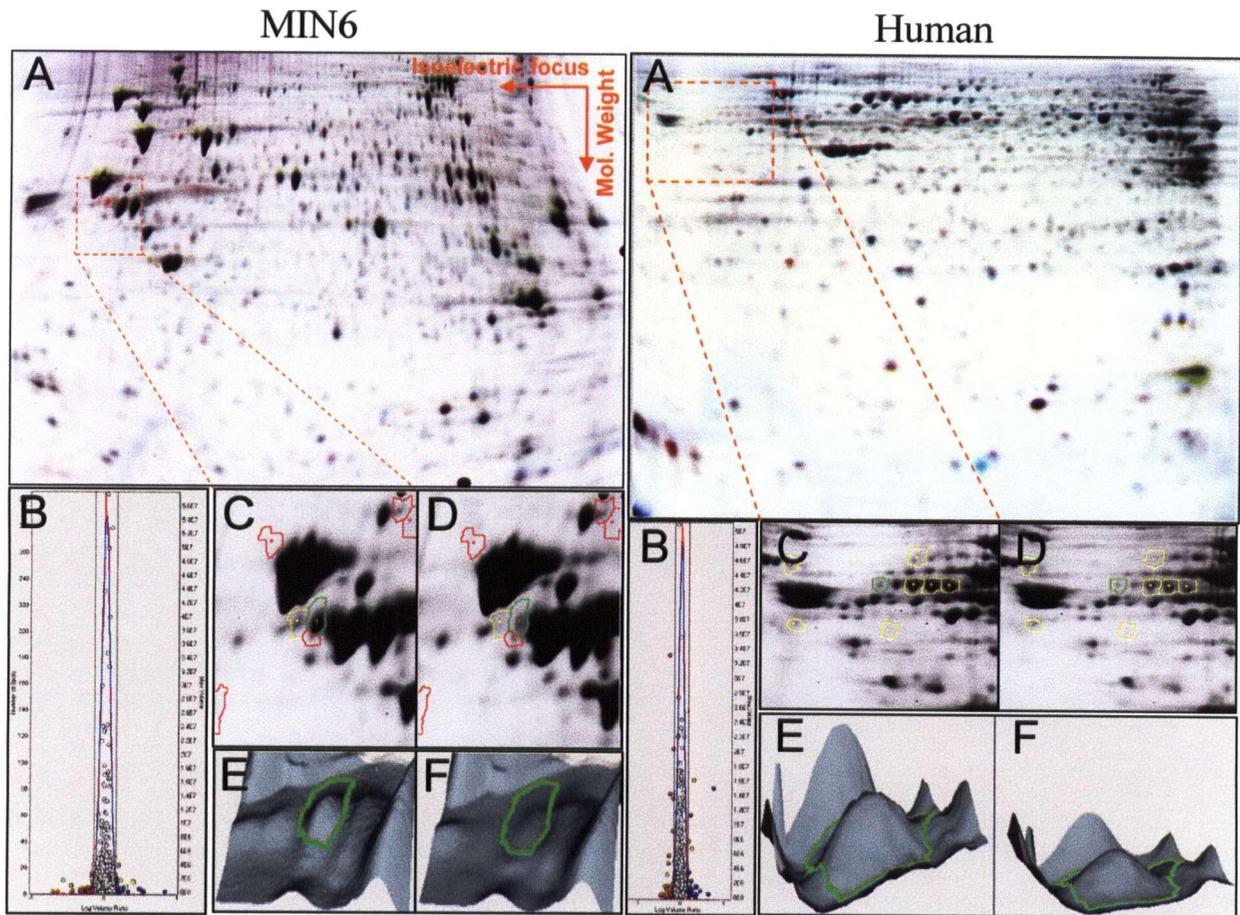
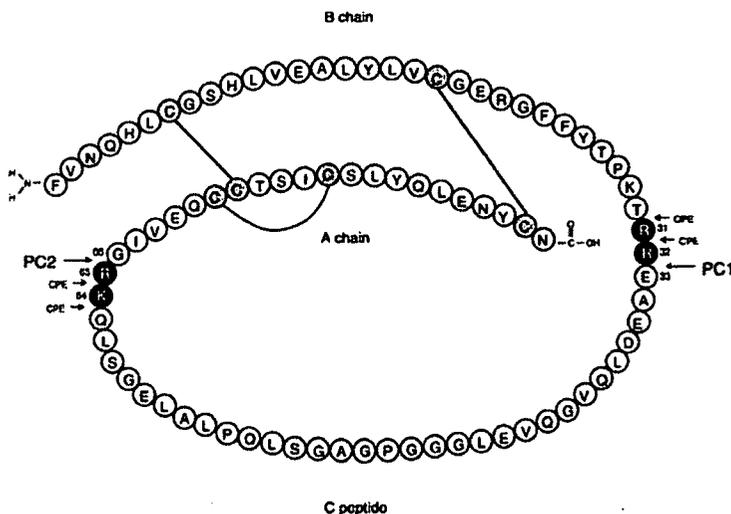


Figure 5. Cy-dye fluorescent analysis of palmitate-induced changes in the MIN6 and human islet cell proteomes (A) On these 12% 2D gels, red spots are decreased with 24 hours of 250 μ M palmitate, while blue spots are increased. **(B)** Normalization of all spot volumes across treatments reveals significantly different spots (>2 S.D.). **(C, D)** Close-up and 3D rendering **(E, F)** of the most altered spots in control (left) and palmitate-treated cells (right).

1.8 Carboxypeptidase E

CPE, also known as carboxypeptidase H or enkephalin convertase, is an enzyme that processes prohormones into biologically active hormones. CPE modifies des-64/65 proinsulin (or des-31,32 proinsulin) to produce mature insulin in the β -cell. CPE performs the final steps in insulin processing in the secretory granules by trimming away c-terminal dibasic amino acid residues (Figure 6).



Mackin R.B. 1998. Cell. Mol. Life Sci. 54: 696-702

Figure 6. Diagram of human proinsulin. Cleavage sites for PC1, PC2, CPE are indicated. Black circles represent the two pairs of basic amino acids used for proteolytic processing, while grey circles represent cysteine residues which participate in disulphide bonding.

CPE was originally discovered in adrenal chromaffin secretory granules as an enzyme that had a particular affinity for enkephalin containing peptides, which led to its original name, enkephalin convertase (88, 89). Enkephalin convertase (or CPE) was initially described as a carboxypeptidase B-like enzyme in reference to its ability to remove basic amino acids from the carboxyl terminus of trypsin fragments (90). However, enkephalin convertase activity displayed regional variations in the brain, which paralleled the variations in endogenous enkephalin levels, suggesting that this enzymatic activity was peptide specific (89). Previously thought to be primarily involved in enkephalin biosynthesis, enkephalin convertase activity was highest in the anterior pituitary, although enkephalin concentrations were highest in the posterior pituitary (91). Furthermore, similar carboxypeptidase activity was also found in rat insulinoma cells (92, 93).

These results suggested that while involved in enkephalin production, enkephalin convertase was involved in the production of other peptide neurotransmitters and hormones in addition to enkephalin. Carboxypeptidase E has now been localized to the brain and many endocrine tissues, including the pancreas and is responsible for the removal of basic c-terminal amino acids from a wide range of peptide hormone precursors.

CPE is a member of the metallocarboxypeptidase gene family, of which there are 13 known members. The metallocarboxypeptidase gene family is divided into two subgroups based on the conservation of 14 amino acid residues, including Zn²⁺-binding residues and those involved in substrate binding and catalytic activity (94). The members within each subgroup share 30-70% sequence similarities, while sharing only 15-25% sequence similarities with members from the other group (95). The first subgroup of the metallocarboxypeptidase gene family includes carboxypeptidases that are involved in the digestion of foods, such as the pancreatic carboxypeptidases A and B (CPA1, CPA2, CPB), as well as mast cell CPA and CPU (plasma CPB). Carboxypeptidase E belongs to the second subgroup, which includes CPM, CPN, CPD, CPZ. Three other proteins in this group, CPX1, CPX2, and AEBP1, lack carboxypeptidase activity but are thought to act as binding proteins (96-101). The metallocarboxypeptidases are responsible for many biological functions, including the digestion of food, and the processing of specific neuroendocrine peptide hormones and neurotransmitters (102-106). CPE can be differentiated from other cellular proteases with c-terminal exopeptidase activity by its distinctive enzymatic characteristics, such as its low pH optimum corresponding to the pH inside the secretory granules. Within the secretory granules, CPE is present in both soluble and membrane-associate forms. In its membrane-bound form CPE has been suggested to work as a sorting receptor, guiding hormones to the regulated secretory pathway (107-110). While this remains a debated topic, the importance of CPE in proper insulin production and secretion has been well established. The importance of CPE *in vivo* is supported by the pathology in the naturally occurring CPE mutant mouse. The CPE^{fat/fat} mouse has a spontaneous point mutation ⁷²⁹TCT to ⁷²⁹CCT within the coding region of the CPE gene located on chromosome 8, as shown in Figure 7.

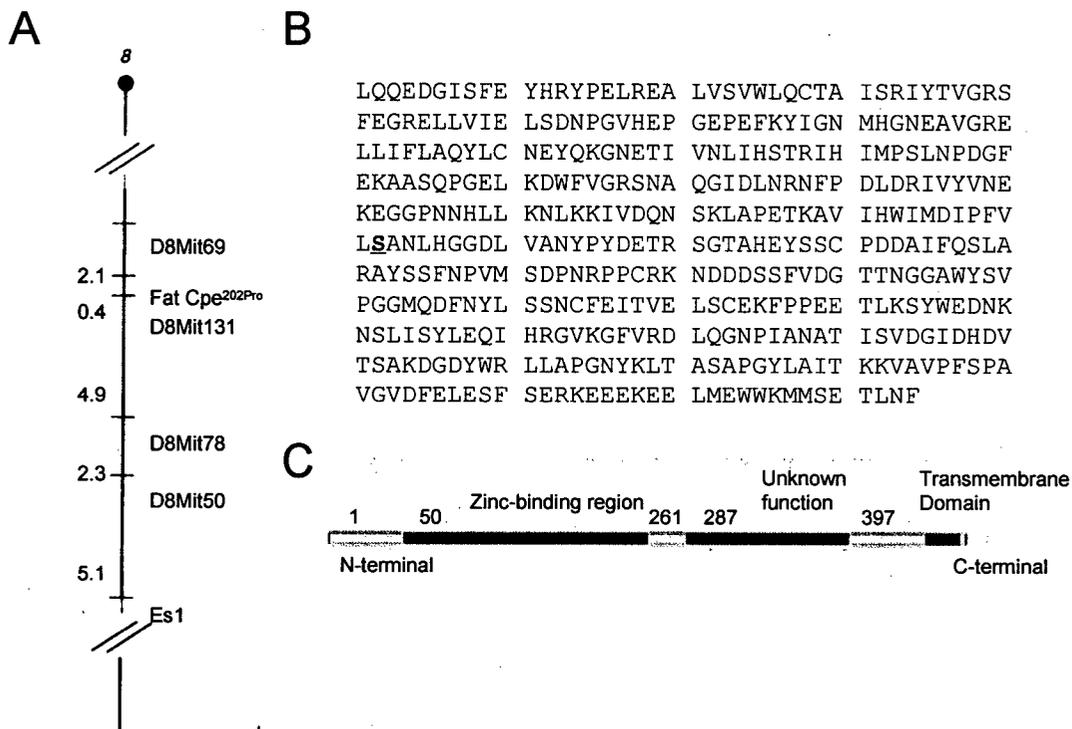


Figure 7. Location of the mutation in the mouse CPE gene and protein sequence. (A) Location of the CPE fat mutation on chromosome 8 of the mouse. Relative distances are located on the left. The most-likely gene order is: centromere, D8Mit69, fat, Cpe, d8Mit131, D8Mit78, D8Mit50, Es1. (B) Location of the serine to proline mutation on serine 202 (S) in the CPE^{fat/fat} mouse (Source: Naggert et al. 1995. Nature Genetics 10: 135-141). (C) Schematic of the CPE protein (Source: Transduction Laboratories, Lexington, KY).

While the mRNA is still translated, this genetic point mutation leads to a Ser202 to Pro alteration in the protein, leading to the inactivation of the enzyme and its subsequent degradation within hours of synthesis (111). Consequently, the CPE^{fat/fat} mouse shows a complete lack of CPE activity in the pancreas, pituitary, adrenal, brain, and intestines. Due to its role in processing mature insulin in the β -cell, approximately 80% of the insulin in the serum of CPE mutant mouse is composed of inactive proinsulin and their extended diarginyl forms (112). In addition to proinsulin, CPE is involved in the processing of many other hormones, including proinsulin, proglucagon, pro-gonadotropin-releasing hormone, procholecystokinin, pro-thyrotropin-releasing hormone, proopiomelanocortin, prodynorphin, proenkephalin, chromogranin A and B, secretogranin II, and progastrin. Therefore, the lack of CPE in these mice leads to prohormone-processing defects (102, 113, 114), resulting in many endocrine disorders including maturity-onset obesity, infertility, diabetes. In addition, the CPE^{fat/fat} mouse also possesses disproportionate levels of proinsulin: insulin, also known as hyperproinsulinemia (102, 113, 114). Furthermore, mice with a targeted deletion of CPE have an identical phenotype to the

CPE^{fat/fat} mouse, indicating that the *fat* allele is the functional equivalent of a null allele (113, 114).

Although CPE is generally considered to be a house keeping enzyme, a few studies have suggested additional roles for CPE. For example, CPE has been shown to play a key role in responding to ischemia in the brain. Rats exposed to transient global ischemia, followed by 8 hours of reperfusion showed increases in CPE mRNA and protein levels, which returned to normal following 24 hours of reperfusion (115). Similar experiments in the CPE^{fat/fat} mouse resulted in an increase in terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) positive cells in the cortex compared to wildtype animals (116), suggesting that CPE may be important not only in insulin processing and secretion, but in cell survival under stressful conditions as well.

1.8 Carboxypeptidase E in human type 2 diabetes

There is evidence from one study that CPE itself may be a human type 2 diabetes susceptibility gene. Mutations in CPE have been identified in Ashkenazi families with type 2 diabetes and these mutations have correlated with an early onset of type 2 diabetes in affected individuals (117). Furthermore, several research groups have also investigated the role of CPE (referred to as CPH in these studies) in the classification of diabetes in humans. While not abundant in all type 2 diabetics, autoimmune antibodies directed at CPE have been found in subjects with latent autoimmune diabetes in adulthood (LADA) (118). Paradoxically, these subjects did not exhibit autoantibodies directed at the more typical autoantigens GAD (glutamic acid decarboxylase) or IAA (islet autoimmune antibodies)(118). These findings suggested that CPE may serve as a marker for detecting a specific class of diabetes in humans.

