

Selective Inhibition of Eukaryotic Translation Initiation Factor 2 α Dephosphorylation Potentiates Fatty Acid-induced Endoplasmic Reticulum Stress and Causes Pancreatic β -Cell Dysfunction and Apoptosis^{*[5]}

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Free fatty acids cause pancreatic β -cell apoptosis and may contribute to β -cell loss in type 2 diabetes via the induction of endoplasmic reticulum stress. Reductions in eukaryotic translation initiation factor (eIF) 2 α phosphorylation trigger β -cell failure and diabetes. Salubrinal selectively inhibits eIF2 α dephosphorylation, protects other cells against endoplasmic reticulum stress-mediated apoptosis, and has been proposed as a β -cell protector. Unexpectedly, salubrinal induced apoptosis in primary β -cells, and it potentiated the deleterious effects of oleate and palmitate. Salubrinal induced a marked eIF2 α phosphorylation and potentiated the inhibitory effects of free fatty acids on protein synthesis and insulin release. The synergistic activation of the PERK-eIF2 α branch of the endoplasmic reticulum stress response, but not of the IRE1 and activating transcription factor-6 pathways, led to a marked induction of activating transcription factor-4 and the pro-apoptotic transcription factor CHOP. Our findings demonstrate that excessive eIF2 α phosphorylation is poorly tolerated by β -cells and exacerbates free fatty acid-induced apoptosis. This modifies the present paradigm regarding the beneficial role of eIF2 α phosphorylation in β -cells and must be taken into consideration when designing therapies to protect β -cells in type 2 diabetes.

Type 2 diabetes develops in individuals who fail to compensate for insulin resistance by increasing pancreatic insulin output. This relative insulin deficiency results from pancreatic β -cell dysfunction (1) and/or death (2). Although genetic predisposition to the disease is an important risk factor, the rapid rise in the prevalence of type 2 diabetes over the past decades underscores the importance of environmental determinants in its pathogenesis (3). Western diets rich in saturated (animal) fats cause elevated plasma lipid levels. They are associated with obesity and insulin resistance in peripheral tissues, thereby increasing the demand on pancreatic β -cells. Concomitantly, β -cells are exposed to increased levels of free fatty acids (FFA)³ and other products, either of dietary origin or released from the enlarged fat depots. Chronic exposure to high concentrations of FFA causes β -cell apoptosis (4) and may thus contribute to the increased β -cell apoptosis rates and progressive β -cell loss in type 2 diabetes (2).

We have recently proposed that endoplasmic reticulum (ER) stress is one of the molecular mechanisms implicated in FFA-induced pancreatic β -cell apoptosis (5), and this was confirmed by others (6, 7). At approximately the same time, ER stress was proposed as a mechanism linking high fat diet-induced obesity with insulin resistance (8). Alterations of normal ER function and environment lead to accumulation of unfolded proteins and activate a specific ER stress response, also known as the unfolded protein response (UPR) (9). This ER stress response is mediated through three pathways under the control of the ER membrane proteins double-stranded RNA-activated kinase-like kinase (PERK), activating transcription factor (ATF)-6, and inositol requiring-1 α (IRE1 α), with the latter leading to splicing and activation of X-box-binding protein-1 (XBP-1). To decrease ER protein accumulation and recover ER function, the UPR: 1) attenuates translation via activation of PERK and subsequent phosphorylation of the eukaryotic translation initiation factor 2 α (eIF2 α) at Ser⁵¹; 2) increases the folding capacity of the ER by up-regulation of ER chaperones, such as BiP, via

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S10.

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³ The abbreviations used are: FFA, free fatty acid(s); ER, endoplasmic reticulum; UPR, unfolded protein response; PERK, double-stranded RNA-activated kinase-like kinase; ATF, activating transcription factor; IRE1 α , inositol requiring-1 α ; XBP-1, X-box-binding protein-1; eIF2 α , eukaryotic translation initiation factor 2 α ; sal, salubrinal; CPA, cyclopiazonic acid; BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

the transcription factors ATF-6 and XBP-1; and 3) degrades misfolded proteins via the ER-associated degradation pathway (10). However, when ER stress is prolonged or excessive, the pro-apoptotic transcription factor CHOP and other components of the apoptotic machinery are activated (11). The mechanisms linking ER stress and apoptosis remain incompletely understood (10, 12, 13).

Because of their high rate of protein synthesis, β -cells are particularly susceptible to changes in ER homeostasis (14, 15). Regulation of mRNA translation via phosphorylation of eIF2 α is crucial to maintain the integrity of the β -cell ER. Thus, homozygous mutations of *PERK* trigger β -cell death and diabetes in the human Wolcott-Rallison syndrome (16, 17) and in a knock-out mouse model (14). Even a partial reduction in eIF2 α phosphorylation, as in *Eif2s1^{+/tm1Rjk}* mice heterozygous for an alanine substitution at Ser⁵¹, is sufficient to trigger β -cell failure and diabetes when coupled to a high fat diet (15). By screening small molecules that protect cells from ER stress, we have previously identified a selective inhibitor of eIF2 α dephosphorylation, named salubrinal (sal) (18). Sal, in concentrations ranging from 10 to 75 μ M, protects the rat pheochromocytoma cell line PC-12 against apoptosis induced by the ER stressors tunicamycin, brefeldin (18), and cyclopiazonic acid (CPA) (present data). Based on these and previous observations (19–22), it has been suggested that eIF2 α phosphorylation is cytoprotective during ER stress.

The susceptibility of pancreatic β -cells to ER stress and apoptosis induced by FFA (5–7) but also by synthetic ER stress inducers and cytokines (23, 24) prompted us to explore potential therapies to modulate the β -cell response to ER stressors and increase its defense mechanisms. We presently evaluated the protective effects of sal against FFA-induced β -cell apoptosis. Unexpectedly, sal-induced eIF2 α phosphorylation was pro-apoptotic in pancreatic β -cells, and it potentiated the deleterious effects of the FFA oleate and palmitate. Our findings, together with previous observations (19–22), indicate that both deficient and excessive eIF2 α phosphorylation are poorly tolerated by pancreatic β -cells and trigger the apoptotic program. This modifies the present paradigm regarding the putative beneficial role of eIF2 α phosphorylation in β -cells.

