Carboxypeptidase E mediates palmitate-induced β-cell ER stress and apoptosis

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Obesity is a principal risk factor for type 2 diabetes, and elevated fatty acids reduce β-cell function and survival. An unbiased proteomic screen was used to identify targets of palmitate in β-cell death. The most significantly altered protein in both human islets and MIN6 β-cells treated with palmitate was carboxypeptidase E (CPE). Palmitate reduced CPE protein levels within 2 h, preceding endoplasmic reticulum (ER) stress and cell death, by a mechanism involving CPE translocation to Golgi and lysosomal degradation. Palmitate metabolism and Ca^{2+} flux were also required for CPE proteolysis and β-cell death. Chronic palmitate exposure increased the ratio of proinsulin to insulin. CPE null islets had increased apoptosis *in vivo* and *in vitro*. Reducing CPE by ≈30% using shRNA also increased ER stress and apoptosis. Conversely, overexpression of CPE partially rescued β-cells from palmitate-induced ER stress and apoptosis. Thus, carboxypeptidase E degradation contributes to palmitate-induced β-cell ER stress and apoptosis. CPE is a major link between hyperlipidemia and β-cell death pathways in diabetes.

2D difference gel electrophoresis proteomics | free fatty acids | hyperproinsulinemia | mechanisms of β-cell lipotoxicity | type 2 diabetes

There is a strong association between type 2 diabetes and obesity. High levels of circulating lipids, including free fatty acids, are a prominent clinical feature of type 2 diabetes and represent an important risk factor for this disease (1, 2). But exactly how elevated lipids might lead to diabetes remains unresolved. Fatty acids increase basal insulin secretion (3) and the relative levels of circulating proinsulin (4). Chronic exposure to the free fatty acid palmitate has been shown to impair glucose-stimulated insulin release (i.e., lipotoxicity) (5–10). β-Cell apoptosis can be initiated by high levels of palmitate (6, 7, 11–14), which may account in part for alterations in insulin secretory function (13). A number of studies have established palmitate targets in the β-cell, including lipid metabolism (15, 16), mitochondrial function (17–23), and prosurvival transcription factors such as Pdx1 (24, 25). Recently, a role for endoplasmic reticulum (ER) stress in lipotoxicity has been demonstrated in multiple cell types, including β-cells (11, 26, 27). The effects of palmitate on β-cell survival are likely mediated by a number of mechanisms.

In the present study, we conducted unbiased proteomic screens using human islets and MIN6 β-cells to elucidate targets of palmitate. Carboxypeptidase E (CPE) was the most significantly changed protein in both screens. Mice lacking CPE develop hyperproinsulinemia and hyperglycemia (28), but the involvement of this protein in β-cell apoptosis has not been reported. Palmitate caused the rapid intracellular redistribution and degradation of CPE via mechanisms that required palmitate metabolism, K_{ATP} channel closure, Ca^{2+} influx, and protease activity. We further showed that CPE levels control β-cell ER stress and apoptosis. Thus, CPE is a critical link between saturated free fatty acids, a major type 2 diabetes risk factor, and β-cell dysfunction.

Results

Analysis of the β-Cell Proteome During Palmitate-Induced Apoptosis. Palmitate, but not oleate, induced dose-dependent apoptosis, illustrated by DNA laddering [supporting information (SI) Fig. S1*A*]. A high-throughput, real-time assay of β-cell death demonstrated that palmitate-induced propidium iodide incorporation was significant at ≈12 h. Neither the rate nor the amplitude of palmitate-induced death differed between 5 mM glucose and 25 mM glucose (Fig. S1*B*). Palmitate did not require high glucose to induce ER stress in MIN6 cells (Fig. S1*C*) or human islets (Fig. S1*D*; see also Fig. S4*B*). We have shown previously that palmitate could induce apoptosis in mouse islets cultured in 5 mM glucose (29). To better understand the mechanism of β-cell lipotoxicity, we undertook proteomic analyses of MIN6 cells and human pancreatic islets exposed to palmitate. Strikingly, the gel features most reduced by palmitate in both MIN6 cells and human islets were identified as CPE (Fig. 1 and Figs. S2 and S3). Significant changes were also found in protein spots identified as key metabolic enzymes (NADH dehydrogenase, phosphoglycerate mutase, ATP citrate lyase), and components of the proteasome (UCHL1, proteosome subunit α3) (Tables S1 and S2). Because of its known role in insulin processing and diabetes, we focused primarily on CPE in this study.