1.9 Linking CPE and β -cell death in diabetes

Exposure of the β -cell to saturated FFAs is a stressful cellular condition consistently leading to the induction of ER-stress as well as apoptosis. However, despite these consistencies, it remains unclear how FFAs kill pancreatic β -cells. As saturated fatty acids are increasingly more common in the diet and have been linked to β -cell dysfunction and apoptosis, research examining the effects of these fats on β -cell death is critical. To gain insight this mechanism and the potential targets of saturated FFAs, two proteomics screens were conducted

that identified CPE as the most reduced spot in both palmitate-treated MIN6 cells and human islets. Notably, CPE is vital in the production of functional insulin, plays a key role in the β -cell secretory pathway, and has been shown to play a role in cell death in the brain. Furthermore, induction of ER-stress in palmitate-treated cells has been recognized but the mechanism by which this occurs has not been established. Increases in protein-folding demands have been associated with ER-stress (119-121) and CPE lies in the secretory pathway where it is involved in the processing of active insulin. Therefore, it is possible that a reduction in CPE could theoretically increase β -cell ER-stress. This could conceivably connect key features of type 2 diabetes, namely β -cell apoptosis and hyperproinsulinemia, to a major risk factor, elevated circulating FFAs.

The purpose of this study, therefore, was to investigate the possibility that the palmitate-associated decrease in CPE was involved in ER-stress, potentially due to a backlog of unprocessed proteins in the secretory pathway, ultimately leading to β -cell death. In this study, we aimed to: 1) confirm the effects of palmitate on CPE protein levels and establish the mechanisms by which palmitate decreases CPE and 2) determine the consequence of decreased or increased CPE on β -cell apoptosis.

2.0 Materials and Methods

2.1 Reagents

Chemicals were from Calbiochem (La Jolla, CA), Sigma Aldrich (St. Louis, MO), and BioRad (Hercules, CA). The following drugs were used, nifedipine, E64-D, diazoxide, thapsigargin (Sigma, St. Louis, MO) and ALLM (Calbiochem, La Jolla, CA). The following antibodies were used for Western blots: Carboxypeptidase E zinc binding region (Transduction Laboratories C61820; Lexington, KY), CPE n-term (Serotec AHP672, Kiddington, UK), CPE c-term (Chemicon AB5314; Billerica, MA), calpain-10 (Abcam Ab10820; Cambridge, MA), GADD153/CHOP (Santa Cruz F-168; Santa Cruz, CA), β -actin (Novus Biologicals NB600-501; Littleton, CO), cleaved caspase 3 (Cell Signaling 9664; Danvers, MA).

Table 1. Antibodies and dilutions used for Western blots.

Antibody (Ab)	Company	Source/Type	1°Ab dilution	2°Ab dilution
CPE	Transduction Laboratories C61820	Mouse/monoclonal	1:3000	1:6000
CPE n-term	Serotec AHP672	Rabbit/polyclonal	1:1000	1:3000
CPE c-term	Chemicon AB5314	Rabbit/polyclonal	1:1000	1:3000
CHOP	Santa Cruz F-168	Rabbit/polyclonal	1:500	1:1000
Cleaved Caspase 3	Cell Signaling 9664	Rabbit/monoclonal	1:1000	1:2000
Calpain-10	Abcam Ab10820	Rabbit/polyclonal	1:1000	1:2000
β -Actin	Novus Biologicals NB600-501	Mouse/ monoclonal	1:3000	1:6000

2.2 Cell culture and treatment of β -cells

2.2a MIN6 cell line

MIN6 cells, between passages 6 and 30, were obtained from stocks in Dr. Tim Kieffer's laboratory (Life Sciences Centre, UBC) under Material Transfer Agreement from Dr. Jun-ichi Miyazaki (Osaka, Japan). The MIN6 cells were cultured in 25 mM DMEM (Gibco, Grand Island, NY) with 2% penicillin-streptomycin (Gibco, Grand Island, NY) and 10% fetal bovine serum (Gibco, Grand Island, NY). Prior to treatment, MIN6 cells were seeded on 12 well culture plates (BD Falcon, Franklin Lakes, NJ) to ensure a final treatment confluency of approximately 80%.

For palmitate and 2-bromopalmitate treatments, palmitic acid (Nu-Chek Prep N-16-A; Elysian, MN) or 2-bromopalmitate (Sigma Aldrich, St. Louis, MO) were dissolved in 65 mM NaOH and complexed with 20% essentially fatty-acid free bovine serum albumin (BSA) stock solution (Sigma Aldrich, St. Louis, MO). The complex was added to DMEM containing 10% fetal bovine serum and 2% penicillin-streptomycin for a final molar ratio of 6:1 palmitate to BSA. A vehicle control was prepared in parallel containing all reagents, with the exception of palmitic acid. The medium was prepared fresh for each treatment.

Treatments with various drugs were prepared immediately before each experiment and were combined with palmitate media prior to their addition to the cells. Unless otherwise specified, cells were treated with drug for a 24 hour incubation period in MIN6 cells or for 48 hours in the case of human islets.

2.2b Human islets

Human islets were kindly provided by Dr. Garth Warnock (Ike Barber Transplantation Unit, Department of Surgery, Vancouver General Hospital) as part of the MSFHR-funded Centre for Human Islet Transplant and Beta-cell Regeneration. Islets were isolated from both men and women, ranging from 20 to 70 years of age without a history of diabetes. Human islets were cultured in 11.1 mM glucose RPMI 1640 (Gibco, Grand Island, NY) containing 10% FBS and 2% penicillin-streptomycin at 37°C under 5% CO₂.

2.2c Mouse islets

Following death by cervical dislocation and exposure of the mouse pancreas, the bile duct was ligated and the pancreas was inflated by injecting 2-5 mL collagenase (0.375 mg/mL, Sigma Type XI) in Hanks solution (137 mM NaCl, 5.4 mM KCl, 4.2 NaH₂PO₄, 4.1 KH₂PO₄, 10 mM HEPES, 1 mM MgCl₂, and 5 mM glucose) through the pancreatic duct. The pancreas was then removed and placed in a 50 mL BD Falcon (Franklin Lakes, NJ) tube containing 2 mL collagenase for 13 minutes in a 37°C waterbath. Following this incubation, the tube was shaken and the homogenous solution was then washed with Hanks solution containing 1 mM CaCl₂. The mixture was centrifuged at 1200 rpm for 30 seconds and the supernatant was aspirated. This step was repeated twice. The islets were then resuspended in RPMI 1640 (Gibco, Grand Island, NY) and poured into a pre-wet 70 µm nylon filter (BD Biosciences, Franklin Lakes, NJ). The

captured islets were rinsed into a dish with complete RPMI 1640 containing 10% FBS. The islets were then hand picked and counted and placed in 11.1 mM glucose RPMI 1640 containing 10% FBS and 2% penicillin-streptomycin. Islets were cultured at 37°C under 5% CO₂ overnight. Where applicable, islets were treated with 1.5 mM palmitate as previously described (Section 2.2a).

2.3 RT-PCR

Gene expression of palmitate-treated MIN6 cells was semi-quantitatively examined using reverse transcriptase PCR. Following 24-hour palmitate, RNA was isolated from MIN6 cells using RNEasy (Qiagen, Mississauga ON) according to the manufacturer's protocol. Following RNA isolation, cDNA was prepared using DNTPs (Invitrogen, Burlington ON), Superscript III (Invitrogen, Burlington ON), and Taq Polymerase (Fermentas, Hanover MD). PCR was performed using 35 cycles and primers for mouse CPE (Integrated DNA Technologies CPE fwd:13774680, CPE rev: 13774679) with the following sequences: Forward: 'TCC CTG TCG CAA GAA TGA CGA; Reverse: 'TGA AGG TCA CGG ACA AAC CCT. Cycle tests were performed on the primers to determine optimal primer annealing and amplification of gene products and to ensure that the amplification used was in the linear range.

2.4 Protocol for Western blot

Thirty to 40 µg of protein for MIN6 cells or 15 µg of protein for primary islets were combined with sample buffer containing sodium dodecyl sulphate (SDS) and the samples were boiled at 95° for 5 minutes and promptly put on ice. The total volume of each sample was loaded into the wells of the gel along with a protein ladder. Protein samples were run on a 12% SDS-acrylamide gel at 120 V. For 2 gels, the voltage used was 125 V. Following the gel run, a polyvinylidene fluoride membrane (Biorad, Hercules, CA) cut to the size of the gel was immersed in methanol for a few seconds to activate the membrane. The membrane and the two blot papers were then immersed in transfer buffer. The transfer was run at 22 V for 1.5 hours using a Trans-blot semi-dry transfer apparatus (Bio-Rad, Hercules, CA). Following the transfer, the membrane was blocked for 1 hour with a blocking solution, such as I-Block (Tropix, Bedford MA). After blocking, the membrane was incubated in the desired primary antibody in I-block for 1 hour at room temperature or overnight at 4°C. After the primary antibody, the membrane was

washed 3 times for 5 minutes each with I-Block. The secondary antibody was added to I-block in the appropriate dilution and probed for 1 hour at room temperature. After the secondary antibody, the membrane was washed 3 times for 5 minutes each with Wash II (1X PBS with 0.2% Tween 20). Chemiluminescent substrate (Amersham, Piscataway NJ) was added in a 1:1 ratio of reagent 1 and reagent 2 and incubated for 2 minutes. The membrane was wrapped in cling wrap and put in a developing cassette. In the dark room, a film was placed on the membrane, exposed for the desired time and developed.

2.5 Immunofluorescence of MIN6 cells

MIN6 cells were seeded onto glass coverslips for a desired confluency of 75% and incubated overnight. The MIN6 cells were incubated with the appropriate treatment and were fixed with 4% paraformaldehyde at room temperature for 10 minutes. Following subsequent incubations with glycine and phospho-buffered-saline (PBS), the cells were permeabilized for 10 minutes with 0.1% Triton-X and blocked with DAKO Protein Block Serum-Free (Dako, Carpinteria, CA) for 30 minutes. The procedures for the addition of primary and secondary antibodies have been described in immunofluorescent staining of tissue sections (Section 2.8c).

2.6 Real-time measurement of cell death in MIN6 cells

Use of a high-content imaging system, the Cellomics KineticScan (Cellomics Inc., Pittsburg, PA), allowed for the real-time measurement of cell death in MIN6 cells treated with palmitate, 2-bromopalmitate, and a variety of other drugs. Cell death was measured using propidium iodide, a dye that is unable to cross the plasma membrane in viable cells but is membrane-permeant in dying cells. Upon entry into a dying cell, propidium iodide binds to DNA and fluoresces brightly. Using the Cellomics reader in Dr. T.M. Underhill's laboratory (Life Science Institute, UBC), cell death was measured over the course of 24 hours. In preparation of these experiments, MIN6 cells were seeded to the desired confluency (approximately 80%) in a 96 well Cellomics Viewplate (Packard, Meriden, CT), centrifuged for 1 minute at 1000 rpm, and left to grow overnight. In a separate 96 well plate (BD Falcon, Franklin Lakes, NJ) without cells, the desired treatments to be added to the cells were prepared containing 2.5 ng/ μ l of light sensitive propidium iodide. Once the treatment plate was prepared, the plate containing the MIN6 cells was flicked upside down to remove medium. The contents of the treatment plate

were quickly transferred to the MIN6 cell plate using a multi-channel pipettor. The plate was then loaded into the Cellomics machine where cells were kept incubated at 37° at 5% CO₂. Using the Cellomics machine, fluorescence from propidium iodide was captured using for 4 capture fields every 30 minutes with the fluorescent microscope embedded in the machine. Data analysis was performed using a custom macro written by Dr. Dan Luciani in Microsoft Excel.

2.7 Over-expression of CPE in MIN6 cells

Two CPE plasmids, pcDNA22 (under the control of the CMV promoter) and pcDNA48 (under the control of the rat insulin promoter) were kindly supplied by Dr. L.D. Fricker. The pcDNA was transformed to 50 µl of thawed DH5α competent cells (Invitrogen, La Jolla, CA). Two nanograms of DNA were added to the cells and mixed gently by tapping. The mixture of DNA and cells were incubated on ice for 30 minutes. The cells were then heat-shocked for 1 minute at 42°C. The tubes were then placed on ice for 2 minutes and 950 µl of pre-warmed S.O.C. (Super Optimal Catabolite repression) medium (Invitrogen, La Jolla, CA) was then added to the tubes. The tubes were then incubated at 37°C for 1 hour. Following incubation, the tubes were spun at 10,000 rpm for 1 minute and the 20 µl of the resulting supernatant was spread onto a LB (lysogeny broth) plate containing 100 µg/ml ampicillin. The plates were left in the 37°C incubator for a few hours then flipped upside down overnight to avoid condensation. After growing overnight, the plates were stored at 4°C. To ensure the colonies grown contained the correct plasmids, one colony was scraped from each plate and placed into tubes containing 5 mL of LB broth and 12.5 µl ampicillin (Gibco, Grand Island, NY). The tubes were shaken at 37°C for 8 hours or overnight. Following incubation, 1 mL of this mixture was used to isolate DNA using the Qiagen mini-prep and the Qiagen protocol (Qiagen, Mississauga, ON). Once the DNA was isolated from the cells, a 1 in 50 dilution was used to measure the DNA. Using the quantified DNA, restriction digests were performed using EcoR1 and BamH1 restriction enzymes (New England Biolabs, Ipswich, MA) and 10X Tango buffer (New England Biolabs, Ipswich, MA) to confirm that the correct plasmid was being used. Once the identity of the plasmid was confirmed, the DNA was used for its transfection into MIN6 cells using Lipofectamine 2000 (Invitrogen, La Jolla, CA). To transfect the MIN6 cells, the cells were plated on 12 well plates (BD, Franklin Lakes, NJ) and the cells were placed in 1 mL of medium (as described in Section 2.2a). In 1.5 ml tubes, 25 µl of Opti-MEM (Invitrogen, La Jolla, CA) was

mixed with 1 μ l of Lipofectamine 2000 and incubated at room temperature for 5 minutes, while in another tube, 25 μ l of Opti-MEM was mixed with 1 μ g of cDNA from the desired plasmid or from an empty pcDNA 3.1 vector (Invitrogen, La Jolla, CA). The contents of the two tubes were mixed and incubated at room temperature for 20 minutes. This final mixture was added dropwise to each well containing the MIN6 cells and incubated for 4 hours. After the 4 hours, another 1 mL of medium was added to each well to further dilute the Lipofectamine 2000 solution. Following 18 hours of incubation, the Lipofectamine 2000 reagents were removed and the cells were passaged. Once seeded and left to attach overnight, the MIN6 cells were then ready for treatments.

2.8 *In vivo* studies

2.8a Intra-peritoneal glucose tests

Mice were starved overnight (approximately 16 hours), weighed, and blood glucose levels were measured from the tail vein using a BD glucose meter (BD Logic, Franklin Lakes, NJ). The mice were then injected in intra-peritoneal cavity with 18% glucose at 2 g/kg body weight. Blood glucose was then measured at six time points: 0, 15, 30, 60, 90, and 120 minutes.

2.8b Preparation of tissue sections

Mice were randomly selected for pancreatic tissue samples. Before sacrificing by cervical dislocation, mice were starved for 4 hours, weighed, and plasma glucose was measured. Blood was taken via heart puncture and coagulation was prevented with a heparin-saline solution. The blood was separated by centrifugation at 10,000 rpm for 10 minutes at 4°C and plasma was stored at -80°C. The pancreata were excised, weighed, placed in a histology cassette and incubated overnight at 4°C in a 4% paraformaldehyde solution for fixation. Following fixation, the pancreata were stored in 70% ethanol and brought to the Morphological Services laboratory (UBC, Department of Pathology and Laboratory Medicine) where they were sliced into sections 10 μ m thick and embedded in paraffin.