EXPERIMENTAL PROCEDURES

Culture of Primary Autofluorescence-activated Cell Sorting-purified β -Cells and INS-1E Cells—Male Wistar rats (Charles River Laboratories Belgium, Brussels, Belgium) were housed and used according to the guidelines of the Belgian Regulations for Animal Care. The Ethical Committee for Animal Experiments of the Université Libre de Bruxelles approved the protocol. Rat islets were isolated by collagenase digestion followed by hand picking under a stereomicroscope. For β -cell isolation, the islets were dispersed, and the β -cells were purified by autofluorescence-activated cell sorting (FACStar; Becton Dickinson and Co., Sunnyvale, CA) (25, 26). The preparations used in the present experiments contained $87 \pm 1\%$ β -cells ($n = 11$). Purified β -cells were precultured overnight in Ham's F-10 medium with 10 mM glucose, 2 mM glutamine, 50 μ M 3-isobutyl-1-methylxanthine, 50 units/ml penicillin, 50 μ g/ml streptomycin, 5% heat-inactivated fetal bovine serum, and 0.5% char-

coal-absorbed bovine serum albumin (BSA) (27). During FFA exposure, the cells were cultured in the same medium with 1% BSA and no serum. The rat insulin-producing INS-1E cell line (a kind gift from Prof. C. Wollheim, Centre Medical Universitaire, Geneva, Switzerland) was cultured in RPMI 1640 (with GlutaMAX-I) containing 5% fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 50 μ M 2-mercaptoethanol (28) for 48 h prior to FFA exposure. The PC-12 cell line was cultured in F-12K Nutrient Mixture Kaighn's modification medium supplemented with 15% horse serum, 2.5% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Oleate and palmitate (sodium salt; Sigma) were dissolved in 90% ethanol, heated to 60 °C, and used in a 1:100 dilution (at a final concentration of 0.5 mM) in RPMI 1640 with 1% BSA and 1% fetal calf serum (5, 29, 30). Sal and derivative-3 were dissolved in Me₂SO. The control condition contained similar dilutions of ethanol and Me₂SO as appropriate.

Assessment of β -Cell Viability—The percentage of viable, apoptotic, and necrotic cells was determined after 12–72 h of exposure to sal with or without FFA. Autofluorescence-activated cell sorting-purified β - and INS-1E cells were incubated for 15 min with the DNA binding dyes propidium iodide (5 μ g/ml; Sigma) and Hoechst 33342 (5 μ g/ml; Sigma), as previously described (5, 31). This method is quantitative and has been validated by systematic comparison with electron microscopy (31) or determination of DNA strand breaks (5).

Western Blot Experiments—For Western blot analysis of eIF2 α phosphorylation on serine 51 residue (P-eIF2 α) or total eIF2 α , used as a loading control, 5×10^4 FFA-treated INS-1E cells were lysed in 15 μ l of whole cell extract buffer (150 mM NaCl, 50 mM NaF, 1 mM MgCl₂, 1 mM CaCl₂, 1% Nonidet P-40, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and 1 $\times$ complete protease inhibitor mixture (Roche Applied Science)) for 15 min. The total cell lysates of two duplicate wells were pooled and centrifuged (15 min at 14,000 rpm), and the supernatant was used for Western blot analysis. For the detection of ATF-4 and CHOP protein, 5×10^5 cells were collected in ice-cold phosphate-buffered saline, washed, and lysed by boiling in Laemmli sample buffer. The protein samples were separated on 10–12% SDS/PAGE gels, blotted onto a nitrocellulose membrane (Hybond-ECL; Amersham Biosciences), and probed with rabbit antibodies against P-eIF2 α (1:1000 dilution; Cell Signaling, Beverly, MA), eIF2 α (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), ATF-4 (1:2500 dilution; anti-CREB-2/ATF4 from Santa Cruz Biotechnology), GADD153/CHOP (1:250 dilution; Sigma-Aldrich), or β -actin (1:1000 dilution; Cell Signaling) as primary antibodies and a horseradish peroxidase-labeled donkey anti-rabbit antibody (1:3000 dilution; Santa Cruz Biotechnology) as secondary antibody. The protein-specific signals were detected using chemiluminescence, quantified using Biomax1D image analysis software (Kodak), and expressed as optical densities. The membranes were reused after stripping the primary antibody by incubation in 2% (w/v) SDS, 50 mM Tris/HCl, 150 mM NaCl, 100 mM 2-mercaptoethanol, pH 7.4, for 30 min at 50 °C in a shaking water bath.

Measurement of Protein Biosynthesis—For the measurement of protein synthesis in INS-1E cells, 6×10^4 cells were treated

with sal and/or FFA for 12–48 h. The last 2 h of the incubations were performed in 200 μ l of Krebs-Ringer buffer containing 1% BSA, 11 mM glucose, sal, and/or FFA and 10 μ Ci/ml L-(4,5- 3 H)-leucine (NET135H; PerkinElmer Life Sciences). Excess nonincorporated [3 H]leucine was removed by washing five times with Hanks' solution containing 10 mM nonradioactive L-leucine. The cells were extracted in 1 ml of buffer (11% acetic acid in water with 0.25% BSA), and the proteins were precipitated in 10% trichloroacetic acid. The content of 3 H-labeled protein was determined by measuring the radioactivity using a liquid scintillation analyzer (Packard, Meriden, CT) (32). Protein biosynthesis in INS-1E cells exposed to salubrinal for 24–48 h was expressed per total protein content, measured using the Bio-Rad protein assay and protein assay standard II (Bio-Rad GmbH, München, Germany), to correct for differences in cell number in the experimental conditions.

Insulin Measurements— β -Cell insulin release to the medium over a 24–72-h culture period was measured as previously described (33, 34). In brief, an anti-insulin antibody and 125 I-labeled insulin were added to the medium and incubated for 48–72 h at 4 °C. Two ml of a charcoal-dextran suspension was added to separate the antibody-insulin complex. After a 30-min incubation at 4 °C, the samples were centrifuged at 800 $\times$ g for 25 min. The radioactive content of the pellet was examined in a γ -counter. Insulin content was determined on acid ethanol-extracted INS-1E cells and expressed per total protein content.

Analysis of ER Stress Markers mRNA Expression by Real Time PCR—Poly(A) $^+$ RNA was isolated from INS-1E cells and reverse transcribed as previously described (35). The real time PCR amplification reaction was done in a volume of 20 μ l containing 2.5–5 mM MgCl $_2$, 0.5 μ M forward and reverse primers, 2 μ l of SYBR Green PCR master mix (Qiagen, Hilden, Germany), and 2 μ l of cDNA. Standards for each gene were prepared using appropriate primers in a conventional PCR. The samples were assayed on a LightCycler instrument (Roche Applied Science), and their concentrations were calculated as number of copies using the standard curve approach (36). The expression level of the gene of interest was corrected for that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The specific primers for spliced *XBP-1* were F 5'-GAGTCCGCAG-CAGGTG-3' and R 5'-GCGTCAGAATCCATGGGA-3'; for *GADD34* they were F 5'-AGGGATGTGGAGAAGCA-GAG-3' and R 5'-AAAGATCTGAGCCGCTTCTG-3', yielding PCR fragments of 65 and 123 bp, respectively. The sequence of *GAPDH*, *CHOP*, *ATF-4*, total *XBP-1*, and *BiP* primers have been reported elsewhere (5, 24).