Palmitate Causes Rapid CPE Degradation. The reduced protein spots identified as CPE in the 2-D gels were the approximate size of full-length CPE, rather than fragments. To exclude the possibility that the observed decrease in relative abundance of the gel features was due solely to posttranslational modifications that alter the isoelectric point, we performed Western blot analysis. These experiments demonstrated that palmitate significantly and dose dependently decreased total CPE protein in both MIN6 cells and human islets (Fig. 2*A* and Fig. S4*A* and *B*), with the strongest inhibition seen at a 6:1 palmitate-to-albumin ratio, a dose similar to what would be expected in obese or diabetic patients (30, 31). Similar results were seen when GAPDH or tubulin were used as loading controls (not shown). A loss of CPE was also detected with antibodies to the C-terminal and N-terminal regions (Fig. S4*C*), suggesting loss of the entire protein. A related enzyme, carboxypeptidase D, was not reduced by palmitate in low glucose (Fig. S4 *D* and *F*). Prohormone convertase 1/3 and prohormone convertase 2 were

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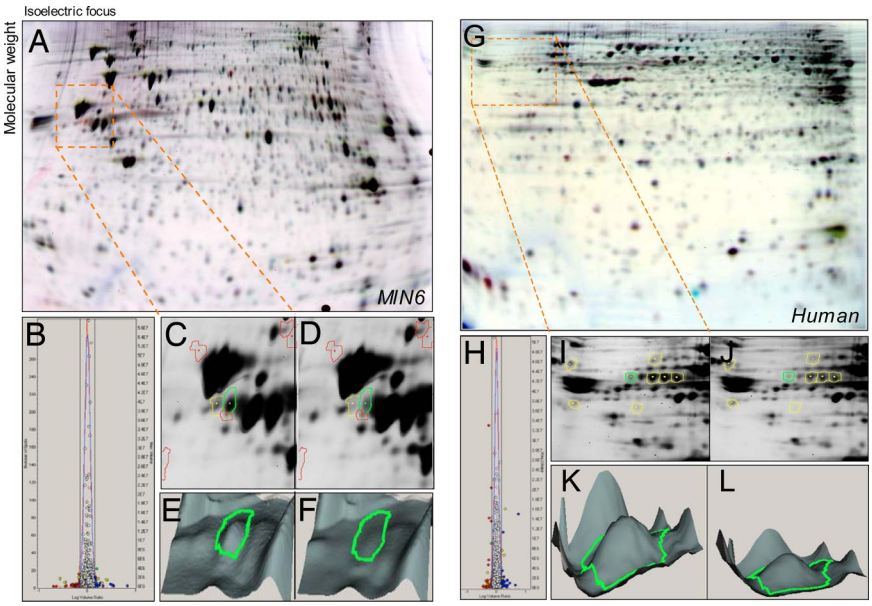

Fig. 1. Fluorescence-based analysis of palmitate-induced changes in the MIN6 and human islet cell proteomes under conditions of β-cell apoptosis and ER stress. (*A*) In 2D gels of MIN6 cells treated with palmitate for 24 h, red spots represent decreased protein species, whereas blue spots show increased proteins. (*B*) Normalization of all spot volumes across treatments revealed significantly different spots (>2 SD). (*C–F*) *C* is a close-up of orange box in *D* and 3D rendering (*E* and *F*) of the most altered gel feature between control (*Left*) and palmitate-treated (*Right*) cells. (*G–L*) The identical proteomics screen performed on human islets also identified CPE as the most changed gel feature.

not significantly altered by 24-h culture in palmitate or glucose (Fig. S4 *D*, *G*, and *H*). Together, these findings indicate that palmitate specifically reduces the total levels of CPE protein.

We tested whether altered CPE translation or transcription were involved in the loss of CPE. As expected, cycloheximide alone decreased CPE protein synthesis, indicating that a large fraction of CPE is degraded in less than 24 h. The co-incubation of cells with palmitate and cycloheximide resulted in a further decrease in CPE expression compared to the effects of cycloheximide alone (Fig. S4*I*), suggesting that palmitate caused CPE degradation. RT-PCR of palmitate-treated MIN6 cells suggested that palmitate did not affect steady-state CPE mRNA (Fig. S4 *J* and *K*). CPE levels were not significantly altered by high glucose, indicating that CPE was not lost via exocytosis (Fig. S4 *A–D*, and *I*). In our hands, palmitate is a weak insulin secretagogue compared to glucose (Fig. S5). We used a protease inhibitor to directly test the hypothesis that CPE was degraded in response to palmitate. Indeed, the cysteine protease inhibitor E64D prevented the palmitate-induced reduction in CPE protein (Fig. 2*B*). These data strongly support the concept that palmitate caused CPE proteolysis.