2.8c Immunofluorescent staining of paraffin embedded pancreas sections

Paraffin-embedded pancreatic sections were deparaffinized in xylene for 30 minutes. The sections were then re-hydrated for 20 minutes in 100% ethanol, followed by 10 minutes in 95% ethanol, and 10 minutes in 70% ethanol. The slides were washed in 1X PBS for 10 minutes on a shaker. In order to facilitate antibody binding, the slides were incubated in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) in the microwave at high power for 5 minutes. This procedure was repeated using fresh buffer 2 to 5 times depending on the tissue fixation. Following washes and antigen retrieval, the pancreatic sections were circumscribed using a hydrophobic pen (ImmEdge™) and allowed to dry. The sections were incubated with DAKO Protein Block Serum-Free (Dako, Carpinteria, CA) at room temperature in a humid chamber for 30 minutes. Removal of the blocking agent was followed by overnight incubation of the slides with the desired primary antibody, diluted in Dakocytomation Antibody Diluent (Dako, Carpinteria, CA). The slides were then washed 3 times in 1X PBS for 10 minutes on a shaker and then incubated for one hour in the dark with the desired secondary antibody diluted in Dakocytomation antibody diluent. The slides were washed again in 1X PBS 3 times and were mounted in VECTASHIELD Mounting Medium for Fluorescence with DAPI (Vector Labs, Burlingame, CA). Cover-slips were applied to slides and sealed with clear nail polish.

2.8d High-fat feeding of C57Bl6/J mice

To investigate the effects of a 16-week high-fat diet on CPE levels, 6 week old C57Bl6/J mice (n=7) were fed a “Western” diet (Harlan Teklad TD88137, Madison, WI) where 40% of the calories were derived from fat. Before feeding began, mice were weighed and given an intra-peritoneal glucose tolerance test (IPGTT). Mice were then randomly separated into a high-fat diet group and a chow diet group and fed for 16 weeks on that diet. Following the 16 weeks, the mice were once again weighed and challenged with an IPGTT. Following overnight fasting, the islets of these mice were isolated, cultured and used for Western blots.

2.8e TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) staining of paraffin embedded pancreas sections

Analysis of apoptosis in tissue sections was performed using an *in situ* cell death detection kit, fluorescein (Roche Applied Science, Laval, QC) according to manufacturer's instructions. Tissue sections were counterstained with DAPI present in the mounting medium (Vectashield, Burlingame, CA).

2.9 Statistical analysis

Data are presented as mean \pm standard error of the mean (SEM). Statistical analysis was performed using SigmaStat (Systat, San Jose, CA). For larger sample interactions, the Student-Newman-Keuls ANOVA was used. For differences between two samples, a t-test was used. $P < 0.05$ was considered to be of significant difference.

3.0 Results

3.1 Palmitate decreases total CPE protein levels in MIN6 cells and human islets

The apoptotic effects of palmitate on pancreatic β -cells have been established in many previous studies (13, 23, 25, 36, 48, 122, 123) and in preliminary experiments (Figure 1). However, it remained unclear exactly how palmitate elicits its pro-apoptotic effects on various cell types, including the pancreatic β -cell. A proteomics screen undertaken on MIN6 cells and human islets treated with palmitate for 24 hours indicated that CPE was the most reduced protein spot on a gel with over 2000 separated protein species. Since proteins are often represented in many locations on 2D gels due to post-translational modifications that change their mobility, it was important not to unambiguously interpret these findings as a decrease in the total levels of CPE protein. The present studies were undertaken to confirm the observation that CPE was altered in palmitate-treated β -cells and to examine the mechanism and consequence of this effect.

First, to establish the dose-dependence and glucose-dependence of this effect, MIN6 cells and human islets were treated with two different ratios of palmitate to BSA, at both high and low glucose (Figure 8A, C). The normal physiological ratios of free fatty acids to albumin are approximately 2:1, while in type 2 diabetic human subjects the ratio of fatty acids to albumin can approach 6:1 (124-126). CPE was significantly decreased in MIN6 cells using a 6:1 ratio of palmitate to BSA in both low and high glucose while a 2:1 ratio did not alter CPE protein expression (Figure 8B). Although, too few human samples ($n=2$) were analyzed to directly compare, this result suggests the possibility that human islets may be more sensitive to palmitate *in vitro* than MIN6 cells. To keep treatments consistent, remaining experiments used the 6:1 palmitate to BSA ratio. Furthermore, unless otherwise indicated, all experiments used an antibody that targets the zinc-binding region of CPE (Figure 8E). However, to confirm that the observed results were not epitope-specific and to examine other parts of the CPE protein, antibodies targeting the c-terminus and n-terminus were used to probe for CPE in MIN6 cells treated with 6:1 palmitate:BSA for 24 hours (Figure 8D, E). A decrease in total CPE protein was observed in both the c-terminus and n-terminus antibodies, indicating the effect of palmitate on CPE was not antibody or epitope specific. These results also suggest that the entire protein was decreased, rather than just a fragment of CPE.

We also considered the possibility that palmitate might alter the subcellular location of the CPE protein, which is normally found predominantly in secretory vesicles in addition to the Golgi and ER. Using fluorescence microscopy we examined the subcellular location of c-terminal CPE in palmitate-treated MIN6 cells, mouse islets and humans islets but did not observe marked differences (Figure 9). Similar results were seen with the antibody to n-terminal CPE. Together, these findings indicate that the primary effect of palmitate is to reduce the total levels of CPE protein in the β -cell.

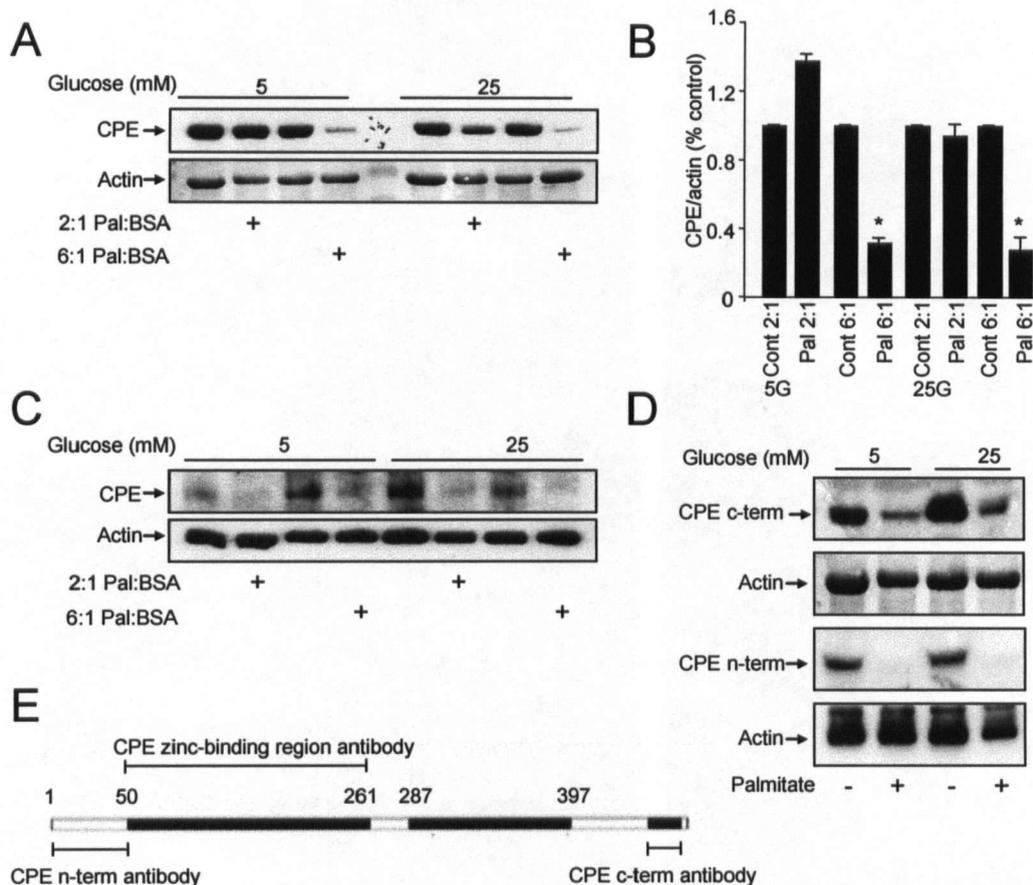


Figure 8. The effects of varying the palmitate:BSA ratio on MIN6 cells and human islets. (A, B) MIN6 cells treated for 24 hours with varying FFA ratios of palmitate to BSA in 5 mM or 25 mM glucose. MIN6 cells treated with 6:1 palmitate:BSA ratios show a 60% reduction in CPE expression at both low and high glucose ($n=4$). * denotes a significant difference from the control ($P<0.05$). No difference in CPE expression was observed between low and high glucose treatments. (C) Human islets treated for 48 hours with varying palmitate:BSA ratios of ($n=2$). (D) MIN6 cells treated for 24 hours with palmitate show a decrease in CPE using two different antibodies; one antibody targeted the c-terminus while another targeted the n-terminus, showing the reduction in CPE is not due to protein fragmentation. (E) Targets of the CPE antibodies on the CPE protein.

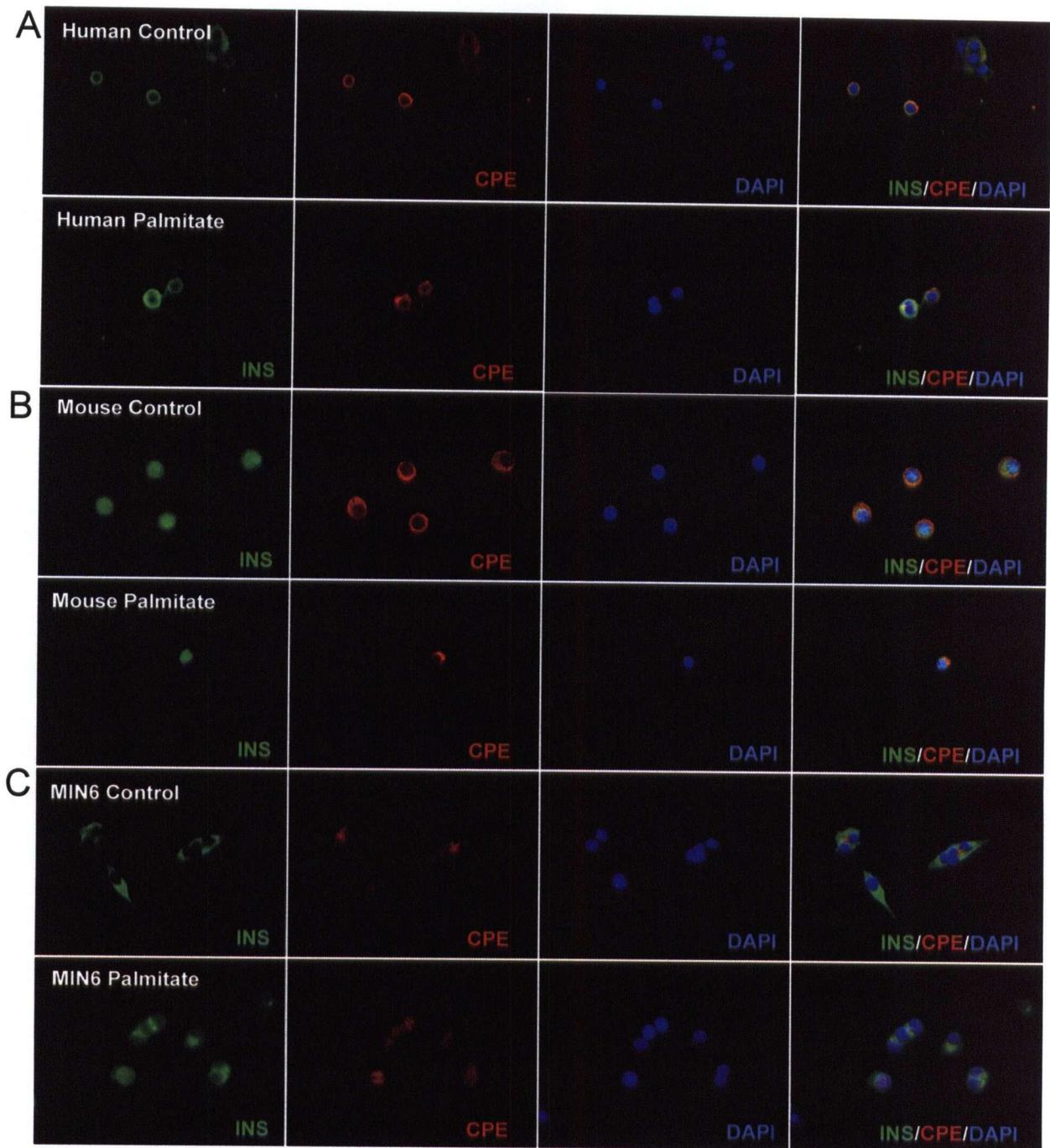


Figure 9. The effect of palmitate on CPE in different cells types. (A, B) Dispersed human and mouse islet cells and MIN6 cells (C) were palmitate-treated for 48 and 24 hours respectively and were triple-stained for insulin (green), c-terminus CPE (red) and DAPI (blue). Sub-cellular localization of CPE did not differ between palmitate-treated cells and control cells.

3.2 Time-course of palmitate-induced decrease in total CPE

Due to the apoptotic effects of palmitate, it was essential to determine whether or not the decrease in CPE protein was due to an increase in cell death. A 24-hour real-time measurement of cellular propidium iodide incorporation in palmitate-treated MIN6 cells indicated that a significant rise in cell death began at approximately 12.5 hours (Figure 10A). Cell death continued to rise steadily throughout the 24-hour observation period. Time course studies of palmitate-treated MIN6 cells indicated that a significant decrease in CPE protein was apparent at 2 hours, as shown by Western blot. ER-stress, as indicated by the induction of the CHOP protein, only showed a significant increase at 6 hours, with no effect at 2 hours (Figure 10B, C). Thus, the palmitate-dependent decrease in CPE occurred well before the increase in the ER-stress marker CHOP. To further investigate the relationship between CPE protein and ER-stress, MIN6 cells were treated with thapsigargin, a drug known to induce ER-stress by blocking the ER Ca^{2+} pump and depleting ER Ca^{2+} stores (Figures 2, 3). Following 24-hour incubation, total CPE protein was measured and found not to differ between thapsigargin-treated cells and the controls (Figure 10D). Together, these results further suggest that neither ER-stress nor apoptosis caused the decrease in CPE. These findings support the idea that the palmitate-induced decrease in CPE lies upstream of ER-stress and apoptosis.