Analysis of UPR Promoter Activity—To evaluate the functionality of the transcriptional activation of the IRE1 and ATF-6 pathways by sal and FFA, we used an UPR luciferase reporter construct kindly provided by Prof. Prywes (Columbia University, New York, NY). This element functions as binding site for both *XBP-1* (37) and *ATF-6* (38). INS-1E cells were cotransfected with this luciferase reporter construct and with pRL-CMV (Promega, Madison, WI) as internal control using Lipofectamine 2000 (Invitrogen) (39). Luciferase activities were assayed with the dual luciferase reporter assay system (Promega) as previously described (39, 40).

Statistical Analysis—The data are presented as the means $\pm$ S.E. Comparisons were performed by two-sided paired or ratio *t* test or by analysis of variance followed by paired *t* test with the Bonferroni correction for multiple comparisons. A *p* value <0.05 was considered statistically significant.

RESULTS

Salubrinal Does Not Protect β -Cells from ER Stress—In a first series of experiments we confirmed that sal protects the pheochromocytoma cell line PC-12 from ER stress-mediated apoptosis induced by the SERCA 2 blockers thapsigargin and CPA. In good agreement with previous findings (18), sal (75 μ M) reduced thapsigargin- or CPA-mediated apoptosis in PC-12 cells (supplemental Fig. S1). Sal *per se* was not toxic to PC-12 cells (supplemental Fig. S1).

We next examined whether sal protects β -cells from ER stress. At variance with the PC-12 cells, sal exerted a dose-dependent pro-apoptotic effect on insulin-producing INS-1E cells (Fig. 1A) and primary β -cells (Fig. 1B), whereas derivative-3 (an inactive analogue of sal, which does not induce eIF2 α phosphorylation) (18) did not affect β -cell viability after 72 h. Sal toxicity in INS-1E cells was already detectable after 24 h (supplemental Fig. S2). Again in contrast to the PC-12 cells, sal did not protect primary β -cells or INS-1E cells from the synthetic ER stress inducers CPA or thapsigargin (supplemental Fig. S2). Neither was protection observed against the combination of cytokines interleukin-1 β and interferon- γ , previously shown to cause β -cell ER stress (23, 24). On the contrary, sal induced a minor increase in cytokine-induced apoptosis in primary β - and INS-1E cells (supplemental Fig. S2).

Salubrinal Synergizes with FFA to Induce β -Cell Apoptosis—We have previously shown that the FFA oleate and palmitate induce ER stress and apoptosis in β -cells, by mechanisms different from cytokines and synthetic ER stress inducers (5, 23, 29). We therefore examined whether sal protects β -cells against FFA-induced apoptosis. Exposure of INS-1E (Fig. 1C) or primary β -cells (Fig. 1D) to oleate or palmitate in the presence of sal unexpectedly potentiated the pro-apoptotic effect of the FFA. The inactive compound derivative-3 did not affect FFA-induced apoptosis (Fig. 1). The potentiating effect of sal was most marked for oleate, augmenting cell death after 72 h by 4.5- and 3-fold in INS-1E and primary β -cells, respectively, as compared with oleate alone. This effect was already detected in INS-1E cells within 12–24 h of exposure, increasing oleate-induced apoptosis more than 3-fold in the presence of sal (after 24 h; supplemental Fig. S3). Following these short term exposures, no additive effect was observed between sal and palmitate (supplemental Fig. S3). No potentiation was seen with the non-metabolizable fatty acid methyl-oleate, which is not toxic to β -cells. The percentage of apoptosis in INS-1E cells following a 72-h exposure to methyl-oleate (0.5 mM) plus sal (75 μ M) did not exceed that observed with sal alone (data not shown). On the other hand, an equimolar mixture of oleate and palmitate, which is not toxic *per se* (29, 41), became highly pro-apoptotic in the presence of sal (57 $\pm$ 12% INS-1E cell apoptosis for the FFA mixture with sal *versus* 3 $\pm$ 1% without sal; *p* < 0.01, *n* = 6). Similarly, sal increased the percentage of apoptotic primary β -cells from 15 $\pm$ 2% to 91 $\pm$ 2% (both after 72 h in the presence

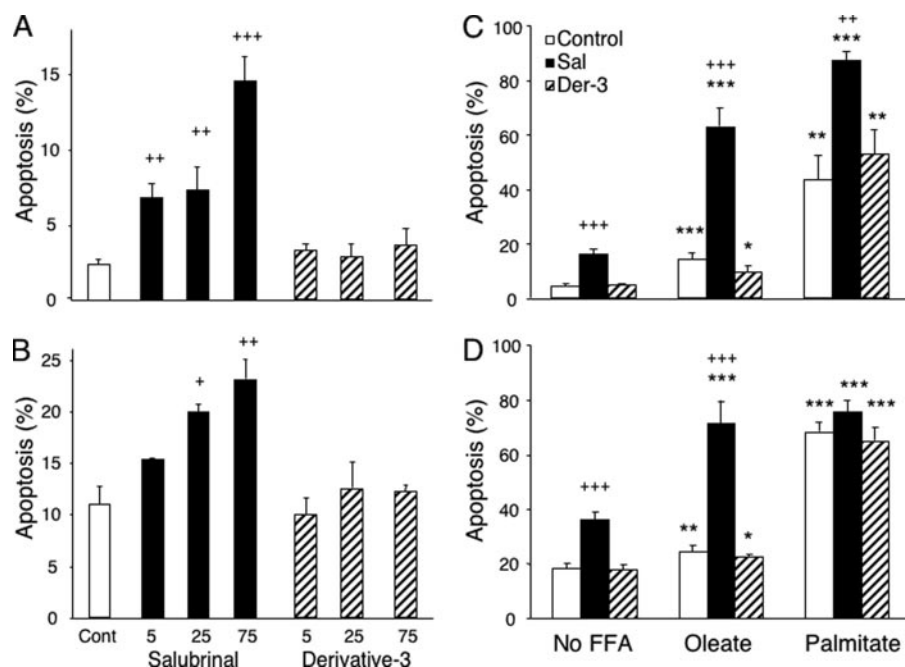


FIGURE 1. Salubrinal induces apoptosis and potentiates FFA-induced β -cell apoptosis. INS-1E cells (A and C) and primary rat β -cells (B and D) were cultured for 72 h in the presence or absence of sal (black bars) or derivative-3 (Der-3; hatched bars) alone, at a concentration of 5, 25 or 75 μ M (A and B) or in combination with oleate or palmitate (C and D; 0.5 mM FFA plus 75 μ M sal or derivative-3). The results are the means $\pm$ S.E. of 3–15 experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for the comparison FFA versus no FFA. +, $p < 0.05$; ++, $p < 0.01$; +++, $p < 0.001$ for the comparison between sal and control.