Soluble and membrane-bound forms of CPE reside in secretory granules, Golgi, and ER (32, 33). In the present study, we examined the subcellular location of CPE in the presence and absence of palmitate. In control conditions, CPE was found both within and outside of secretory granules. Palmitate caused CPE to be lost from insulin granules (Fig. 2*C*). In the presence of palmitate, there was a dramatic accumulation of CPE in Golgi and lysosomes (Fig. 2 *D* and *E*). Experiments were repeated with similar results with other CPE antibodies (not shown). Thus, the loss of CPE protein is associated with its redistribution to Golgi and lysosomes, where it is presumably degraded by E64D-sensitive proteases. Taken together with the rapidity of the CPE loss (see below), these results suggest that CPE is targeted for degradation in palmitate-treated β-cells.

Mechanisms of CPE Degradation, ER stress, and β-Cell Death. Because of the apoptotic effects of palmitate, it was essential to determine whether the decrease in CPE protein was secondary to an increase in ER stress and cell death. The palmitate-induced loss of CPE, already maximal at 2 h, preceded CHOP induction (6 h; Fig. 3*A* and *B*) and the beginning of widespread cell death (12 h, Fig. S1*B*). In addition, CPE was unaffected by thapsigargin (Fig. 3*C*), a drug known to induce β-cell ER stress and death. Together, these results further show 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.

Next, the role of cellular palmitate metabolism in the loss of CPE was investigated by using the nonmetabolizable palmitate homolog 2-bromopalmitate. Incubation of both MIN6 cells (Fig. 3 *E* and *F*) and human islets (not shown) with bromopalmitate at low or high glucose did not significantly decrease CPE protein. CHOP expression was induced by palmitate and, to a lesser extent, bromopalmitate (Fig. 3*G*). This indicated that some ER stress can occur under conditions where CPE is not significantly altered, especially in high glucose conditions. Cell death in the presence of bromopalmitate was significantly retarded compared with palmitate-treated cells at both low and high glucose (Fig. 3*H*). Taken together, these data illustrate that palmitate metabolism is important for the loss of CPE and maximal β-cell death.

Palmitate is known to increase Ca^{2+} influx into β-cells (17), and we have confirmed this in MIN6 cells and human β-cells (J.D.J., unpublished data). It would be expected that palmitate metabolism, but not activation of the FFA receptor GPR40, would increase the β-cell ATP to ADP ratio, close K_{ATP} channels, and activate voltage-gated Ca^{2+} channels in the plasma membrane. Diazoxide, a K_{ATP} channel activator, blocked palmitate-induced MIN6 cell death (Fig. S4 *L* and *M*), further implicating metabolism in the toxic effects of palmitate. Incubation of MIN6 cells with a dose of nifedipine known to completely block β-cell voltage-gated Ca^{2+} currents (34) prevented the loss of CPE protein, attenuated CHOP induction (Fig. 3 *I–K*), and prevented palmitate-induced cell death (Fig. 3*L* and Fig. S4 *N*). These results suggest that the effects of palmitate on CPE, as well as its effects on ER stress and cell death, are Ca^{2+}-dependent.

Hyperproinsulinemia is found in type 2 diabetics, and some studies have suggested that this condition can predict the onset of diabetes (35, 36). Mice lacking functional CPE also exhibit elevated plasma proinsulin levels (28). After 24 h, palmitate, but not bromopalmitate, significantly increased the ratio of proinsulin to insulin that had been secreted into the medium by human islets (Fig. 3*D*), suggesting a defect in insulin processing. Acute palmitate treatment did not alter the secreted proinsulin-to-insulin ratio (Fig. S5 *B–D*). These findings are consistent with the idea that a chronic reduction in CPE accounts for the defect in proinsulin processing.