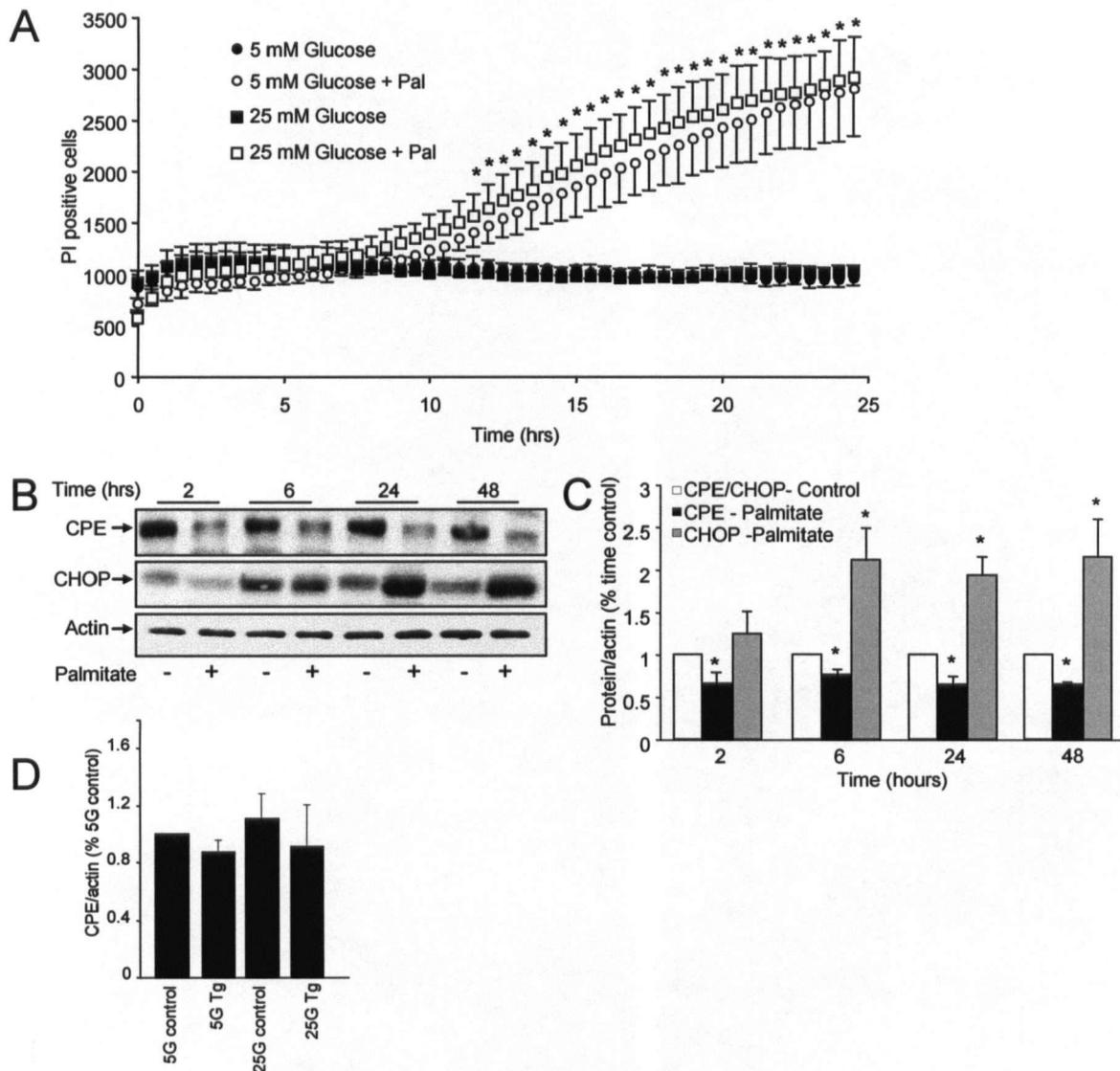


Figure 10. The effect of palmitate on the expression of CPE, CHOP, and β -cell death over time. (A) The effect of palmitate (Pal) on cell death in MIN6 cells at low (5 mM) and high glucose (25 mM) becomes significantly different from the control at 12.5 hours during a 24-hour treatment (n=3). * denotes a significant difference compared to the control ($P < 0.05$). (B) MIN6 cells incubated with palmitate at 25 mM glucose at various time points show a decrease in CPE as early as 2 hours (n=3). (C) CPE expression is significantly reduced by 40% as early as 2 hours and remains reduced during a 48-hour treatment. CHOP expression is not affected at 2 hours, but is significantly up-regulated by 2.25-fold at 6 hours and remains up-regulated during 48-hour treatments with palmitate. (D) Twenty-four hour incubation of MIN6 cells with 1 μ M thapsigargin (Tg) at low glucose (5G) and high glucose (25G) does not affect CPE expression, suggesting ER-stress does not down-regulate CPE production (n=3).

3.3 Post-translational effects of palmitate on CPE protein

To further explore the role of palmitate in CPE protein reduction, we tested whether the reduction in CPE was due to decreased CPE gene expression or protein biosynthesis. Cycloheximide is a drug that targets the activity of the 60S ribosome to inhibit protein translation. As previously observed, palmitate decreased total CPE protein. As expected, cycloheximide also decreased CPE protein synthesis (Figure 11A, B), suggesting that the half-life of the majority of the CPE protein is less than 24 hours. The co-incubation of palmitate and cycloheximide in all cases resulted in a further decrease in CPE expression compared to the effects cycloheximide-alone, although this did not achieve statistical significance. Assuming protein synthesis was completely blocked, these results suggest that perhaps the further decrease seen in these treatments was a result of palmitate targeting CPE for degradation. In addition, CHOP was also induced in the presence of palmitate, as previously seen. However, upon incubation of the MIN6 cells with cycloheximide alone and together with palmitate, CHOP expression was additionally decreased compared to palmitate and control treatments (Figure 11A, C). As cycloheximide is an inhibitor of protein biosynthesis, a global decrease in all cellular proteins, including CHOP, was expected. While this was the case, cleaved caspase 3 was significantly increased in cycloheximide-treated MIN6 cells and was further increased when cycloheximide and palmitate were combined. These results were expected as inhibition of protein synthesis is known to have adverse effects on cell survival.

We also examined the effect on palmitate on CPE steady-state mRNA levels. Reverse-transcriptase PCR of palmitate-treated MIN6 cells suggested that palmitate did not affect CPE mRNA either low or high glucose (Figure 11D, E). This indicated that it was unlikely that palmitate was acting on CPE at the level of gene transcription. Taken together with the rapid time course, these results suggest the possibility that CPE is targeted for degradation in palmitate-treated β -cells.

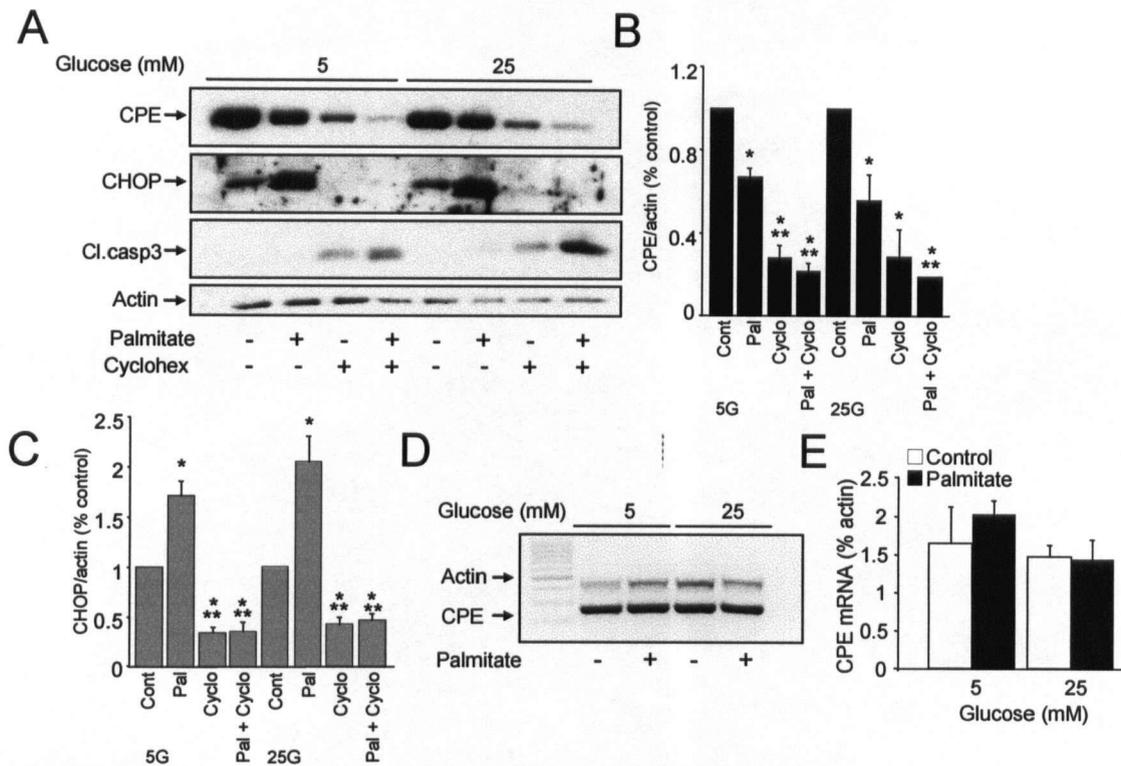


Figure 11. The effects of palmitate on protein biosynthesis and gene expression. (A) MIN6 cells were treated with palmitate (Pal) and 10 μ M cycloheximide (Cyclo) for 24 hours to examine their effects on CPE and CHOP expression (n=3). (B) At both low and high glucose, CPE protein was significantly reduced compared to the control by 30%, 70%, and 75% in palmitate-, cyclo-, and cyclo+pal-treated cells. No significant difference, however, was found between the cyclo- and cyclo+pal-treated cells. * denotes a significant difference from control; ** denotes a significant difference from palmitate treatment ($P<0.05$). (C) At low and high glucose, CHOP protein was upregulated by 1.75-fold in palmitate-treated cells compared to the control, while CHOP expression was significantly reduced in cyclo- and cyclo+pal-treated cells compared to palmitate and control cells. (D) RT-PCR of 24-hour palmitate-treated MIN6 cells show no differences in gene expression at either low or high glucose (n=3). (E) Densitometric analysis of (D).

3.4a Cellular palmitate metabolism is required to reduce CPE protein levels

Over the past several decades, it has generally been thought that the metabolism of FFAs is necessary to mediate their effects on target cells. However, the discovery of a medium to long-chain FFA-activated G-protein coupled receptor has presented a new mechanistic paradigm for understanding the actions FFAs (41, 45). The FFA G-protein coupled receptor, GPR40, is preferentially expressed in the β -cell and has been proposed to be necessary for insulin secretion (41, 45). In our experiments, through the use of the non-metabolizable palmitate homolog, 2-bromopalmitate, we aimed to determine whether palmitate metabolism was involved in the reduction of CPE and palmitate-induced cell death. If metabolism were required it would potentially argue against a role for signalling pathways downstream of GPR40.

Incubation of both MIN6 cells and human islets with 2-bromopalmitate at low and high glucose did not significantly decrease total CPE protein compared to palmitate-treated cells (Figures 12A, B and 13A, B). These results suggested that palmitate must be metabolized to cause the decrease in CPE levels. ER-stress, as evidenced by CHOP expression, was induced by both palmitate and 2-bromopalmitate at low and high glucose in MIN6 cells (Figure 12C). This indicated that some ER-stress can occur under conditions where CPE is not significantly altered. After an early wave of apparent necrosis, 2-bromopalmitate treatment elicited a significantly lower magnitude of cumulative cell death over 24 hours compared with palmitate-treated cells at both low and high glucose, suggesting that palmitate is considerably more pro-apoptotic than its non-metabolizable homolog and elicits its actions earlier on (Figure 12D, E).

Similarly, 2-bromopalmitate was not effective at reducing CPE protein levels in human islets (Figure 13A, B). Robust differences in CHOP and cleaved caspase-3 were not discernable with only two experiments, particularly with the large variance in the values (Figure 13C, D). While the human islet experiments should be repeated, the trend observed in the MIN6 cells that 2-bromopalmitate does not significantly affect CPE levels was observed in human islet cells as well. Together, these data suggest that palmitate metabolism is required for the effects on CPE and cell death in β -cells.

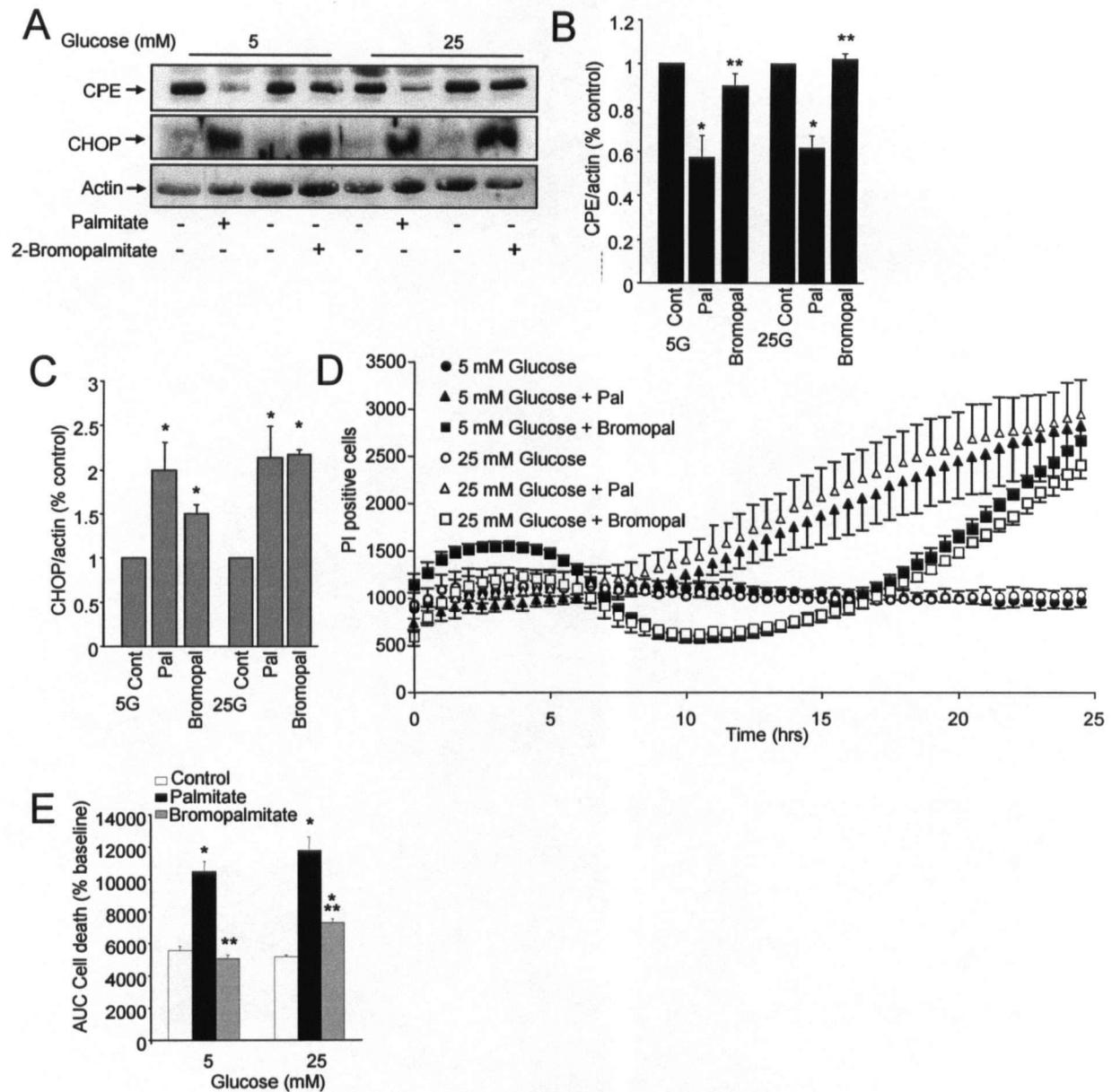


Figure 12. The effect of 2-bromopalmitate on MIN6 cells. (A) MIN6 cells were treated for 24 hours with 2-bromopalmitate at low and high glucose and the expression of CPE and CHOP was examined (n=3). (B) CPE expression was significantly reduced by 40% in palmitate treatments at low and high glucose, while 2-bromopalmitate did not alter CPE expression. * denotes a significant difference from the control; ** denotes a significant difference from palmitate treatments ($P < 0.05$). (C) CHOP expression was significantly increased in both palmitate and 2-bromopalmitate treatments at both low and high glucose. (D) Cell death measurements of MIN6 cells treated with palmitate and 2-bromopalmitate over 24 hours shows palmitate-treated cells undergo cell death more quickly than 2-bromopalmitate-treated cells (n=3). (E) 24-hour cumulative cell death quantitation of (D) shows that palmitate-induced cell death was significantly higher compared to the control and 2-bromopalmitate-treated cells.