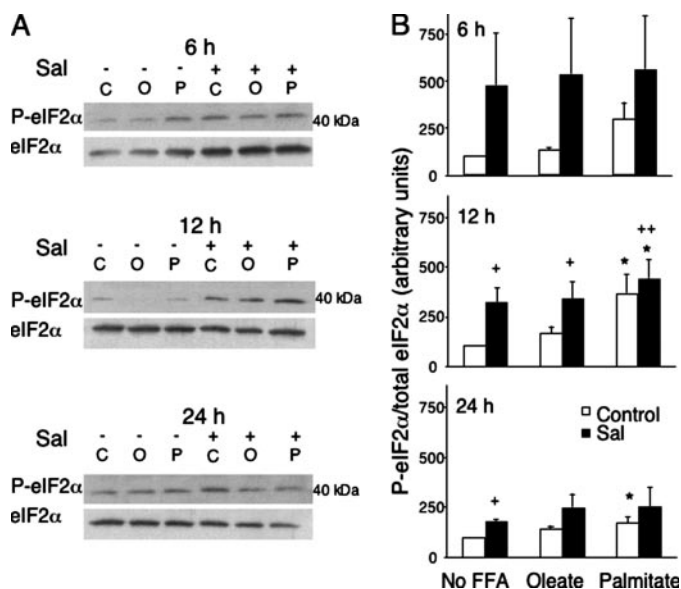


FIGURE 2. Salubrinal and FFA induce eIF2 α phosphorylation. A, INS-1E cells were cultured for 6, 12 and 24 h in the presence or absence of oleate (O), palmitate (P), and/or sal, and Western blot was done using a phospho-specific eIF2 α antibody. C stands for control (no FFA). Total eIF2 α was used as a control for protein loading. One representative experiment for five similar experiments is shown. B, ratio of the mean optical density measurements for phospho- and total eIF2 α protein in INS-1E cells cultured for 6–24 h in the presence or absence of oleate or palmitate, without (control, white bars) or with sal (75 μ M, black bars); $n = 5$; *, $p < 0.05$ for the comparison FFA versus no FFA. +, $p < 0.05$; ++, $p < 0.01$ for the comparison between sal and control.

of 0.5 mM oleate plus palmitate 1:1, $p < 0.001$, $n = 4$). In all of the above described experiments apoptosis was the main mode of cell death, with a negligible necrotic component (data not shown). Because the potentiation of FFA-induced apoptosis by

sal was similar in primary β -cells and well differentiated INS-1E cells and taking into account the large number of cells required, we performed most of the subsequent experiments to elucidate the mechanisms underlying sal effects in INS-1E cells.

Salubrinal Synergizes with FFA to Activate the eIF2 α Branch of the UPR in β -Cells—Sal is a selective inhibitor of eIF2 α dephosphorylation (18) and thereby engages only the PERK-eIF2 α branch of the UPR. We performed a time course analysis of eIF2 α phosphorylation in INS-1E cells exposed to sal and/or FFA. Sal induced eIF2 α phosphorylation after 6 h, with a maximum at 24–36 h, and it was maintained up to 72 h (supplemental Fig. S4). Palmitate, but not oleate, increased eIF2 α phosphorylation (Fig. 2). In the presence of sal, phosphorylation of eIF2 α was observed with both FFA, but to no greater extent than with sal alone (Fig. 2).

Taken together with the above-described pro-apoptotic effects of sal, these results suggest that eIF2 α phosphorylation induced by sal accounts at least in part for its pro-apoptotic activity in pancreatic β -cells.

We next examined whether eIF2 α phosphorylation resulted in some of its known downstream effects in the UPR, namely inhibition of protein synthesis and activation of ATF-4. Sal induced a dose-dependent inhibition of protein synthesis after 12 h (Fig. 3A), reducing protein synthesis by 50% at 75 μ M. Sal maintained protein synthesis inhibited by 50% at 24 h and by 70% after 48 h (supplemental Fig. S5). Palmitate alone, but not oleate, induced a mild (25%) inhibition of protein synthesis after 12 h (Fig. 3B). This effect was significantly potentiated by sal, leading to a 70% inhibition of protein synthesis with both FFA. Derivative-3 (75 μ M), alone or in combination with FFA, did not modify protein synthesis (data not shown). It has been previously reported that prolonged inhibition of protein synthesis can trigger β -cell apoptosis (31). To test whether this could explain the pro-apoptotic effect of sal, we performed dose-response studies with cycloheximide (supplemental Fig. S6), obtaining a rapid inhibition of INS-1E cell protein synthesis by 50 and 85% in the presence of 0.1 and 0.5 μ g/ml cycloheximide, respectively. Maintaining protein synthesis inhibited in this range for 12–24 h did not, however, induce a marked increase in apoptosis ($\sim$ 8% apoptosis after 24 h (supplemental Fig. S6) compared with 22–30% for FFA plus sal (supplemental Fig. S3)), making it unlikely that protein synthesis inhibition is the sole determinant for the early pro-apoptotic effects of sal or sal + FFA.

We next examined whether the inhibition of protein synthesis by sal was paralleled by a decrease of β -cell insulin content

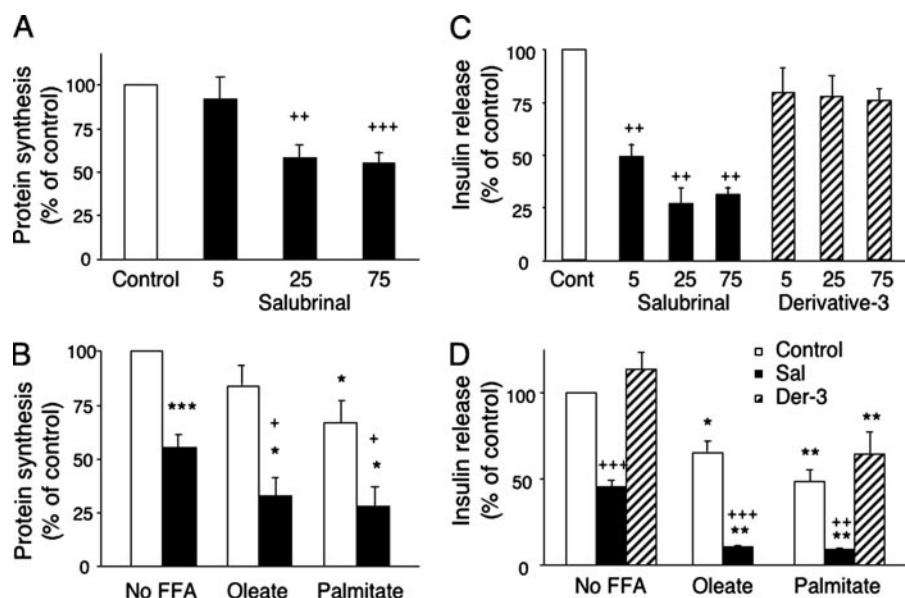


FIGURE 3. Salubrin and FFA inhibit protein synthesis and insulin release. A, dose-dependent inhibitory effects of sal on protein synthesis. INS-1E cells were cultured for 12 h in the presence or absence of 5, 25 or 75 μ M sal. B, synergistic inhibitory effects of FFA and sal on protein synthesis. INS-1E cells were cultured for 12 h in the presence or absence of oleate or palmitate, without (control, white bars) or with sal (75 μ M, black bars). C, dose-dependent inhibitory effects of sal on insulin release to the culture medium by β -cells. Primary rat β -cells were cultured for 72 h in the presence or absence of sal (black bars) or derivative-3 (Der-3, hatched bars) at a concentration of 5, 25, or 75 μ M. D, synergistic inhibitory effects of FFA and sal on insulin release to the culture medium by β -cells. Primary rat β -cells were cultured for 72 h in the presence or absence of oleate or palmitate, without (control, white bars) or with sal (75 μ M, black bars) or derivative-3 (75 μ M, hatched bars). The results represent the means $\pm$ S.E. of three to six experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for the comparison FFA versus no FFA. +, $p < 0.05$; ++, $p < 0.01$; +++, $p < 0.001$ for the comparison between sal and control.

