Glucagon-Like Peptide-1 Agonists Protect Pancreatic β-Cells From Lipotoxic Endoplasmic Reticulum Stress Through Upregulation of BiP and JunB

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OBJECTIVE—Chronic exposure of pancreatic β-cells to saturated free fatty acids (FFAs) causes endoplasmic reticulum (ER) stress and apoptosis and may contribute to β-cell loss in type 2 diabetes. Here, we evaluated the molecular mechanisms involved in the protection of β-cells from lipotoxic ER stress by glucagon-like peptide (GLP)-1 agonists utilized in the treatment of type 2 diabetes.

RESEARCH DESIGN AND METHODS—INS-1E or fluorescence-activated cell sorter–purified primary rat β-cells were exposed to oleate or palmitate with or without the GLP-1 agonist exendin-4 or forskolin. Cyclopiazonic acid was used as a synthetic ER stressor, while the activating transcription factor 4–C/EBP homologous protein branch was selectively activated with salubrinal. The ER stress signaling pathways modulated by GLP-1 agonists were studied by real-time PCR and Western blot. Knockdown by RNA interference was used to identify mediators of the antiapoptotic GLP-1 effects in the ER stress response and downstream mitochondrial cell death mechanisms.

RESULTS—Exendin-4 and forskolin protected β-cells against FFAs via the induction of the ER chaperone BiP and the antiapoptotic protein JunB that mediate β-cell survival under lipotoxic conditions. On the other hand, exendin-4 and forskolin protected against synthetic ER stressors by inactivating caspase 12 and upregulating Bcl-2 and X-chromosome–linked inhibitor of apoptosis protein that inhibit mitochondrial apoptosis.

CONCLUSIONS—These observations suggest that GLP-1 agonists increase in a context-dependent way the β-cell defense mechanisms against different pathways involved in ER stress–induced apoptosis. The identification of the pathways modulated by GLP-1 agonists allows for targeted approaches to alleviate β-cell ER stress in diabetes. ***Diabetes*** **58:2851–2862, 2009**

The prevalence of type 2 diabetes is increasing dramatically as a result of environmental changes, including the adoption of Western diets rich in saturated fats. These diets augment insulin requirements but also cause early pancreatic β-cell dysfunction that is central to the pathogenesis of type 2 diabetes. Three lines of evidence support this hypothesis: *1)* β-Cell function is decreased before clinically detected type 2 diabetes (1,2) (i.e., early in its natural history). *2)* Most type 2 diabetes candidate genes affect β-cell mass and/or function (3). *3)* Type 2 diabetic individuals have decreased β-cell mass (4,5), secondary to increased β-cell apoptosis (4,6). The progressive nature of type 2 diabetes, characterized by "secondary failure" requiring patients on oral agents to switch to insulin, is due to loss of insulin secretory capacity. This has shifted research emphasis to the search for agents that prevent loss of functional β-cell mass by increasing proliferation and/or decreasing β-cell death.

Chronic exposure to free fatty acids (FFAs) causes lipotoxic β-cell dysfunction and death in in vitro and some, but not all, in vivo models tested (7,8) and may contribute to type 2 diabetes development. A growing body of evidence suggests that endoplasmic reticulum (ER) stress is one of the molecular mechanisms underlying both insulin resistance and β-cell failure (9). Saturated and, to a lesser extent, unsaturated FFAs trigger β-cell ER stress and apoptosis (10–14) through changes in ER Ca^{2+} handling and secondary protein malfolding (12).