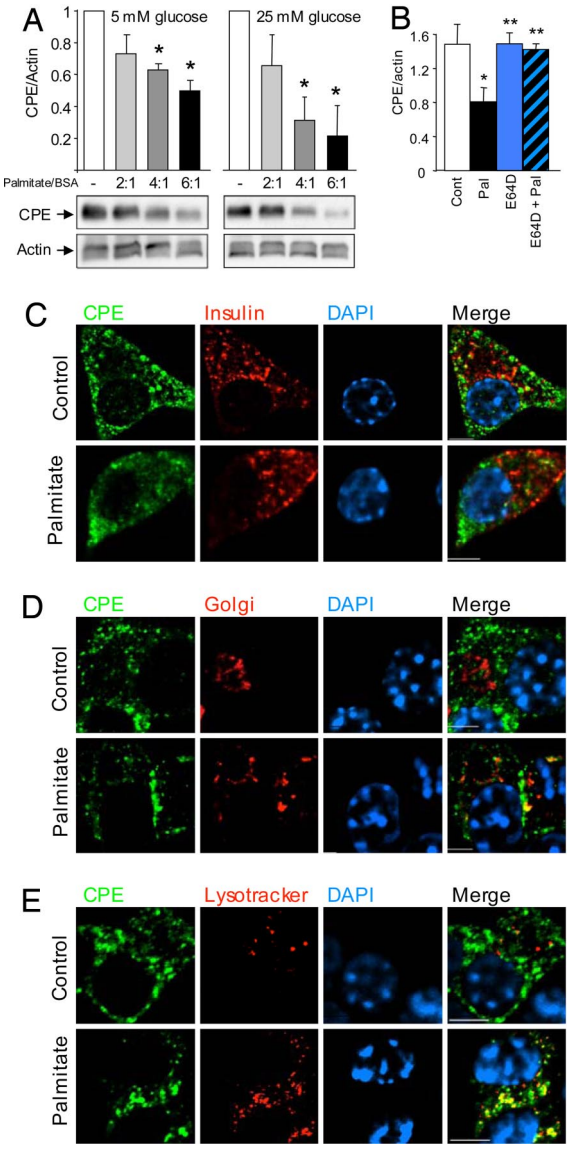

Fig. 2. Palmitate causes CPE degradation. (*A*) CPE levels in MIN6 cells treated for 24 h with different ratios of palmitate to BSA, in 5 mM or 25 mM glucose. The asterisk denotes a significant difference from the control ($n = 4$). (*B*) Quantification of Western blot of MIN6 cells treated with palmitate (6:1) and/or 25 μM E64D for 24 h at 25 mM glucose. The asterisk denotes significant difference from the control; the double asterisk denotes significant difference from palmitate treatment ($n = 3$). (*C–E*) Representative images of the colocalization of CPE with respect to insulin, Golgi marker (GM130), and lysosomes are shown. (Scale bars, 5 μm.)

CPE Regulates Islet Survival *in Vivo* and *in Vitro*. The effects of lipids on CPE levels *in vivo* were assessed by subjecting C57BL/6J mice and their littermate controls to a high-fat diet. Analysis of islets isolated from these mice demonstrated that hyperlipidemia decreased CPE protein expression *in vivo* (Fig. 4*A*). The ratio of CPE-processed mature insulin to total insulin was dramatically decreased in islets from high-fat-fed mice and in MIN6 cells treated with palmitate. This decrease in mature insulin was comparable to that seen in mice lacking CPE (Fig. S6). The studies described above show that CPE protein is decreased under conditions in which FFA induces ER stress and β-cell death. We next used both loss-of-function and gain-of-function experiments to test the hypothesis that CPE is a causal factor in β-cell apoptosis. We took advantage of mice deficient in CPE (Fig. 4*B*) to determine whether CPE plays a role in β-cell survival *in vivo* and *in vitro*. Pancreatic sections from CPE$^{fat/fat}$ mice showed significantly more TUNEL-positive cells in their islets compared to those of littermate controls (Fig. 4 *C* and *D*). Moreover, although CPE$^{fat/fat}$ islets generally covered a larger area, there was a striking loss of normal architecture, with marked cell loss within the islet (Fig. 4*E* and Fig. S6). A caveat with these experiments is that the CPE$^{fat/fat}$ mice exhibit hyperglycemia (Fig. 4*B*) and it is possible that chronic exposure to high glucose could augment β-cell death in this model. To determine whether islets of the CPE$^{fat/fat}$ mice were more susceptible to apoptosis in a controlled *in vitro* setting, we exposed isolated islets to palmitate for 24 h. Even in the absence of palmitate, islets from CPE$^{fat/fat}$ mice showed significantly higher CHOP and caspase-3 activation (Fig. 4 *F* and *G*), consistent with the *in vivo* findings described above. Palmitate increased CHOP expression and cleaved caspase-3 levels in wild-type islets, but the effects of palmitate were not additive to the caspase-3 activation induced by CPE deficiency (Fig. 4 *F* and *G*). In preliminary experiments, we also observed an increase in cleaved caspase-3 in freshly isolated CPE$^{fat/fat}$ mouse islets (data not shown). These results suggested that the islets of mice lacking CPE have higher basal levels of ER stress and apoptosis, both *in vivo* and *in vitro*. The observation that palmitate did not induce further caspase-3-dependent apoptosis in CPE$^{fat/fat}$ islets suggests that the suppression of CPE may play an essential role in palmitate-induced β-cell death.

Next, we sought to further verify the causal link between CPE deficiency and increased β-cell ER stress and apoptosis. Using a combination of plasmid-based RNA interference and fluorescence-activated cell sorting (to enrich for shRNA-GFP expressing cells), we were able reduce CPE protein levels in MIN6 cells by ≈30%. This modest, but significant, decrease in CPE expression was sufficient to significantly increase levels of CHOP and cleaved caspase-3, relative to control cells transfected with scrambled shRNA-GFP (Fig. 4 *H–K*). Together, with the results presented above, this experiment demonstrates that a reduction in CPE similar to that caused by palmitate significantly increases β-cell apoptosis.