and release. Sal decreased INS-1E cellular insulin content by 25 and 35% after 24 and 48 h (supplemental Fig. S5) and by 55% after 72 h ($p < 0.01$). Sal caused a dose-dependent decrease in insulin release to the culture medium, whereas derivative-3 did not affect insulin accumulation (Fig. 3C). In primary β -cells sal reduced insulin release by 70% relative to control (Fig. 3C). In INS-1E cells insulin release to the medium in the presence of 25 and 75 μ M sal was reduced by 90% as compared with the control condition (data not shown). Sal potentiated the oleate- and palmitate-mediated decrease in insulin release by primary β -cells (Fig. 3D), whereas derivative-3 had no effect. Insulin release to the medium by β -cells exposed to CPA, thapsigargin, or cytokines was also lowered by sal but to a lesser extent than that seen with FFA (data not shown).

One of the consequences of prolonged inhibition of protein synthesis is decreased synthesis of I κ B inhibitory molecules, such as I κ B α , and consequent up-regulation of NF- κ B activation (42). Because NF- κ B activation is pro-apoptotic in pancreatic β -cells (43, 44), we used an NF- κ B luciferase reporter containing five NF- κ B-binding sites (45, 46) to examine whether sal activates NF- κ B in INS-1E cells. Although interleukin-1 β and interferon- γ , used as positive control, induced a 15-fold increase in NF- κ B activity (supplemental Fig. S7), sal alone or in combination with FFA did not lead to NF- κ B activation (supplemental Fig. S7 and data not shown), making it unlikely that NF- κ B mediates sal-induced β -cell apoptosis.

In parallel to the inhibition of protein synthesis by eIF2 α phosphorylation, translation of the transcription factor ATF-4 is paradoxically increased, and this is often accompanied by ATF-4 mRNA induction. As previously shown (5), palmitate

and, to a lesser extent, oleate induced ATF-4 mRNA expression after 12 and 24 h (Fig. 4A). ATF-4 was induced at the protein level only by palmitate, after 6 h and more markedly after 12 h (Fig. 4B). Sal alone induced ATF-4 at the mRNA (from 6 h on; Fig. 4A) and at the protein level (detectable at 12 and 24 h; Fig. 4B). There was a synergistic effect between the combination of sal and oleate or palmitate on ATF-4 translation (from 6 h on; Fig. 4B) and ATF-4 mRNA (from 12 h on; Fig. 4A). Sal increased ATF-4 mRNA levels synergistically with oleate or palmitate, by 5- and 2-fold compared with the FFA alone after 12 h, respectively, and by 4- and 6-fold after 24 h (compared with a 2-fold increase by sal alone). ATF-4 protein levels in the presence of oleate plus sal were at least double the levels observed with sal alone. The same observation was made for palmitate plus sal at 6 and 12 h (Fig. 4B). It is noteworthy that although there was no apparent additive

effect between sal and FFA on eIF2 α phosphorylation (Fig. 2), the combination of sal and oleate or palmitate clearly potentiated the inhibition of protein synthesis (Fig. 3B) and ATF-4 induction (Fig. 4) as compared with either agent alone. In the presence of sal, oleate activated this UPR branch as potently as palmitate, whereas in the absence of sal a preferential induction was seen mostly with palmitate.

Downstream of ATF-4, we examined expression of GADD34, a nonenzymatic cofactor of protein phosphatase 1, which dephosphorylates eIF2 α . There was a marked induction of GADD34 by FFA plus sal after 12 and 24 h (supplemental Fig. S8), by $\sim$ 10-fold in the presence of oleate and sal and by 10–30-fold in the presence of palmitate and sal.

Salubrin Does Not Affect FFA-induced Activation of the IRE1 and ATF-6 Branches of the UPR in β -Cells—The two other main pathways of the UPR are activated by IRE1 and ATF-6. IRE1 leads to alternative splicing and activation of the transcription factor XBP-1. In parallel, total expression of XBP-1 is up-regulated at the transcriptional level by spliced XBP-1 and ATF-6 (37). Palmitate and oleate, but not sal alone, induced spliced (Fig. 5A) and total XBP-1 expression (supplemental Fig. S9). Sal potentiated the effects of FFA, predominantly for palmitate at 24 h (6- and 2.5-fold increase in XBP-1s and XBP-1t expression as compared with palmitate alone).

To evaluate the functional consequences of sal- and FFA-induced activation of the IRE1 and ATF-6 pathways, we used an UPR reporter construct. This element functions as binding site for both XBP-1 (37) and ATF-6 (38). Oleate and palmitate, but not sal alone, increased the UPR reporter activity (Fig. 5C). Sal did not significantly amplify the FFA effects on the reporter.