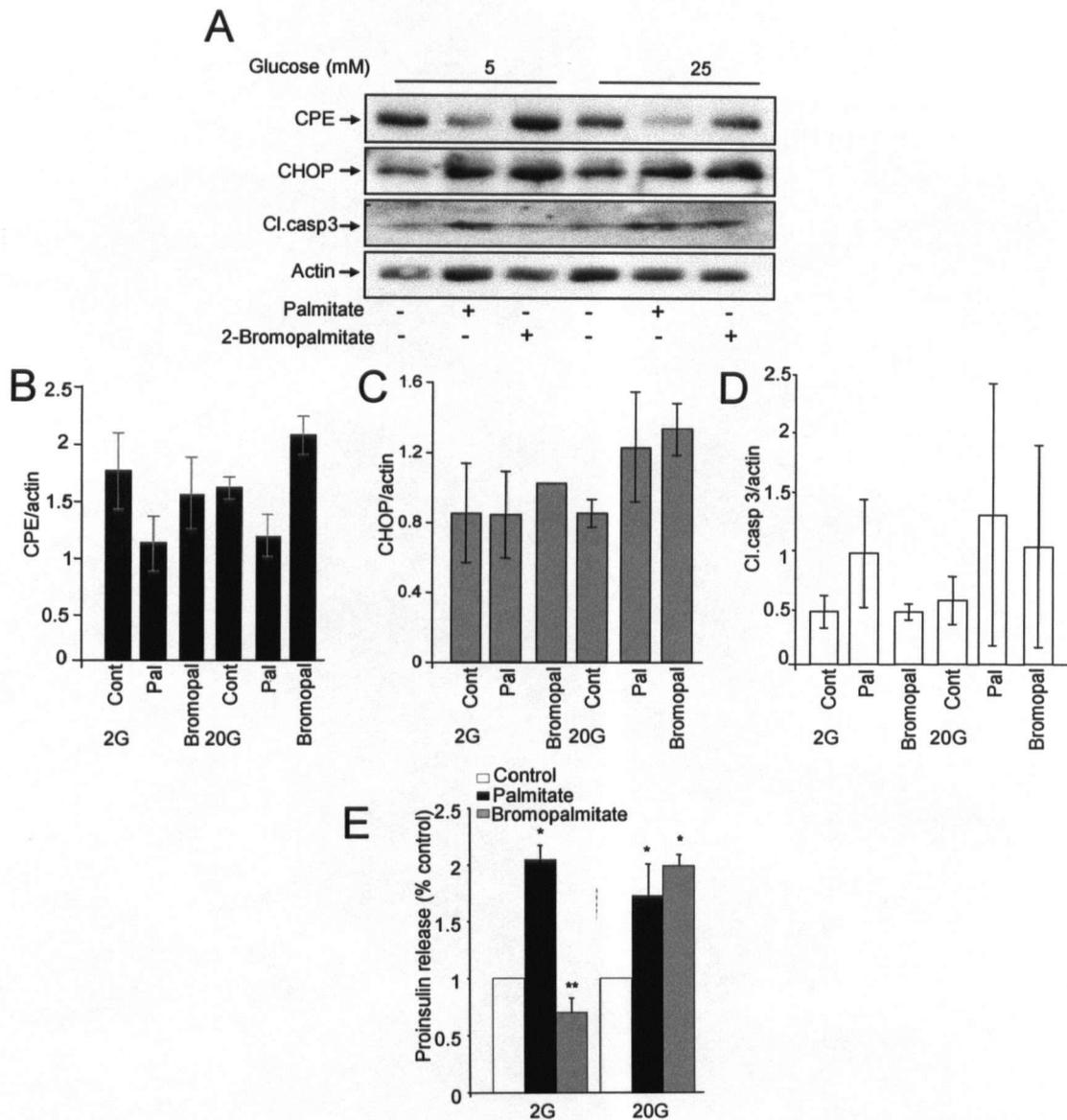


Figure 13. Effect of 2-bromopalmitate on CPE in human islets (A) Human islets treated for 48 hours with palmitate and 2-bromopalmitate and their effects of CPE, CHOP, and cleaved caspase 3 (n=2). **(B)** Compared to the control, CPE expression is reduced by 1.3- fold and 1.5-fold, respectively in palmitate-treated cells at low and high glucose. 2-bromopalmitate does not alter CPE expression to the same degree as the palmitate-treatments (Bars in graph represent the range of the data in the two test samples). **(C)** CHOP expression is up-regulated in palmitate and 2-bromopalmitate-treated cells, particularly at high glucose. **(D)** Cleaved caspase-3 is up-regulated by at least 2-fold in palmitate-treated cells at both low and high glucose, while 2-bromopalmitate only becomes cytotoxic at high glucose. The range in the results, however, weakens this observed trend. **(E)** Proinsulin measurements from human islet cell secretions treated the palmitate and 2-bromopalmitate over 48 hours (n=3).

3.4b The effect of FFAs on proinsulin secretion in human islet cells

Previous studies have implicated FFAs in altering proinsulin to insulin ratios in diabetes possibly due to a down-regulation of prohormone convertase 1/3 activity (127). Interestingly, hyperproinsulinemia is common in type 2 diabetes (128-131) and has been reported in the CPE^{fat/fat} mouse (114). The elevated levels of circulating proinsulin, which has been reported to have 10% of the biological activity of insulin (132-134) may contribute to hyperglycemia, possibly due to β -cell dysfunction and insufficient insulin processing. In this study, we examined the effect of palmitate on proinsulin secretion in human islets to determine whether insulin processing was altered (Figure 13E). Interestingly, palmitate significantly increased proinsulin levels at both low glucose and high glucose compared to the control, suggesting impaired insulin processing. Conversely, 2-bromopalmitate did not evoke the release of proinsulin at low glucose but was equally effective at high glucose. These results demonstrate that palmitate requires metabolism to disrupt proinsulin processing in low glucose. In the low glucose condition, these findings are consistent with the idea that reduction in CPE accounts for the defect in proinsulin processing. The effects of bromopalmitate at high glucose suggest that another proinsulin processing enzyme may be involved under these conditions.

3.5 Ca²⁺-dependence of palmitate-induced CPE degradation and cell death

Intracellular Ca²⁺ signals have been implicated in the effects of palmitate downstream of its metabolism, as well as after the activation of the GPR40 receptors. The above observation that 2-bromopalmitate was unable to reduce CPE indicated that the metabolism of palmitate is likely required. Palmitate metabolism, but not activation of GPR40, would be expected to increase the ATP to ADP ratio in the β -cells and close K_{ATP} channels. This, in turn, would be expected to open voltage-gated Ca²⁺ channels in the plasma membrane. Therefore, we investigated the role of Ca²⁺ in the palmitate-associated CPE reduction, as well the potential role of Ca²⁺ in mediating palmitate-induced apoptosis. Upon co-incubation of palmitate with K_{ATP} channel opener, diazoxide, MIN6 cell death was significantly blocked, while diazoxide alone had no effect on cell death (Figure 14A, B). This result demonstrated that K_{ATP} channels are required downstream of palmitate metabolism to initiate β -cell apoptosis.

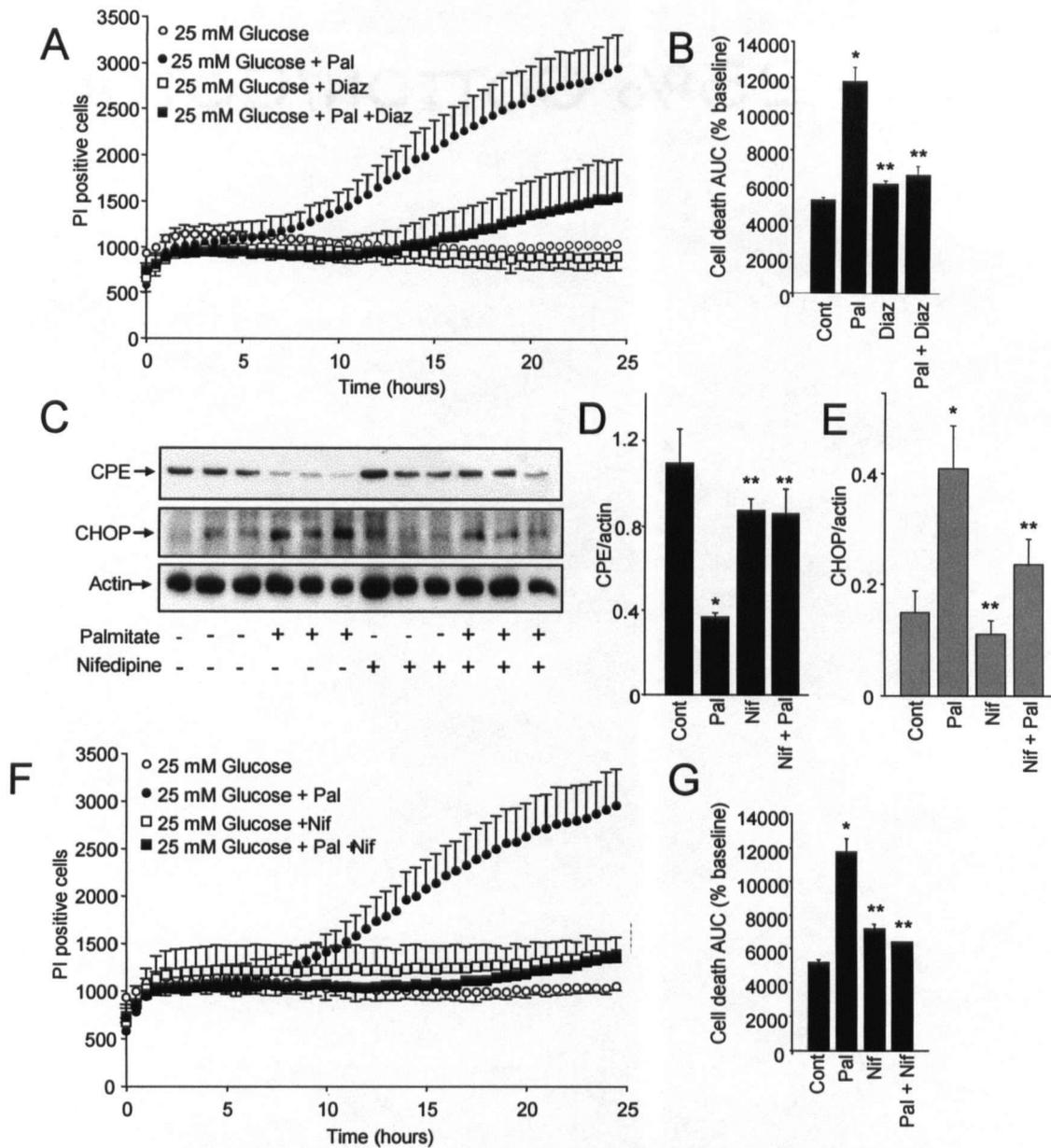


Figure 14. The effect of cytosolic Ca²⁺ modulators on palmitate-treated MIN6 cells. (A) Measurement of cell death in MIN6 cells incubated with palmitate (Pal) and 100 μ M of a K-ATPase activator, diazoxide (Diaz) for 24 hours (n=3). (B) 24-hour cumulative cell death quantitation of (A). Addition of diazoxide to palmitate treatments significantly reduces death of MIN6 cells. * denotes a significant difference from control; ** denotes a significant difference from palmitate treatments ($P < 0.05$). (C) Western blot of MIN6 cells treated with palmitate (Pal) and 10 μ M of L-type Ca²⁺ channel blocker, nifedipine (Nif) for 24 hours at 25 mM glucose (n=3). (D) Compared to the control (Cont) and the Nif treatment, CPE expression is significantly reduced by at least 50% in palmitate-treated cells. (E) Compared to the control and cells treated with nifedipine, CHOP expression is significantly increased by ~2-fold in palmitate-treated cells. Palmitate-treated MIN6 cells are rescued from ER-stress upon incubation with nifedipine ($P < 0.05$). (F) Measurement of cell death in MIN6 cells incubated with palmitate and 10 μ M nifedipine shows a reduction in cell death (n=3). (G) 24-hour cumulative cell death quantitation of (F).

Both palmitate metabolism and K_{ATP} channel closure are known to increase Ca^{2+} influx via voltage-gated Ca^{2+} channels in β -cells. Thus, we examined the role of Ca^{2+} influx on CPE levels and apoptosis in palmitate-treated MIN6 cells using a concentration of nifedipine known to block all voltage-gated Ca^{2+} currents in β -cells (135, 136). Incubation of MIN6 cells with nifedipine alone did not alter CPE expression (Figure 14C, D), but co-incubation of nifedipine and palmitate prevented the decrease in CPE total protein, suggesting the effects of palmitate on CPE protein are strictly dependent on Ca^{2+} influx from the extracellular space. In addition, while nifedipine alone did not induce CHOP expression, co-incubation of palmitate and nifedipine prevented the induction of CHOP (Figure 14C, E). Similarly, using our real-time cell death assay, we demonstrated that nifedipine could completely prevent palmitate-induced cell death during the 24-hour test period (Figure 14F, G). These results suggested that the effects of palmitate on CPE, as well as its effects of ER-stress and cell death, are Ca^{2+} -dependent.

3.6 Role of calpains in CPE protein levels

The previous experiments have shown that palmitate treatment leads to a rapid reduction in CPE protein and that this likely occurs at the post-translational level. We have also demonstrated that this requires palmitate metabolism and Ca^{2+} influx. Together, these observations pointed to the possibility that Ca^{2+} -dependent proteases may be involved. The calpain family of Ca^{2+} -dependent proteases are well known mediators of protein degradation (137-139). In addition, it has been shown that FFA-induced apoptosis in primary mouse islets can be prevented with a calpain inhibitor (ALLM) or the targeted deletion of the calpain-10 gene (87, 140). To determine whether calpains were playing a role in the degradation of CPE, MIN6 cells were treated for 24 hours with two calpain inhibitors, E64D (*trans*-epoxysuccinyl-L-leucylamido (4-guanidino)-butane) and ALLM (N-Acetyl-Leu-Leu-Met-CHO; calpain inhibitor II) (86, 87). Co-incubation of E64D with palmitate reduced the palmitate-induced decrease in CPE protein (Figure 15A, B), suggesting a requirement for the calpains. E64D alone, however, increased ER-stress, as indicated by CHOP expression, which is not unexpected of a drug that would slow protein clearance in the cell (Figure 15A, C). Interestingly, this was apparently insufficient to trigger significant cell death over the 24-hour period in separate experiments, as evidenced by the real-time imaging of propidium iodide incorporation. These studies also showed that palmitate-induced cell death was insensitive to E64D (Figure 15D, E). These results suggest that the molecular target(s) of the cysteine protease inhibitor E64D, which include many

isoforms of the calpains and the proteasome, play a role in CPE degradation, but are redundant for palmitate-induced cell death under these conditions.