Finally, we determined whether an increase in CPE might be able to rescue β-cells from palmitate-induced death, by exploring the effects of overexpressing CPE in palmitate-treated MIN6 cells. Using a vector with a CMV promoter, we were able to transiently overexpress CPE by 1.5 fold (Figs. 4*L* and S7*A*), whereas only minor over-expression was observed with the rat insulin promoter (data not shown). Palmitate treatment caused a similar relative reduction of CPE in CPE overexpressing and untransfected cells. However, the absolute level of CPE remained higher in the palmitate-treated CPE overexpressing cells compared to the empty-vector transfected cells. CPE overexpression itself caused an increase in ER stress, as might be expected with the increased ER protein load. For this reason, the results of palmitate treatment were normalized to their respective controls. 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 (Fig. 4 *M* and *N*). Together, these findings suggest that increased CPE levels can limit the deleterious effects of palmitate on β-cells and suggest a role for CPE in the control of β-cell ER stress and apoptosis.

Discussion

Pathways involved in fatty acid-induced ER stress and β-cell apoptosis were investigated in this study. An unbiased proteomics screen elucidated several unexpected targets for the saturated fatty acid palmitate. CPE was the most decreased protein in two independent screens using MIN6 cells and human islets. 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, and to cause diabetes in humans and animals (37, 38). In the CPE$^{fat/fat}$ mouse strain, a single point mutation