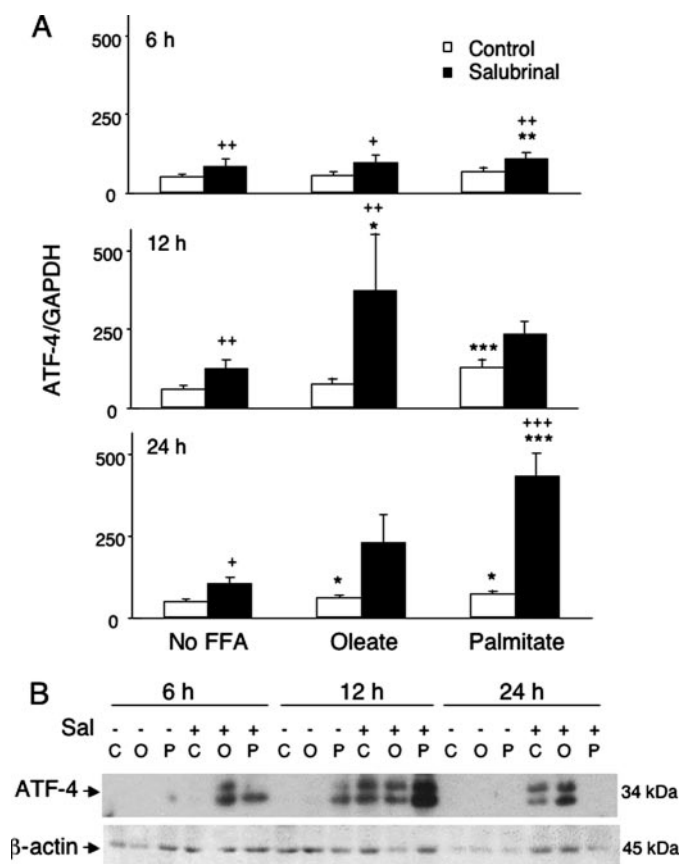


FIGURE 4. Salubrial induces ATF-4 and augments FFA-induced ATF-4 expression. A, ATF-4 mRNA expression was analyzed by real time reverse transcription-PCR and normalized for the expression of the housekeeping gene GAPDH. INS-1E cells were cultured for 6–24 h in the presence or absence of oleate or palmitate, without (control, white bars) or with sal (75 μ M, black bars). The results represent the means $\pm$ S.E. of five independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for the comparison FFA versus no FFA. +, $p < 0.05$; ++, $p < 0.01$; +++, $p < 0.001$ for the comparison between sal and control. B, ATF-4 protein expression was analyzed by Western blot. INS-1E cells were cultured for 6, 12, or 24 h in the presence or absence of oleate (O) or palmitate (P), with (+) or without (–) sal (75 μ M). C stands for control (no FFA). β -Actin was used as a housekeeping protein. One representative experiment for two similar experiments is shown.

One of the downstream effects of ATF-6 activation is up-regulation of the ER chaperone BiP at the transcriptional level. Although FFA induced BiP, as previously reported (5), sal alone did not (Fig. 5B). The potentiation pattern for BiP was similar to that seen with total and spliced XBP-1, namely a 2.2-fold induction of BiP with the combination of sal and palmitate after 24 h (Fig. 5B). Taken together, these data demonstrate that oleate and palmitate activate the IRE1 and ATF-6 branches of the UPR. Sal has no effect *per se*, but leads to a late (24 h) potentiation of palmitate but not oleate. These data are consistent with previous observations in PC-12 cells, where sal did not induce XBP-1 or BiP protein (18).

Salubrial Synergizes with FFA to Induce CHOP in β -Cells—One of the mediators of ER stress-induced apoptosis is the transcription factor CHOP (47). CHOP expression is regulated by several transcription factors, among them ATF-4 and ATF-6 (48). Palmitate and, less so, oleate induced CHOP in INS-1E cells at the mRNA (Fig. 6A) and protein level (Fig. 6B) from 12 h on. Sal up-regulated CHOP early on (6 h) and potentiated the effects of FFA on CHOP mRNA and protein from 12 h on. The

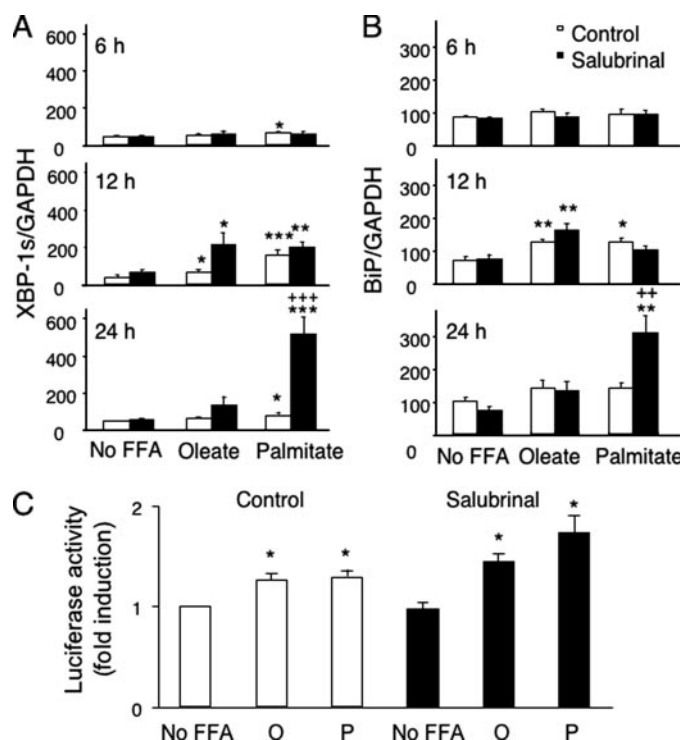


FIGURE 5. Salubrial minimally affects FFA-mediated induction of XBP-1s and ATF-6 in β -cells. A, FFA, but not sal, induces XBP-1 mRNA splicing. XBP-1s mRNA expression was analyzed by real time reverse transcription-PCR and normalized for the expression of the housekeeping gene GAPDH. B, FFA, but not sal, induces BiP expression. BiP mRNA expression was analyzed by real time reverse transcription-PCR and normalized for the expression of the housekeeping gene GAPDH. INS-1E cells were cultured for 6–24 h in the presence or absence of oleate or palmitate, without (control, white bars) or with sal (75 μ M, black bars). C, FFA, but not sal, induces UPR reporter activation. INS-1E cells were cotransfected with the UPR luciferase reporter and the internal control pRL-CMV (encoding Renilla luciferase). After overnight transfection, the cells were exposed for 16 h to oleate or palmitate, without (control, white bars) or with sal (75 μ M, black bars) and assayed for firefly and Renilla luciferase activities. The results were normalized for Renilla luciferase activity. The results are the means $\pm$ S.E. of five independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for the comparison FFA versus no FFA. +, $p < 0.05$; ++, $p < 0.01$; +++, $p < 0.001$ for the comparison between sal and control.

CHOP levels in the presence of oleate or palmitate were increased by sal by 15- and 3-fold, respectively, at 12 h and by 15- and 27-fold after 24 h (Fig. 6). The pattern of CHOP induction was very similar to that observed for ATF-4 (Fig. 4), suggesting that CHOP up-regulation is largely the result of the activation of the eIF2 α branch of the UPR. As for ATF-4, sal augmented CHOP expression by oleate to a level comparable with that observed with palmitate, whereas in the absence of sal, palmitate was more potent than oleate. The observed sal- and FFA-induced CHOP expression (Fig. 6) preceded induction of β -cell apoptosis, which increases significantly by 24 h (supplemental Fig. S3 and Fig. 7A).