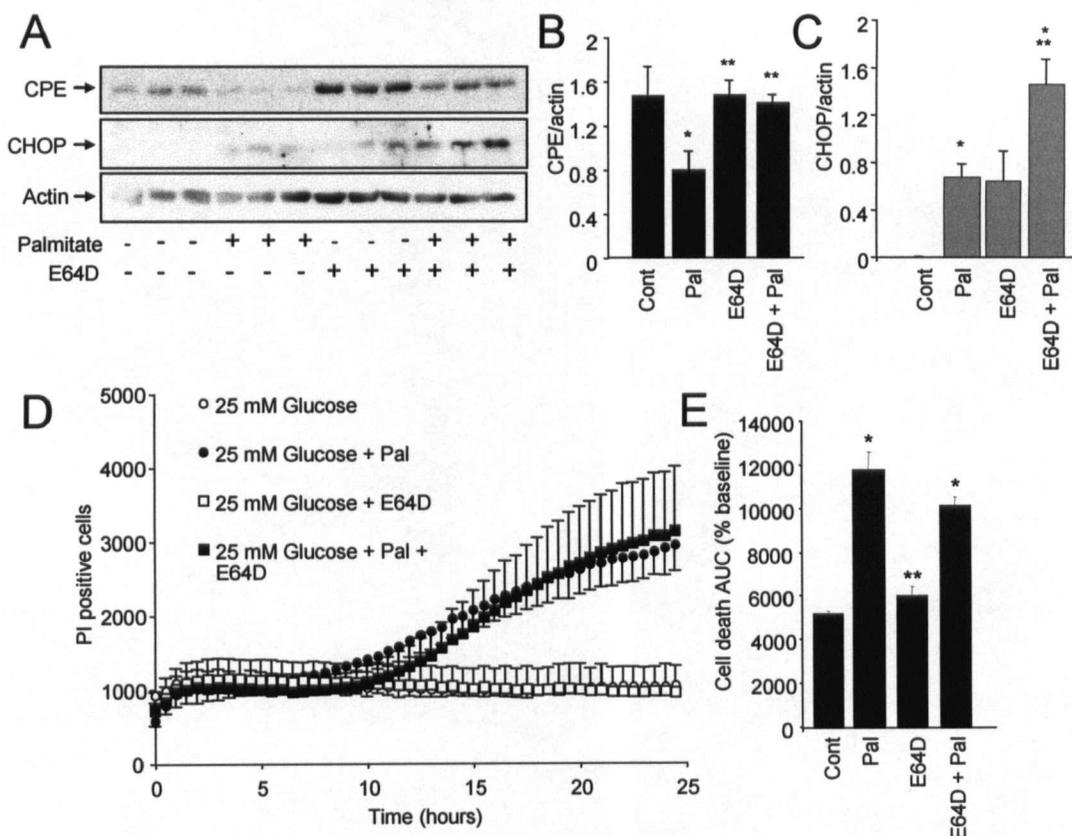


Figure 15. Effects of the calpain inhibitor, E64D, on palmitate-treated MIN6 cells. (A) Western blot of MIN6 cells treated with palmitate (Pal) and 25 μ M E64D for 24 hours at 25 mM glucose (n=3). (B) Incubation of MIN6 cells with the calpain inhibitor E64D significantly prevents a ~2-fold decrease in CPE expression in palmitate-treated MIN6-cells. * denotes a significant difference from the control; ** denotes a significant difference from palmitate treatments. ($P < 0.05$). (C) Incubation of MIN6 cells with the calpain inhibitor E64D significantly induces CHOP on its own and co-incubation of palmitate and E64D increases CHOP expression by 2-fold. (D) Measurement of cell death over 24 hours in MIN6 cells treated with palmitate and E64D at 25 mM glucose shows reduced cytotoxic effects (n=3). (E) 24-hour cumulative cell death quantitation of (D) shows that cell death in Pal and E64D+Pal treatments is significantly higher than control and E64D alone treatments.

Incubation of MIN6 cells with the second calpain inhibitor, ALLM, caused massive ER-stress (Figure 16A, C) and cell death (Figure 16D) that was additive to that of palmitate. Furthermore, ALLM did not prevent CPE degradation (Figure 16A, B). These results suggest that the tested concentration of ALLM may have been cytotoxic to the cells, where the detrimental effects of ALLM superceded its potential effects on inhibiting CPE reduction. Thus, it is difficult to unequivocally determine whether calpains are involved in the effects of palmitate with these findings.

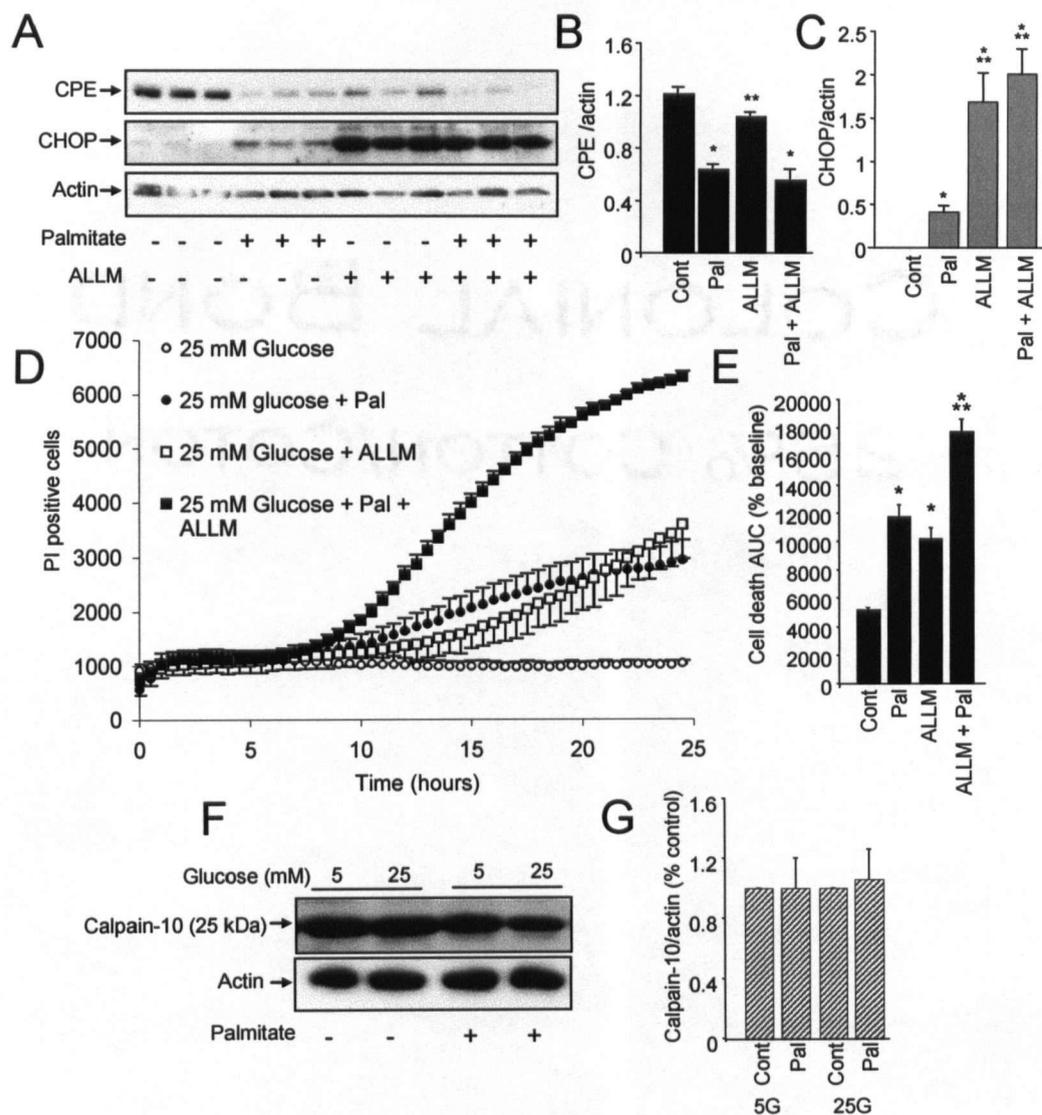


Figure 16. The effects of calpain inhibitor, ALLM, on palmitate-treated MIN6 cells. (A) Western blot of MIN6 cells treated with palmitate (Pal) and 100 μ M ALLM for 24 hours at 25 mM glucose (n=3). (B) CPE expression is significantly reduced by 1.75 fold in palmitate- and ALLM+Pal-treated cells compared to the control and ALLM-alone treatments. * denotes a significant difference from the control; ** denotes a significant difference from palmitate treatments ($P < 0.05$). (C) CHOP expression is significantly increased in palmitate-, ALLM-, and ALLM+Pal-treated cells compared to the control and ALLM-alone treatments. (D) Measurement of cell death in MIN6 cells treated with palmitate and ALLM at 25 mM glucose shows increased cytotoxicity in ALLM- and ALLM+Pal treated cells (n=3). (E) 24-hour cumulative cell death quantitation of (D). Cell death in Pal, ALLM, and ALLM+Pal treatments is significantly higher than control, showing that ALLM is cytotoxic alone and that the combination of ALLM+Pal exacerbates cell death. (F) Representative calpain-10 Western blot from palmitate-treated MIN6 cells at low (n=4) and high glucose (n=6). (G) No significant difference was found in calpain-10 expression between control and palmitate treatments at either low and high glucose

In addition, because palmitate-induced apoptosis was absent in islets from calpain-10 knockout mice (87) and calpain-10 has been implicated as a type 2 diabetes susceptibility gene, we investigated the effect of palmitate on calpain-10 protein expression in MIN6 cells. In our studies, Western blots consistently showed several bands upon probing for calpain-10 (data not shown). While each band likely corresponded to the various splice variants of calpain-10 (141), no single band showed consistent changes during palmitate treatments. Given that previous studies on calpain-10 knock-out mice have shown the absence of a calpain-10 band at approximately 25kDa in islets (87), we used this band to search for differences between control and palmitate treatments (Figure 16F, G). Overall, we found no consistent differences in calpain-10 expression in palmitate treated cells compared to the controls at either low or high glucose. While these results do not show any differences in calpain-10 expression, they do not rule out the involvement of other calpains in CPE degradation or the possibility that the changes in CPE expression must be captured at a specific time point.

3.7 CPE^{fat/fat} islets exhibit increased apoptosis *in vivo* and *in vitro*

Many of the studies described above show that CPE protein is decreased under conditions of FFA-induced ER-stress and β -cell death. This association points to a possible role of CPE in apoptosis, but required both loss-of-function and gain-of-function experiments to test this hypothesis. We took advantage of mice lacking functional CPE to determine whether CPE plays a role in β -cell survival *in vivo* and *in vitro*. CPE^{fat/fat} pancreatic sections showed an elevated number of TUNEL positive cells in the islets of the mutant mice compared to the controls (Figure 17B, C). These results suggested that under basal conditions, CPE^{fat/fat} mice exhibit increased islet apoptosis compared to their littermate controls. Insulin and glucagon staining of the CPE^{fat/fat} islets in pancreatic tissue sections revealed marked defects in islet architecture compared to their controls. Although the islets were generally larger, the center of the CPE^{fat/fat} islets showed a lack of cells, suggesting partial atrophy (Figure 17A). To determine if islets of the CPE^{fat/fat} were more susceptible to stressful conditions *in vitro*, islets were exposed to palmitate at high glucose for 24 hours. Even without palmitate treatment, the islets of the CPE^{fat/fat} mice showed significantly higher CHOP and caspase-3 activation. Palmitate increased CHOP and cleaved caspase 3 levels in wildtype islets, and the effects of palmitate were not additive to the ER-stress and apoptosis induced by the CPE mutation (Figure 17D, E, F).

In preliminary experiments, we also observed an increase in cleaved caspase-3 in $CPE^{fat/fat}$ mouse islets that were isolated and collected immediately (i.e. no overnight culture)(data not shown). These results suggested that the islets of mice lacking CPE are more susceptible to ER-stress and apoptosis at basal levels both *in vivo* and in the controlled *in vitro* environment. The observation that palmitate did not induce further ER-stress and apoptosis in $CPE^{fat/fat}$ islets suggests the possibility that palmitate-induced cell death may require CPE.

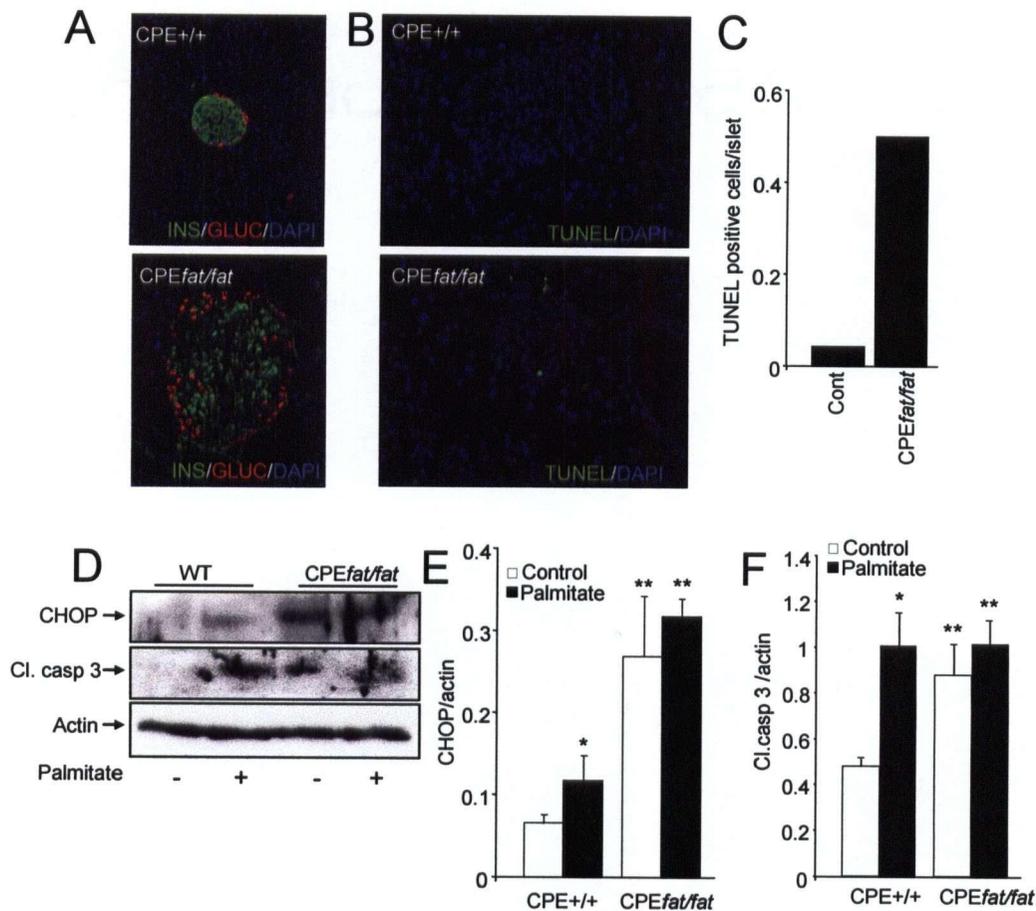


Figure 17. ER-stress and cell death in the islets of $CPE^{fat/fat}$ mice. (A) Representative insulin (green), glucagon (red), and DAPI (blue) staining in the islets of $CPE^{fat/fat}$ mice and wild-type controls. Islets appear approximately 5-times larger in mutant mice compared to control and show disrupted architecture. (B, C) TUNEL staining in islets of pancreatic sections of $CPE^{fat/fat}$ mice and wild-type controls show increased TUNEL-positive cells (n=2). (D) CHOP and cleaved caspase 3 in 24 hour palmitate-treated glucose islets at 20 mM from $CPE^{fat/fat}$ mice and wild-type controls (n=3). (E,F) Both CHOP and cleaved caspase 3 expression are significantly increased in islets from $CPE^{fat/fat}$ mice compared to the control both with and without palmitate. * denotes a significant difference between palmitate and control within the same genotype. ** denotes a significant difference between genotypes within the same treatment ($P < 0.05$).