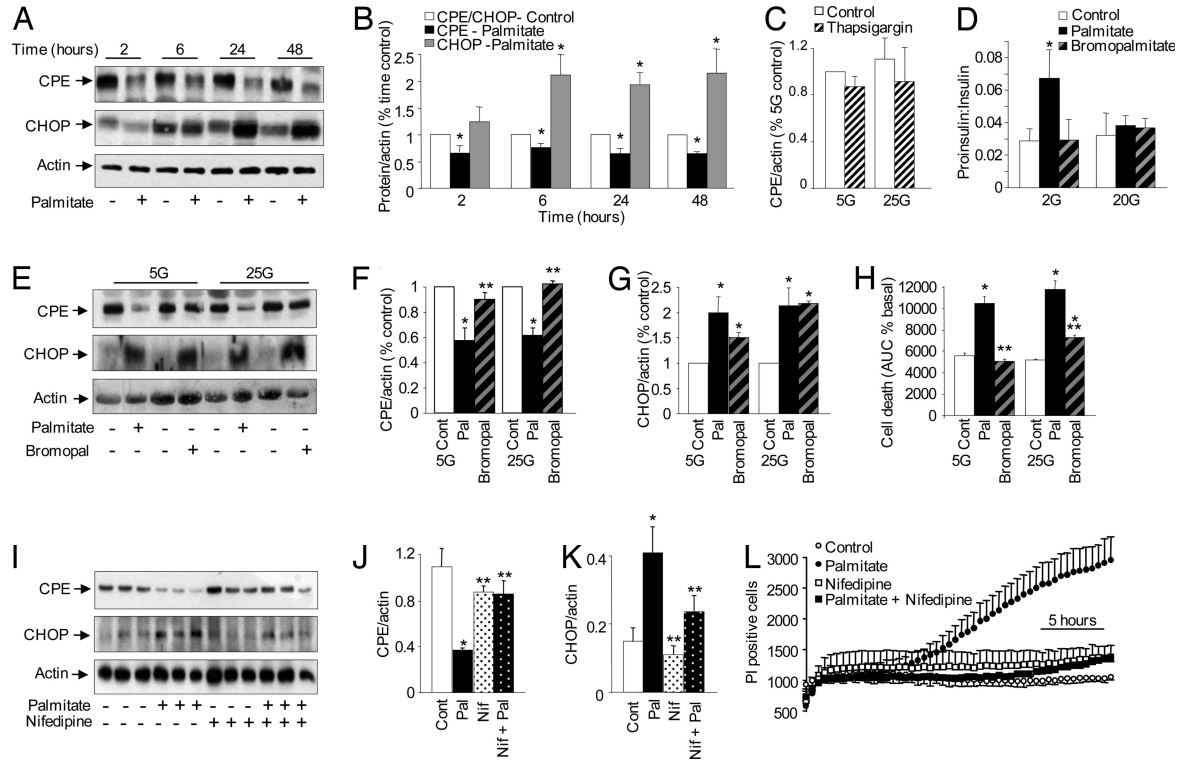

Fig. 3. Ca^{2+}-dependent mechanisms of palmitate-induced CPE degradation, ER stress, and β-cell death and dysfunction. (*A* and *B*) MIN6 cells incubated with palmitate at 25 mM glucose at specific time points ($n = 3$). (*C*) Twenty-four-hour incubation of MIN6 cells with 1 μM thapsigargin did not affect CPE expression ($n = 3$). (*D*) The molar ratio of proinsulin to insulin secreted from human islets treated with palmitate and 2-bromopalmitate over 48 h ($n = 5$). (*E–G*) CPE and CHOP expression in MIN6 cells treated for 24 h with palmitate or 2-bromopalmitate at low or high glucose ($n = 3$). (*H*) Propidium iodide incorporation in MIN6 cells treated with palmitate or 2-bromopalmitate over 24 h ($n = 3$). (*I–K*) Western blot and quantification of CPE and CHOP in MIN6 cells treated with palmitate and 10 μM nifedipine for 24 h at 25 mM glucose ($n = 3$). (*L*) Propidium iodide incorporation in MIN6 cells at 25 mM glucose with palmitate and 10 μM nifedipine ($n = 3$). The asterisk denotes a significant difference from control; the double asterisk denotes a significant difference from palmitate treatments.

in CPE is sufficient to produce an animal with multiple disorders including obesity and diabetes (28, 39). Importantly, one study found CPE polymorphisms associated with type 2 diabetes in humans (40). The finding that changes in CPE protein levels may mediate the adverse effects of the saturated fatty acid palmitate on β-cell function and may contribute to the pathogenesis of diabetes is an important advancement in our understanding of the molecular pathways involved in the progression of this disease.

The rapid loss of CPE in palmitate-treated cells, and the insensitivity of CPE levels to thapsigargin treatment, placed CPE upstream of ER stress and apoptosis in the β-cell. The findings that the loss of functional CPE leads to apoptosis *in vivo* and *in vitro*, and that CPE overexpression partially rescues β-cells from palmitate-induced ER stress and death, demonstrate a previously unappreciated role for CPE in programmed cell death. FFAs have been shown to induce ER stress (i.e., expression of CHOP, spliced XBP-1, ATF4, and eIF2α) in multiple studies (11, 26, 27, 41–43), and ER stress is known to be induced under situations of protein overload in the secretory pathway (44, 45). Our results suggest that a component of palmitate-induced β-cell death can be explained by the loss of CPE, an event that is sufficient to cause ER stress potentially because of a backlog of unprocessed proinsulin in the secretory pathway.

Our studies also defined, to a large extent, the mechanism by which palmitate reduces CPE levels. The results point to a rapid CPE degradation that requires palmitate metabolism, ATP synthesis, plasma membrane depolarization, and Ca^{2+} influx. We and others (46, 47) have found that palmitate is more cytotoxic than 2-bromopalmitate. These results argue against a prominent role in β-cell death for the G protein-coupled FFA receptor GPR40, which nevertheless appears to be involved in β-cell Ca^{2+} signaling and insulin secretion (48, 49). This conclusion is supported by studies of GPR40 transgenic and knockout mice. Overexpression of GPR40 in mice led to overt diabetes, but morphological analysis of the pancreas showed no evidence of reduced β-cell mass (49). Although GPR40 knockout mice were protected from developing diabetes following a high-fat diet *in vivo*, islets of these mice were not rescued from palmitate-induced apoptosis *in vitro* (49, 50).

We and others have established that palmitate increases cytosolic Ca^{2+} levels in primary β-cells and β-cell lines (17, 51, 52). In our experiments, blocking Ca^{2+} influx with nifedipine abolished the palmitate-associated decrease in CPE and the induction of ER stress, and prevented palmitate-induced death in MIN6 cells. Cell death was also prevented by co-incubation of cells with diazoxide, a K_{ATP} channel activator that prevents ATP-dependent membrane depolarization and subsequent Ca^{2+} influx through L-type Ca^{2+} channels. Our results show that nifedipine can prevent the palmitate-associated reduction in CPE, and other studies have also investigated the potential link between Ca^{2+} and CPE. Notably, local Ca^{2+} has been implicated in the control of CPE stability in the trans-Golgi network (53, 54). 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 degradation of CPE.

Efforts to pinpoint the exact protease(s) responsible for the loss of CPE were only partially successful. E64D is a general inhibitor of cysteine proteases, and its targets include both lysosomal proteases and calpains. This drug prevented the palmitate-induced loss of CPE, a finding that agrees well with our data showing CPE