DISCUSSION

A high fat diet and obesity are important environmental factors for the development of type 2 diabetes, via the induction of pancreatic β -cell lipotoxicity and insulin resistance. Following our observation that FFA cause ER dilation and apoptosis in pancreatic β -cells (29), we demonstrated that FFA induce an ER stress response in β -cells (5). It is conceivable that chronic ER stress contributes to insidious β -cell loss *in vivo* and conse-

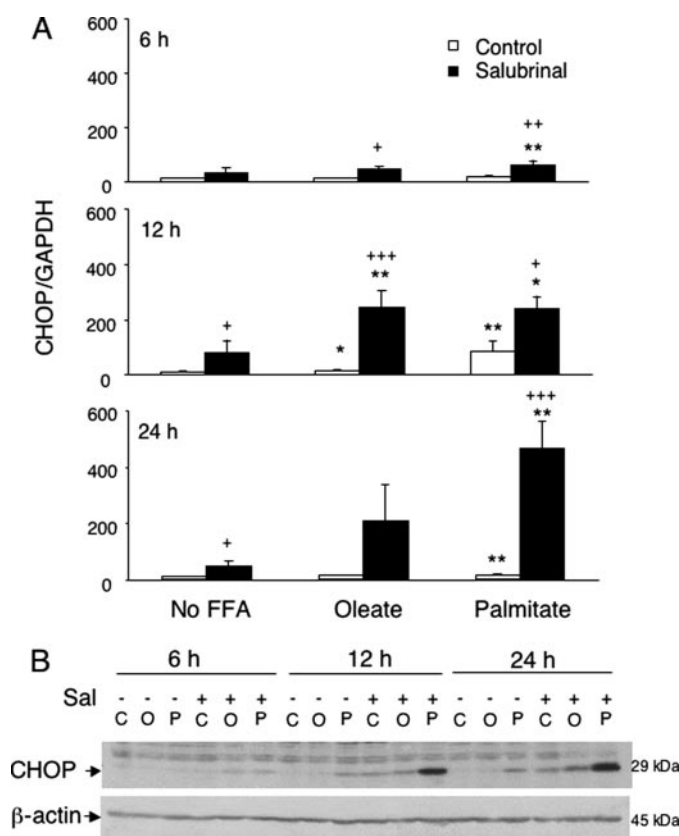


FIGURE 6. Salubrial induces CHOP and augments FFA-induced CHOP expression. *A*, CHOP mRNA expression was analyzed by real time reverse transcription-PCR and normalized for the expression of the housekeeping gene GAPDH. INS-1E cells were cultured for 6–24 h in the presence or absence of oleate or palmitate, without (control, white bars) or with sal (75 μ M, black bars). The results represent the means $\pm$ S.E. of five independent experiments. *, $p < 0.05$; **, $p < 0.01$ for the comparison FFA versus no FFA. +, $p < 0.05$; ++, $p < 0.01$; +++, $p < 0.001$ for the comparison between sal and control. *B*, CHOP protein expression was analyzed by Western blot. INS-1E cells were cultured for 6, 12, or 24 h in the presence or absence of oleate (O) or palmitate (P), with (+) or without (–) sal (75 μ M). C stands for control (no FFA). β -Actin was used as a housekeeping protein. One representative experiment of three similar experiments is shown.

quent development/aggravation of type 2 diabetes in genetically predisposed individuals. Against this background, the development of therapies to prevent or halt β -cell apoptosis by enhancing β -cell defense mechanisms is of great interest. Sal is a selective inhibitor of eIF2 α dephosphorylation that was recently developed as a protective agent against ER stress-mediated apoptosis (18). In the present study, we examined the potential protective effects of sal against ER stress-mediated β -cell apoptosis. We unexpectedly found it to be deleterious to β -cells. Sal specifically potentiated oleate- and, to a lesser extent, palmitate-induced β -cell apoptosis, although it did not affect β -cell apoptosis induced by synthetic ER stressors. Sal and FFA synergized to phosphorylate eIF2 α and induce ATF-4 and CHOP (see time course summary in Fig. 7A and supplemental Fig. S10), whereas sal did not affect the IRE1- and ATF-6-controlled ER stress pathways. In addition to their pro-apoptotic effects, FFA and sal induced β -cell dysfunction. As expected, given its effect on eIF2 α phosphorylation, sal decreased protein synthesis, an effect much enhanced by FFA. FFA and sal also markedly impaired β -cell insulin release. This

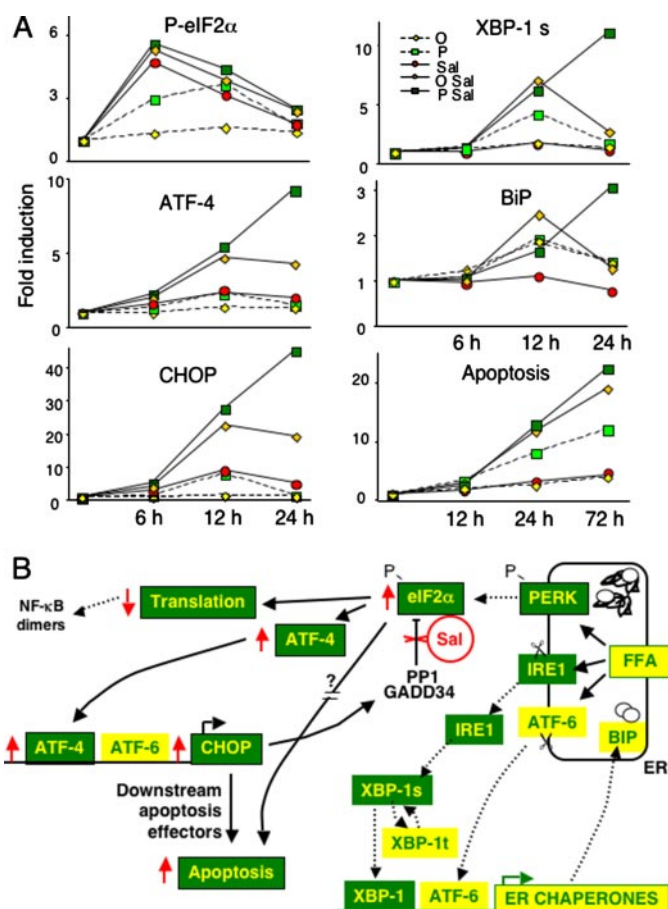


FIGURE 7. Time course and proposed model for the potentiating effect of salubrial on FFA-induced ER stress and apoptosis. *A*, time course of oleate-induced (O, yellow symbols) or palmitate-induced (P, green symbols) ER stress and apoptosis without (hatched lines) or with sal (solid lines). Sal alone is represented by red circles. Phospho-eIF2 α protein (corrected per total eIF2 α protein), ATF-4, CHOP, spliced XBP-1, and BiP mRNA expression (corrected for GAPDH mRNA) and apoptosis rates are derived from previous figures and expressed as fold increase relative to the control. *B*, proposed model for sal-mediated potentiation of FFA-induced ER stress. ER stress responses similar for oleate and palmitate are shown in yellow boxes, responses predominant for palmitate alone are shown in green boxes, and potentiation of FFA effects by sal is indicated by red arrows. The hatched lines indicate minor or absent sal effects.

may be due to the inhibition of insulin synthesis and progressive decrease in insulin content over time (supplemental Fig. S5) and/or result from the arrest of synthesis of proteins operating in the exocytosis machinery.