3.8 Over-expression of CPE partially rescues MIN6 cells from palmitate-induced ER-stress and apoptosis

Up to now, the results demonstrated that incubation of MIN6 cells with palmitate leads to a decrease in CPE, a rise in CHOP expression, and an increase in cell death. ER-stress appeared to follow CPE, suggesting that CPE lies upstream of ER-stress. Moreover, CPE-deficient islets show significantly more apoptosis *in vivo* and *in vitro*. To determine whether an increase in CPE might be able to rescue β -cells from death, the effects of over-expressing CPE in palmitate-treated MIN6 cells were explored. Using the CMV promoter, we over-expressed CPE by 1.5 fold (Figure 18A, B). Negligible over-expression was observed with the rat insulin promoter (data not shown). Following palmitate treatments, the over-expressed CPE was similarly reduced when compared to the palmitate-treated untransfected cells (Figure 18A, B). CPE over-expression itself caused an increase in ER-stress, as might be expected with the increased ER protein load. Therefore, to examine the effects of palmitate on ER-stress, the results of palmitate treatment were normalized to their respective controls (empty vector or CPE over-expression) (Figure 18C). When the effects of CPE over-expression alone were controlled for, palmitate-induced CHOP and cleaved caspase 3 activation were significantly reduced in these cells (Figure 18C, D). Together, these findings suggest that CPE can limit the deleterious effects of palmitate on β -cells and suggest a novel role for CPE in the control of β -cell ER-stress and apoptosis.

3.9 The effects of a high fat diet on CPE protein expression in vivo

To mimic the *in vitro* studies *in vivo*, we examined the effects of a high-fat diet on CPE in C57Bl6/J mice that were fed a high-fat diet for 16 weeks. These mice became more obese and slightly more glucose-intolerant than their littermate controls (Figure 19A, B). Following the high-fat diet regimen, the islets were collected, lysed immediately (without overnight culture) and probed for CPE and CHOP protein expression (Figure 19C, D, E). The islets of these mice, after a high fat diet, did not exhibit differences in CPE or CHOP. These results, however, do not conclusively rule out the possibility that high fat diet could alter CPE with a longer study period or higher fat diet. In addition, the immediate analysis of the islets, rather than allowing overnight culture, may have increased basal cell death and masked the effects on CPE.

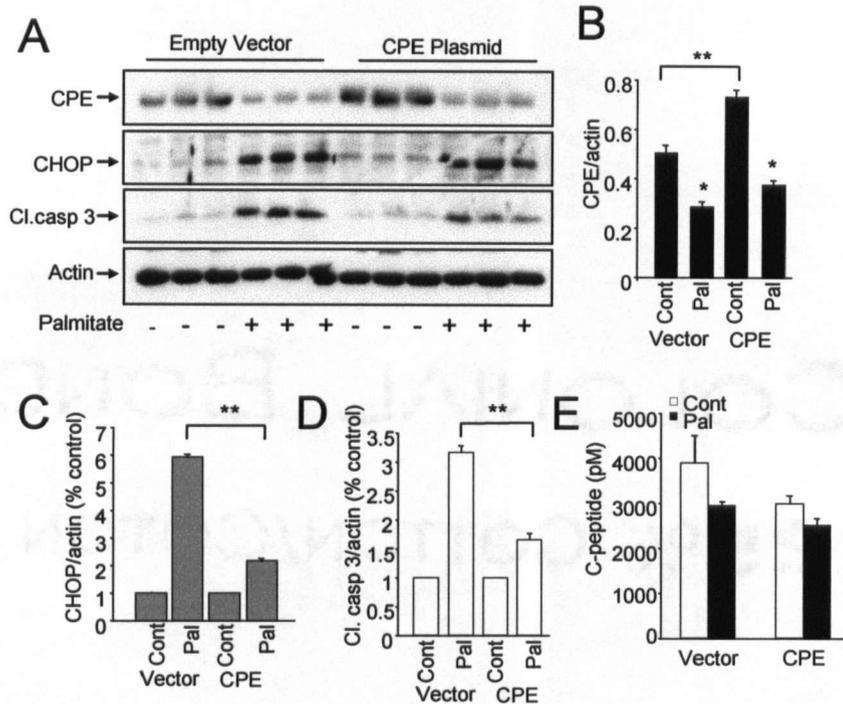


Figure 18. The effect of over-expressing CPE in palmitate-treated MIN6 cells. (A) MIN6 cells were transfected with a CPE plasmid and treated with palmitate for 24 hours at 25 mM glucose (n=3). (B) CPE protein was significantly up-regulated by 1.5-fold in transfected cells. CPE expression is significantly reduced by 40% and 50% in both palmitate-treated control and CPE-over-expressing MIN6 cells, respectively. * denotes a significant difference from the control within the same plasmid; ** denotes a significant difference from the empty vector within the same treatment ($P < 0.05$). (C) CHOP expression is significantly reduced by 3-fold in palmitate-treated MIN6 cells over-expressing CPE compared to palmitate-treated cells containing the empty vector. (D) Cleaved caspase 3 expression is significantly reduced by 2-fold in palmitate-treated MIN6 cells over-expressing CPE compared to palmitate-treated cells containing the empty vector. (E) No difference found in C-peptide measurements from the cell secretions of the 24-hour treated cells.

To further examine the effects of high fat on CPE levels *in vivo*, islets from the *ob/ob* mouse model were obtained. Previous studies have shown that these obese mice have circulating FFAs levels of approximately 1.5 mM (142) and are therefore a good model to test the effect of high circulating FFAs on CPE. These mice were 2-fold heavier than controls (Figure 20A, B), but their glucose-intolerance was not statistically significant in this small group of animals. While showing a trend towards an increase in CHOP (Figure 20C, D), *ob/ob* mice did not show any differences in CPE protein levels. While CPE was not implicated in these limited *in vivo* experiments, they do provide starting points for future studies.

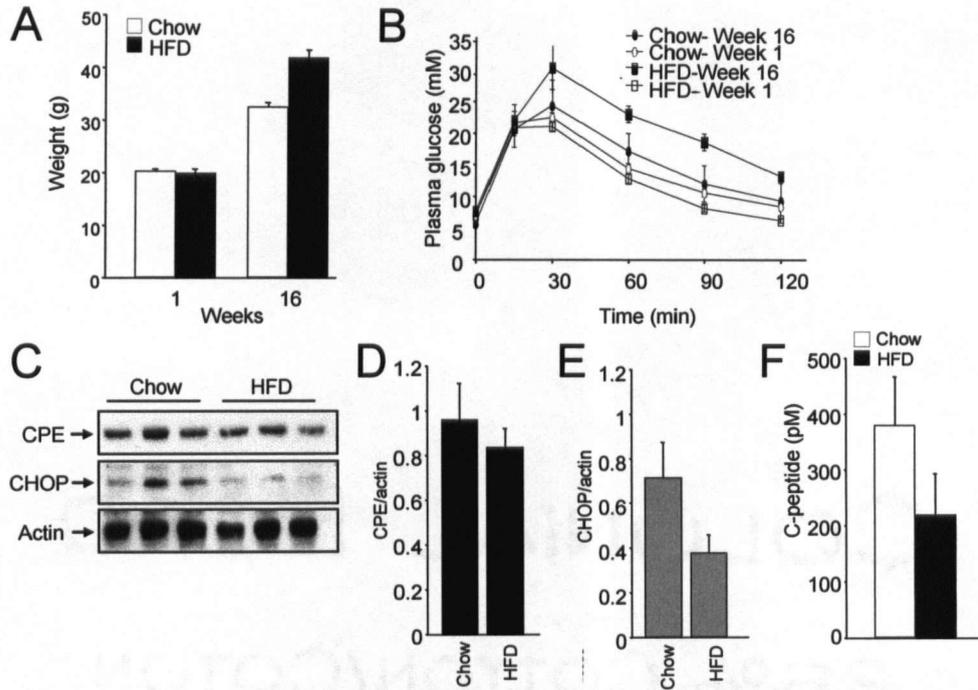


Figure 19. The effect of a high-fat diet on CPE and CHOP protein in C57B16/J mice. (A) Body weights of mice fed a high-fat diet (HFD) for 16 weeks is increased compared to mice fed regular chow ($42.02 \text{ g} \pm 1.27$ vs. $32.12 \text{ g} \pm 0.67$) ($n=6$). **(B)** IPGTT of mice before and after 16 weeks of high fat feeding. **(C)** CPE and CHOP expression in islets freshly isolated from C57B16/J mice on chow or high-fat diets. **(D, E)** No significant differences were found in CPE and CHOP expression between HFD and Chow mice. **(F)** C-peptide measurements of serum from C57B16/J mice on chow or high-fat diets did not differ significantly.

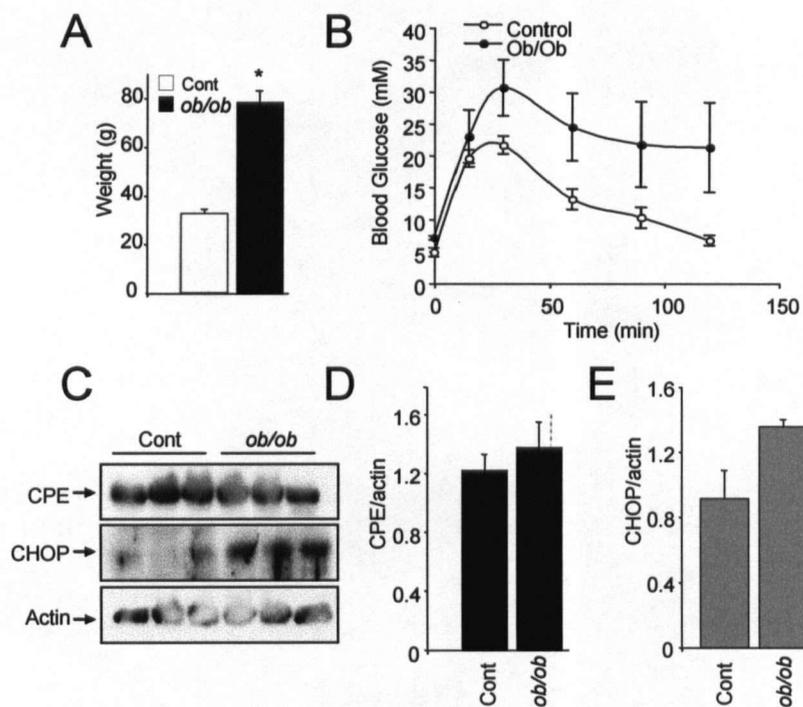


Figure 20. CPE and CHOP protein expression in the islets of *ob/ob* mice
(A) Body weight was significantly different in *ob/ob* mice compared to the wild-type controls ($78.6\text{g} \pm 0.87$ vs. $32.8\text{g} \pm 1.87$) ($n=3$). * denotes a significant difference compared to the control ($P<0.05$). **(B)** IPGTT of mice following an overnight fast ($n=3$). No significant differences in glucose tolerance were found. **(C, D, E)** CPE and CHOP expression in islets isolated from *ob/ob* did not differ significantly from the control mice ($n=3$).

4.0 Discussion

This study investigated pathways involved in fatty acid-induced ER-stress and β -cell apoptosis. Using an unbiased proteomics screen, several potential targets for the saturated fatty acid palmitate were identified. The most significantly altered protein spot on the 2D gels corresponded to CPE. Remarkably, CPE was the most decreased spot in two completely independent screens using MIN6 cells and human islets, suggesting a potentially important and conserved role in the pro-diabetic effects of palmitate. CPE is a key enzyme in the insulin secretory pathway and disruptions in this pathway are known to alter the function and survival of pancreatic β -cells. In the CPE^{fat/fat} mouse strain, a single point mutation (Ser202 to Pro) in the CPE protein is sufficient to produce an animal with multiple disorders including obesity and diabetes (114, 143). The confirmation that CPE is a critical target for the fatty acid palmitate and a potential key player in the pathogenesis of diabetes is an important advance in our understanding of the molecular pathways involved in the progression of this disease.

In this study, we have shown that palmitate not only leads to a decrease in CPE total protein, but also leads to the up-regulation CHOP, a marker for ER-stress, and an increase in β -cell apoptosis. Time course studies indicated that the palmitate-associated decrease in CPE preceded the induction of CHOP as well as cell death, indicating that cell death is not responsible for the observed decrease in CPE protein. In addition, thapsigargin-treated MIN6 cells also showed no change in CPE expression, further confirming that the reduction in CPE protein was not due to the generalized decrease in protein synthesis that accompanies ER-stress.

Saturated FFAs have been shown to induce ER-stress in multiple studies with various cell types (46-49, 61, 144, 145). Moreover, ER-stress has also been shown to be induced under situations of protein overload in the secretory pathway (119-121). Due to the importance of CPE in the β -cell secretory pathway, we were interested in investigating the possibility that the palmitate-associated decrease in CPE could increase the susceptibility of the β -cell to ER-stress, potentially due to a backlog of unprocessed proinsulin in the secretory pathway. If chronically activated, this ER-stress results in β -cell apoptosis. Indeed, tissue sections of the CPE^{fat/fat} mice showed an increase in TUNEL positive cells in pancreatic islets and exhibited islet atrophy, suggesting elevated β -cell apoptosis *in vivo*. We also found that the isolated islets of CPE^{fat/fat} mice had significantly increased CHOP and cleaved caspase 3 expression compared to the islets of wild-type mice. Treating CPE^{fat/fat} islets with palmitate did not further increase ER-stress or

apoptosis compared to untreated islets. The observation that the effects of the mutation in CPE and palmitate were not additive suggests that either 1) the reduction in CPE is required for palmitate-induced cell death, or 2) the absence of CPE may be so harmful that the addition of palmitate cannot worsen ER-stress or cell death. Conversely, over-expression of CPE in palmitate-treated MIN6 cells showed decreased expression of cleaved caspase 3 and CHOP compared to palmitate-treated control cells, suggesting the addition of CPE could reduce the effects of palmitate on cell death and ER-stress. Together, the finding that the lack of or addition of CPE to the β -cells could alter survival during palmitate-treatments suggests that CPE might play a role in modulating ER-stress and cell death in type 2 diabetes (Figure 21).