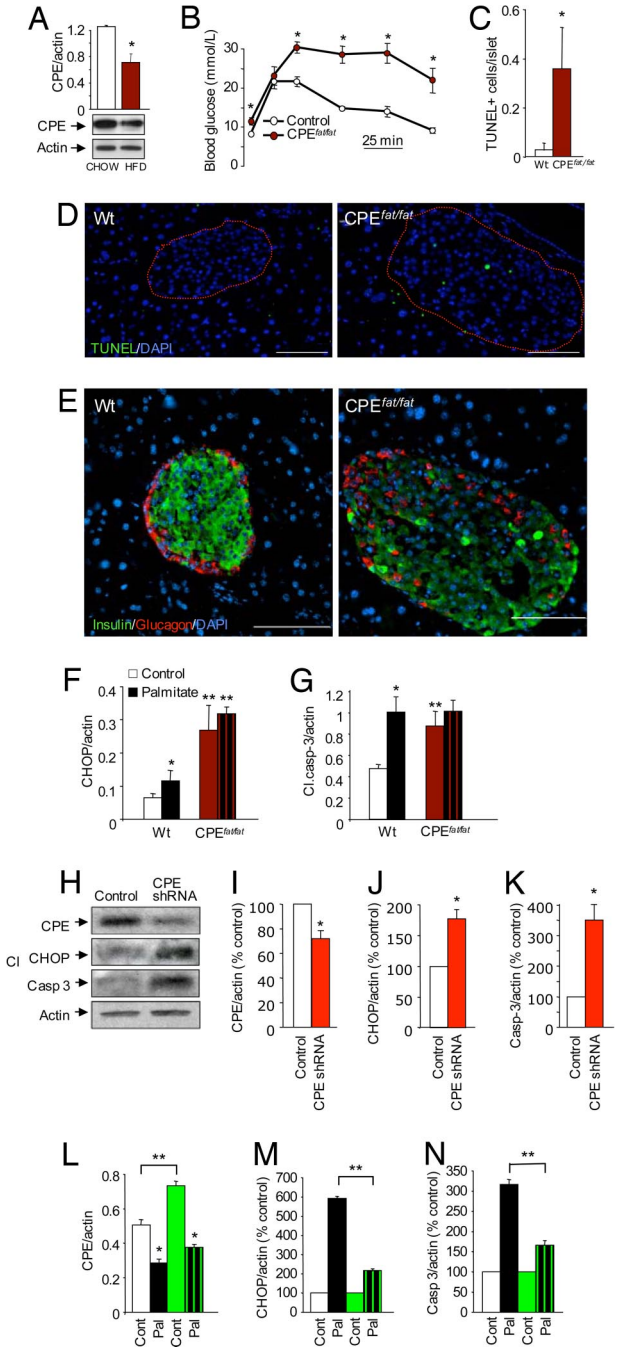

Fig. 4. *In vivo* and *in vitro* role of CPE in β-cell death. The ratio of CPE-processed mature insulin to total insulin was dramatically decreased in islets from high-fat-fed mice and in MIN6 cells treated with palmitate. This decrease in mature insulin was comparable to that seen in mice lacking CPE (Fig. S6). (*A*) Reduced CPE protein expression in isolated islets from C57BL/6J mice fed high-fat diet (40% calories from fat) for 6 months ($n = 3$). (*B*) Homozygous null CPE$^{fat/fat}$ mice were significantly heavier (28 ± 2 g vs. 41 ± 3 g) and exhibited significantly impaired i.p. glucose tolerance compared with littermate controls ($n = 4$). (*C* and *D*) Increased TUNEL staining in islets of pancreatic sections of CPE$^{fat/fat}$ mice compared with wild-type controls ($n = 3$). (Scale bar, 100 μm.) (*E*) Representative insulin (green), glucagon (red), and DAPI (blue) staining in the islets of CPE$^{fat/fat}$ mice and wild-type controls. Islets from mutant mice had weaker and more heterogeneous insulin staining, as well as disrupted architecture. (*F* and *G*) CHOP and cleaved caspase-3 protein were quantified in isolated islets from control and CPE$^{fat/fat}$ mice treated as indicated for 24 h in 20 mM glucose ($n = 3$). (*H–K*) Significant small hairpin RNA-mediated reduction in CPE protein increases CHOP expression and caspase-3 cleavage, compared with MIN6 cells transfected with a scrambled shRNA control ($n = 3$). (*L–N*) MIN6 cells were transfected with a CPE plasmid (green bars) with palmitate for 24 h at 25 mM glucose ($n = 3$). The asterisk denotes significance between palmitate and control; the double asterisk denotes significance between vectors within the same treatment.

translocation to lysosomes. However, both E64D and another protease inhibitor (ALLM; data not shown) caused ER stress on their own, likely because of a backlog of undegraded proteins, making interpretation of their effects on palmitate-induced β-cell death difficult. An inhibitor of UCHL1, a component of the ubiquitin system and the 2-D gel feature most increased by palmitate in the proteomic analysis, prevented the loss of CPE in response to palmitate in high glucose, but not low glucose (Fig. S7*B*). In our previous study, ALLM and specific deletion of calpain-10 partially reduced apoptosis in primary mouse islets in long-term cultures containing palmitate and 5 mM glucose (29). However, the link between the calpain-10 pathway and the ER stress-associated cascade that CPE participates in remains unclear.