The PERK-eIF2 α pathway is regulated by a negative feedback loop via CHOP-mediated GADD34 induction (49). GADD34 is the nonenzymatic cofactor of serine/threonine protein phosphatase 1, which dephosphorylates eIF2 α . Sal inhibits eIF2 α dephosphorylation by directly or indirectly interfering with the GADD34 protein phosphatase 1 complex (18) and thereby interrupts a feedback mechanism that attenuates signaling in the ER stress response. The marked GADD34 induction by FFA plus sal after 12 and 24 h may partly overcome the effect of sal and lessen eIF2 α phosphorylation over time (Fig. 7). In contrast to its effects in β -cells, sal protects PC-12 cells from ER stress-mediated apoptosis caused by tunicamycin, brefeldin (18), thapsigargin, CPA (supplemental Fig. S1), or mutant α -synuclein overexpression (50). Recently, sal was also shown

to protect murine leukemia cells from ER photodamage-mediated apoptosis (51). In PC-12 cells, sal induced a rapid and robust eIF2 α phosphorylation and up-regulated the downstream proteins GADD-34 and CHOP (18), but it did not induce PC-12 cell apoptosis. The reason for this divergence in cell fate following sal exposure is unknown but suggests that β -cells require a particularly delicate fine tuning of eIF2 α phosphorylation (see below).

It has been proposed that the PERK-eIF2 α branch of the UPR is crucial for the maintenance of cellular functional integrity, allowing recovery from ER stress by inhibiting protein translation to match the cellular protein folding capacity (19, 21). A decrease in eIF2 α phosphorylation potential in mice heterozygous for an eIF2 α Ser⁵¹ to Ala substitution leads to β -cell failure during high fat feeding, as a result of defective trafficking of proinsulin and a reduced number of insulin granules. This ER stress pathway, and eIF2 α phosphorylation specifically, has therefore been suggested to promote β -cell survival (14, 15, 22). In the present study we demonstrate that prolonged or excessive engagement of the eIF2 α branch is pro-apoptotic in β -cells, specifically in the context of FFA-induced ER stress. We therefore propose a paradigm shift for the function of the PERK-eIF2 α pathway in pancreatic β -cells (Fig. 7B). Thus, a marked activation of the PERK-eIF2 α pathway was observed with palmitate, whereas oleate has minimal effects. Sal too engages this pathway and strikingly potentiates the effects of FFA, such that the combination of oleate and sal activated this part of the ER stress response to a similar degree as the combination of palmitate plus sal. This sal-induced augmentation in oleate-mediated ATF-4 and CHOP expression preceded a similar increase in oleate-induced β -cell apoptosis (Fig. 7A). The IRE1 and ATF-6 branches, on the other hand, are activated by palmitate and oleate but not by sal. Moreover, sal did not significantly alter the FFA effects on IRE1 and ATF-6, except for a late synergistic effect with palmitate (24 h), which may result from cross-talk between the pathways. Based on our findings, we suggest that FFA- and sal-mediated apoptosis is a downstream result of the PERK-eIF2 α branch of the ER stress response, culminating in a marked CHOP induction and other effects that remain to be clarified (Fig. 7B). The other ER stress pathways, under the control of IRE1 α and ATF-6, are apparently not involved in FFA-mediated apoptosis and may instead be protective.

It has recently been suggested that the type of kinase phosphorylating eIF2 α is a major determinant of downstream effects, namely cell survival or apoptosis (52). Thus, double-stranded RNA-activated kinase-dependent eIF2 α phosphorylation was shown to be pro-apoptotic in mouse embryonic fibroblasts, whereas PERK-mediated phosphorylation did not lead to cell death (52). Our findings suggest that ER stress-mediated apoptosis in β -cells depends on the ER stressor and the magnitude and duration of eIF2 α phosphorylation, as illustrated by the preferential sensitization of the β -cells to oleate- or oleate plus palmitate-induced apoptosis when eIF2 α phosphorylation is enhanced.

We examined whether the inhibition of protein synthesis could solely account for the pro-apoptotic effects of FFA and sal. Inhibiting cellular protein synthesis to a similar extent by

cycloheximide treatment did not cause a comparably elevated degree of β -cell apoptosis. ER stress and eIF2 α phosphorylation can lead to NF- κ B activation through repression of I κ B protein translation (42). NF- κ B is an important pro-apoptotic transcription factor in β -cells (43, 44), but it was not activated by sal, excluding it as an important contributor for FFA- and sal-induced β -cell death. It is conceivable that protein synthesis inhibition by FFA and sal in β -cells represses translation of specific anti-apoptotic proteins, which remain to be identified. Alternatively, repression may occur at the transcriptional level, as for instance CHOP was reported to inhibit BCL-2 expression (53). CHOP has been shown to be pro-apoptotic in β -cells (54), and it may contribute to sal- and FFA-induced β -cell apoptosis, which was preceded by a marked synergistic induction of ATF-4 and CHOP (Fig. 7A). The potentiation of apoptosis by FFA and sal may also result from the convergence of ER stress and perturbations in mitochondrial function, which deserves further study.

In keeping with the concept that feedback regulation to attenuate eIF2 α signaling is important for β -cell survival is a recent study on the effect of p58 deletion. The ER chaperone p58 is highly expressed in pancreas (55) and is further induced during ER stress. It interacts with and inhibits PERK (56) and double-stranded RNA-activated kinase (57). Blocking of this feedback loop in p58-null mice resulted in diabetes and pancreatic β -cell apoptosis (58). Taken together with our present findings, this suggests that eIF2 α phosphorylation and the activation of the PERK-eIF2 α branch of the UPR in β -cells is subject to very fine tuning, interference with which is deleterious to the β -cell. Perturbing the delicate phosphorylation/dephosphorylation equilibrium by manipulating the kinases and phosphatases activates an apoptosis program in the β -cell.

In conclusion, we show that sal-induced eIF2 α phosphorylation potentiates the pro-apoptotic effects of FFA in pancreatic β -cells through synergistic eIF2 α phosphorylation and induction of ATF-4, the pro-apoptotic transcription factor CHOP, and other downstream effectors that remain to be identified. Our findings demonstrate that excessive eIF2 α phosphorylation is poorly tolerated by β -cells and exacerbates FFA-induced apoptosis. This changes the present paradigm regarding the putative beneficial effect of eIF2 α phosphorylation in β -cells and must be taken into consideration when designing novel therapies to protect β -cells in type 2 diabetes.

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**Selective Inhibition of Eukaryotic Translation Initiation Factor 2 α
Dephosphorylation Potentiates Fatty Acid-induced Endoplasmic Reticulum Stress
and Causes Pancreatic β -Cell Dysfunction and Apoptosis**

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