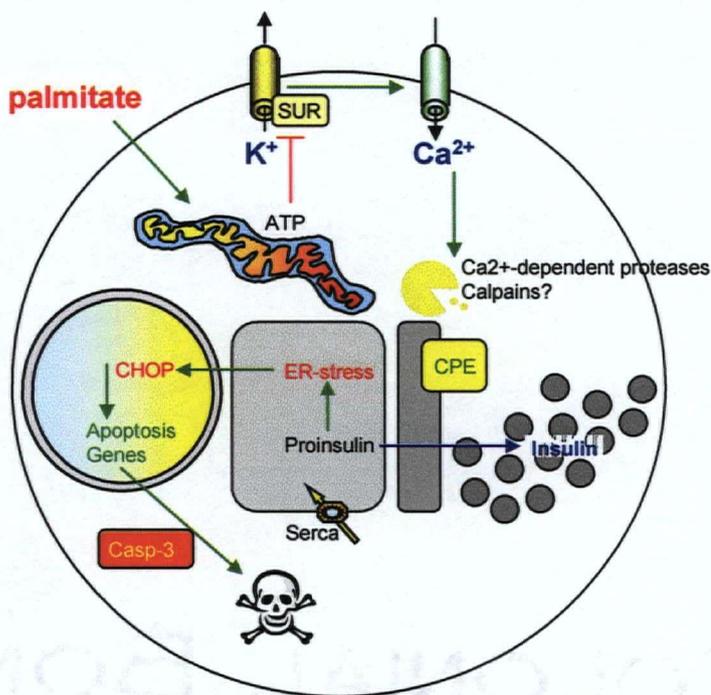


Figure 21. Proposed model for the role of CPE in palmitate-induced ER-stress and apoptosis in pancreatic β -cells. Palmitate leads to alterations in intracellular Ca^{2+} , through metabolism, which potentially activates the Ca^{2+} -dependent proteases, the calpains. Palmitate leads to a decrease in CPE protein levels through degradation. The reduction in CPE leads to a backlog of unprocessed proteins in the secretory pathway. This backlog worsens ER-stress, which has also been induced by altered $[\text{Ca}^{2+}]_i$. The reduced CPE and induced ER-stress somehow work both independently and dependently to lead to apoptosis. Dashed arrows represent that the effect of CPE on apoptosis and ER-stress is not solely dependent on the reduction of CPE.

4.1 Mechanisms of palmitate-induced CPE degradation: Metabolism and Ca²⁺ influx

CPE appears to play a role in FFA-induced ER-stress and β -cell apoptosis, but the exact mechanism by which CPE expression is regulated during palmitate treatments is not clear. Over the years, the effects of palmitate on the pancreatic β -cell have been extensively studied but there is still considerable debate regarding how FFAs elicit their effects on both ER-stress and β -cell death. For some time, the metabolism of FFAs was considered to be of paramount importance in the regulation of insulin secretion and cell death. However, the more recent discovery of FFA-activated G-protein coupled receptors, such as GPR40, has presented a new paradigm for understanding the actions FFAs (41, 45). GPR40, a G-protein coupled receptor, is preferentially expressed in the β -cell and has been shown to be involved in insulin secretion (41, 45) and modulating [Ca²⁺]_i in pancreatic islets (40, 41, 146, 147). Using 2-bromopalmitate, we aimed to clarify the pathways associated with palmitate-induced cell death and CPE reduction and by examining the involvement of metabolic or signalling pathways, potentially through GPR40.

In our studies, we found that in addition to being unable to mimic the palmitate-induced reduction in CPE, 2-bromopalmitate also exhibited reduced cell death over the course of 24 hours compared to palmitate-treated cells. In agreement with other studies (69, 148), palmitate-treatments at both low and high glucose were significantly more cytotoxic than 2-bromopalmitate treatments. The inability of 2-bromopalmitate to decrease CPE and increase cell death at both low and high glucose to the same extent as palmitate suggested that metabolic pathways may have a greater role in inducing cell death than GPR40 signalling pathways. Supporting this conclusion are several studies that show that GPR40 does not play a role in apoptosis (40, 43). For example, while over-expression of the fatty-acid receptor GPR40 in mice (40) led to overt diabetes, morphological studies of the pancreas showed no evidence of reduced β -cell mass. Conversely, high-fat fed GPR40 knockout mice did not develop significant glucose intolerance, and both non-fasted and fasted blood glucose levels were lower than in control littermates (40). While mice lacking GPR40 were protected from developing diabetes following a high-fat diet *in vivo*, knocking out the GPR40 receptor did not protect the islets of these mice from palmitate-induced apoptosis *in vitro*. Since over-expression of GPR40 does not increase the susceptibility of the β -cell to apoptosis (40) and knockout of GPR40 does not protect palmitate-treated cells from cell death (43), these results suggest that palmitate-induced cell death does not

involve GPR40. They do not, however, rule out the role of the GPR40-associated signalling pathways in reducing CPE.

It should be noted that there is controversy regarding the ability of 2-bromopalmitate to activate the GPR40 receptor (39, 149). Therefore the use of 2-bromopalmitate to differentiate between GPR40- or metabolic-dependent pathways is not without question. In our experiments, 2-bromopalmitate did not induce proinsulin secretion in low glucose, but was as effective as palmitate at 20 mM glucose in human islets. Others have also shown that 2-bromopalmitate is not associated with rises in intracellular calcium, which are necessary for insulin secretion (150). While the findings of others make it difficult to conclusively establish whether palmitate primarily acts through GPR40 or metabolism to reduce CPE, our experiments with Ca^{2+} regulators, diazoxide and nifedipine suggest that changes in $[\text{Ca}^{2+}]_i$ subsequent to FFA metabolism, but not GPR40 signalling, are important for the pro-apoptotic effects of palmitate in β -cells.

In recent years, it has been established that palmitate increases cytosolic Ca^{2+} levels, particularly in mouse β -cells (68, 70) and α -cells (151). In addition, others have shown that palmitate can lead to repetitive Ca^{2+} transients and plateau-like rises in $[\text{Ca}^{2+}]_i$ in mouse islets and HIT-T15 cells (150). In our experiments, blocking Ca^{2+} influx with nifedipine abolished the palmitate-associated decrease in CPE and ER-stress, as well as prevented palmitate-induced cell death in MIN6 cells. Cell death was also prevented upon co-incubation of palmitate with diazoxide, a K_{ATP} channel activator that prevents membrane depolarization and subsequent Ca^{2+} influx through L-type Ca^{2+} channels. Interestingly, while our results show that nifedipine can prevent the palmitate-associated reduction in CPE, other studies have also investigated the potential link between Ca^{2+} and CPE. Interestingly, Ca^{2+} has been implicated in the control of CPE stability in the trans-Golgi network (152, 153). These studies have shown that higher cytosolic concentrations of Ca^{2+} altered protein aggregation and could lead to the thermal destabilization of CPE (152, 153). In addition, preliminary studies on apoptosis showed that palmitate-induced cell death could be reduced by interfering with ER Ca^{2+} flux using thapsigargin (data not shown). Together, these results strongly suggest that increased β -cell Ca^{2+} flux is a requirement for palmitate-induced apoptosis, and that altered Ca^{2+} flux may play a role in the decrease of CPE. Our results examining the effects of CPE, ER-stress, and cell death using Ca^{2+} regulators and 2-bromopalmitate suggest that the Ca^{2+} is a critical factor in determining cell survival and cellular CPE levels.

4.2 Mechanisms of palmitate-induced CPE degradation: Calpains

Throughout our experiments, CPE protein expression was consistently decreased in palmitate-treated MIN6 cells and human islets. And since the involvement of cell death and ER-stress in reducing CPE levels was ruled out, several other possibilities were then considered. As many genes have been shown to be altered by FFAs (154-156), we first tested whether CPE was being altered at the level of protein synthesis and/or gene expression by palmitate. Our results on stable CPE mRNA expression showed no differences in palmitate-treated and control MIN6 cells suggesting that the effects of palmitate on CPE were being exerted post-transcriptionally, potentially through degradation.

It is possible that CPE may be degraded by palmitoyl-protein thioesterases (PPTs), which are lysosomal hydrolases that regulate palmitoylated proteins through degradation (157). To date, it is unclear whether CPE is palmitoylated. If it were PPTs could potentially be responsible for its degradation or the palmitoylation of CPE could serving as a tag for other proteases to target it for degradation. However, in our experiments, we established that the effects of palmitate on CPE and cell death are Ca^{2+} -dependent. And since previous research has implicated the calpains (139, 158) not only in protein degradation but to be strongly activated along with CPE during global ischemia and reperfusion experiments (115, 116, 159, 160) we first investigated the role of calpains in the degradation of CPE.

Interestingly, co-incubation of palmitate and a general calpain and cysteine protease inhibitor, E64D, prevented the palmitate-associated decrease in CPE. These results suggested that the molecular target(s) of E64D, likely cysteine proteases, may play a role in CPE degradation. However, while other groups have reported degradation products during calpain-mediated proteolysis (139, 158), we were not able to detect any additional CPE bands in Western blots from palmitate-treated cells at the time points studied, using the antibodies that were available. This may be a time dependent phenomenon where the presence of degradation products was not captured in our experiments. Additional time points may be necessary to capture these CPE degradation products. And while the exact mechanism has not been completely elucidated, E64D reduced the palmitate-induced decrease in CPE, indicating that calpain activation may be a cause for its reduction. Though E64D restored CPE levels, it was not able to prevent ER-stress or apoptosis, showing that palmitate-induced ER-stress and cell death could be dissociated from the reduction in CPE under these conditions.

Another inhibitor of the calpains, ALLM (a.k.a calpain inhibitor II), had dramatic effects on cell death on its own, however, unlike E64D, ALLM did not restore CPE protein levels. While there are discrepancies in the results between these two calpain inhibitors, the specificities of E64D and ALLM for each calpain family member have not yet been determined. These results suggest the possibility that the calpains targeted by ALLM are not involved in the degradation of CPE, while those of E64D are. However, the finding that ALLM was also cytotoxic on its own may have superceded its potential effects on inhibiting CPE reduction. Thus, with these findings, it is difficult to conclude definitively whether or not calpains are involved in the effects of palmitate.

One member of the calpain family, calpain-10, has been implicated in genetic susceptibility to type 2 diabetes. Clinical studies have shown that calpain-10 polymorphisms are linked to insulin resistance and hyperlipidemia and that calpain-10 gene expression is increased after insulin administration in lipid-infused skeletal muscle cells (84). These previous findings implicate calpain-10 in insulin signaling and lipid homeostasis. Furthermore, *in vitro* studies have also implicated calpain-10 in pancreatic β -cell survival and function. Importantly, palmitate-induced apoptosis was absent in islets from calpain-10 knockout mice (87). A recent study has also suggested that calpain-10 is present in mitochondria (140, 161), potentially strategically located to sense metabolic activity, and possibly explaining why 2-bromopalmitate was not able to reduce CPE levels. But, as of today, despite belonging to the calpain family, the precise function of the calpain-10 protein in the cell is still poorly understood. Thus, the role of calpain-10 as a diabetes susceptibility gene and its involvement in palmitate-induced cell death prompted an investigation into its potential role in CPE protein regulation. In the present studies, no consistent differences were found in calpain-10 protein expression in palmitate-treated islets. However, antibodies directed at calpain-10 recognize several bands that likely correspond to known splice variants (141), but may not recognize the less common variants potentially involved in CPE degradation. Adding calpain-10 to palmitate-treated MIN6 cells to examine its effects on CPE levels was considered, but unfortunately, the calpain-10 protein has not been purified and thus this experiment was not possible. Alternatively, however, co-incubation of MIN6 cells with various other calpains, such as the ubiquitously expressed calpain- μ and calpain- m could be performed to investigate whether similar effects on CPE would occur. While the role of the calpains in targeting CPE for degradation remains unclear in these studies, we did establish that E64D, a known inhibitor of calpains, and the prevention of Ca^{2+} influx could restore CPE levels.

4.3 Physiological consequences of palmitate-induced CPE degradation

In the present study, we found that CPE in MIN6 cells was decreased by the high concentrations of palmitate (6:1 palmitate to BSA) that one might expect in diabetes (124-126). Furthermore, we also found that treatment of human islets with palmitate showed increased secretion of proinsulin compared to control cells. These results agree with clinical studies showing that elevated proinsulin to insulin ratios are commonly observed in patients with diabetes (128-131, 162-164). Moreover, hyperproinsulinemia is a characteristic of the CPE^{fat/fat} mouse (114, 165). Interestingly, genetic loss of CPE also causes alterations in the levels of prohormone convertases (PC) 1/3 and 2 (166), suggesting that CPE may be involved in regulating PC1/3 and PC2. In addition, a previous study which examined the effect of 7-day FFA treatment (1:2 palmitate to oleate) on MIN6 cells found defective insulin processing and implicated alterations in PC2 and PC3 post-translational processing (127). Our study is the first to demonstrate that CPE is a target for FFAs in general and palmitate in particular. Thus, palmitate may target multiple insulin processing enzymes, perhaps CPE first, and ultimately leading to increased secretion of proinsulin. And while proinsulin is not known to be physiologically detrimental, it only has 10% of the biological activity of insulin (132-134), potentially making whole body glucose uptake more difficult.

In this study, we also examined the effect of a 16-week high-fat diet on CPE levels in C57Bl6/J mice. Western blots indicated that there were no differences in CPE expression between chow and high-fat diet (HFD) mice. However, while the HFD mice were glucose intolerant, these mice were not diabetic. Furthermore, serum fatty acid levels were not measured, therefore, we cannot rule out the possibility that these levels were not high enough to mimic the levels of palmitate found in our *in vitro* experiments. Examination of the *ob/ob* mouse, a mouse model known to have circulating levels of total FFA of approximately 1.5 mM (142), also did not reveal differences in islet CPE expression. While together these results suggest that high circulating levels of FFAs may not be enough to reduce CPE levels *in vivo*, more examination of the specific circulating FFAs is required. Because the sera of animals is likely made up of a complex mixture of palmitate, oleate and other FFAs, it would be interesting to examine the effects of combining saturated and unsaturated FFAs on CPE expression *in vitro*.

While changes in CPE protein were not observed in the *in vivo* experiments of this study, the potential link between CPE and the pathogenesis of type 2 diabetes is intriguing. Furthermore, the discovery of various mutations in the CPE gene, both in diabetic mice (112, 114, 143) and

humans (117), suggest that CPE may play a vital role in the susceptibility to type 2 diabetes. In addition, the potential role for CPE as an auto-antigen in certain sub-classes of type 2 diabetes (118, 167, 168), suggests that CPE may serve as another marker for detecting diabetes in humans.

5.0 Conclusion

In this study, it was established that exposure of both MIN6 cells and human islets to palmitate led to a marked decrease in CPE protein. The effects on CPE and cell death required palmitate metabolism and Ca^{2+} influx via L-type Ca^{2+} channels. While the cysteine protease inhibitor E64D reversed the palmitate-induced decrease in CPE, its molecular targets and its role in ER-stress or β -cell death were not resolved. Islets from CPE mutant mice exhibited increased CHOP induction, caspase 3 cleavage and TUNEL compared to control mice. Palmitate effects were not additive in these islets suggesting the possibility that CPE is required for palmitate-induced ER-stress and apoptosis. On the other hand, palmitate-treated MIN6 cells over-expressing CPE showed less ER-stress and apoptosis. Together, these results provide the first evidence that CPE, previously thought to play a house-keeping role, is involved in β -cell death. Overall, the findings of this study are intriguing and suggest novel links between hyperlipidemia, insulin processing, and β -cell death pathways.

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