Our results demonstrated that palmitate, but not bromopalmitate, increased the proinsulin-to-insulin ratio at physiological glucose concentrations. This indicates another important functional consequence of CPE degradation. An elevated proinsulin-to-insulin ratio is a well established clinical finding in diabetes and it has also been suggested to play a possible role in prediabetic states (35, 36). Our results provide a plausible mechanism for this defect. While our work demonstrates effects of FFAs on CPE, a previous study found reduced PC2 and PC1/3 posttranslational processing and increased proinsulin-to-insulin ratio in MIN6 cells treated with FFA for 7 days (4). Interestingly, CPE is required for the processing of prohormone convertases, and hyperproinsulinemic mice lacking CPE have reduced prohormone convertase (PC) 1/3 and 2 activity (55). CPE also reduces the ability of prohormone convertase products to inhibit PC1/3 and 2 (56). Thus, CPE may regulate PC1/3 and PC2 at multiple levels. Because we did not observe significant effects of palmitate on PC1/3 or PC2 at 24 h, it is likely that the loss of CPE precedes the loss of the prohormone convertases. Thus, palmitate may target multiple insulin-processing enzymes, perhaps CPE first, ultimately leading to a relative increase in the secretion of proinsulin, which has only 10% of the biological activity of insulin (57).

In conclusion, it was established in this study that *in vitro* treatment of both MIN6 cells and human islets with palmitate and *in vivo* exposure to a high-fat diet led to a reduction in CPE protein. Previously, CPE has been considered to be a "housekeeping" enzyme; here we demonstrate that CPE can be degraded in response to an extracellular signal in β-cells. Both loss-of-function and gain-of-function approaches suggested that CPE positively controls β-cell survival, via effects on ER stress. We propose a model for β-cell lipoxicity, enabled by our unbiased proteomic screens (Fig. S8). Together, these results provide evidence that CPE is a key link between hyperlipidemia/FFAs, insulin processing, and β-cell apoptosis pathways in type 2 diabetes

Experimental Procedures

Reagents and Animals. A detailed list of reagents can be found in the *SI Text*. CPE$^{fat/fat}$ mice on the C57BL/6J background were from Jackson Laboratories. Intraperitoneal glucose tolerance tests (IPGTT; 2 g glucose/kg body weight) were performed on littermate males after a 12-h fast. In some studies, male C57BL/6J mice were fed a high-fat diet (40% of calories from fat, TD88137; Harlan Teklad) for 6 months and compared to littermates fed normal chow. Baseline phenotypes of these mice are detailed in the Supplement. All studies were approved by the University of British Columbia Animal Care Committee.

Cell Culture. Our human islet, mouse islet, and MIN6 cell culture methods have been described (25, 29, 58) and are detailed in the *SI Text*. Hormone release was assessed by static incubation or perifusion (25). Insulin and proinsulin were assayed with Linco RIA or ELISA, respectively. Immunofluorescent staining is described in *SI Text*.

Cell Death and Apoptosis Assays. The incorporation of propidium iodide was monitored in the incubated chamber of a KineticScan Reader (Cellomics). Propidium iodide fluoresces brightly once it passes through the compromised plasma membrane of dying cells. Additional details on this assay and the TUNEL assay are outlined in the *SI Text*.

Immunoblot and Proteomics. We used standard methods for immunoblots, and our approach to 2-D-DIGE proteomics of human islets has been described (see *SI Text* for expanded details). Briefly, lysates from control and palmitate-treated cells were labeled with Cy dyes and analyzed in the same 2-D gel. Individual ''spots'' were quantified and considered significantly different if they were >2 standard deviations outside of the normalized distribution of gel feature intensities. Significantly different spots were sequenced by using mass spectroscopy.

shRNA-Mediated CPE Knockdown and CPE Overexpression. CPE levels were reduced in MIN6 cells by using a vector expressing shRNA sequences designed against CPE (see *SI Text*) transfected with Lipofectamine 2000 (Invitrogen). GFP-positive cells were sorted by fluorescence-activated cell sorting (BD FACS Vantage SE/DIVA). CPE plasmids, one under the control of the CMV promoter, and another under the control of the rat insulin promoter, were kindly supplied by Dr. L. D. Fricker. CPE DNA (1 μg) or empty vector, was transfected into MIN6 cells by using Lipofectamine 2000 (Invitrogen).

Statistics. Statistical analysis was performed with SigmaStat (Systat). ANOVA (Student–Newman–Keuls) or *t* test was used where appropriate. $P < 0.05$ was considered significant.

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