The unfolded protein response (UPR) or ER stress response is triggered when malfolded proteins accumulate inside the ER (15,16). The three UPR pathways are under the control of double-stranded RNA–activated protein kinase (PKR)-like kinase (PERK), activating transcription factor (ATF) 6, and inositol requiring-1 (IRE1) (online appendix Fig. S1 [available at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0685/DC1). The main purpose of the UPR is to restore ER homeostasis by decreasing ER protein load and increasing ER folding capacity (16). PERK phosphorylates the eukaryotic translation initiation factor 2α (eIF2α), leading to attenuation of global translation and, paradoxically, enhanced expression of selected proteins including ATF4. ATF4 induces C/EBP homologous protein (CHOP; also known as GADD153 or DDIT3) and downstream GADD34. The translational inhibition of the UPR is transitory, as GADD34 acts as a substrate-targeting factor to protein phosphatase 1 (PP1) to dephosphorylate eIF2α (17). Activated ATF6 induces transcription of ER chaperones including BiP (18). IRE1 splices X-box binding protein 1 (XBP1) mRNA, leading to the translation of the active transcription factor XBP1s that induces ER chaperones and ER-associated protein degradation (19). IRE1 degrades mRNAs for some highly translated proteins including insulin (20), thereby decreasing demand on the ER. IRE1 also recruits tumor necrosis factor receptor–associated factor (TRAF) 2 and apoptosis signal–regulating kinase (ASK) 1, which activate JNK (21); it can activate caspase 12 (22) and interact with pro- and antiapoptotic

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Bcl-2 family members, thus contributing to ER stress–mediated apoptosis.

Increased ER stress signaling is found in islets from type 2 diabetic patients, including expression of ATF3, CHOP, and BiP; nuclear localization of CHOP; and β-cell ER expansion (9). The stress response has also been implicated in young-onset diabetes. Proinsulin gene mutations cause insulin malfolding (23), and PERK (24) and Wolfram Syndrome-1 mutations (25) affect proper ER stress signaling, all of which culminate in β-cell loss. Agents that modulate the β-cell ER stress response will therefore have immediate therapeutic application.

We have previously examined the usefulness of salubrinal, a selective inhibitor of eIF2α dephosphorylation, shown to protect against ER stress–mediated apoptosis (26). Unexpectedly, salubrinal-induced eIF2α phosphorylation was proapoptotic in β-cells, and it potentiated the deleterious effects of FFAs (11). Prolonged CHOP induction is proapoptotic in rodent models of ER stress and diabetes (27) and in FFA-treated β-cells (12) and mediates the deleterious salubrinal effects through amplified ATF4-CHOP signaling (11).

Exendin-4 is a glucagon-like peptide (GLP)-1 receptor (GLP-1R) agonist used in the treatment of type 2 diabetes (28). Exendin-4 and forskolin have previously been reported to improve β-cell survival following ER stress induced by the synthetic stressors thapsigargin or tunicamycin (29). This cAMP-protein kinase A (PKA)-dependent protection was apparently mediated through enhanced ATF4-CHOP-GADD34 signaling, resulting in eIF2α dephosphorylation and translational recovery (29).

We demonstrate here that exendin-4 and forskolin protect β-cells against FFA- and salubrinal-induced ER stress and apoptosis, not through ATF4-CHOP-GADD34 feedback signaling but by enhancing cellular defense mechanisms (e.g., BiP, Bcl-2, and JunB). The identification of these ER stress signals modulated by GLP-1 may allow tailored approaches to alleviate ER stress in human diabetes and prevent progressive loss of functional β-cell mass.

RESEARCH DESIGN AND METHODS

Culture of primary fluorescence-activated cell sorter–purified rat β-cells, INS-1E cells, and human islets. Male Wistar rats (Charles River Laboratories, Brussels, Belgium) were housed and used according to the guidelines of the Belgian Regulations for Animal Care; experiments were approved by the local ethics committee. Islets were hand picked following collagenase digestion. β-Cells were purified from dispersed islet cells by autofluorescence-activated cell sorting (FACSAria; BD Bioscience, San Jose, CA) (30). The preparations, containing 91 ± 2% β-cells, were precultured overnight in Ham's F-10 medium containing 10 mmol/l glucose and 5% heat-inactivated FBS (31).

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 2 mmol/l GlutaMAX-I) containing 5% FBS (32) and used at passages 56–75.

With the approval of the ethics committee in Pisa, islets of four multiorgan donors (two female and two male subjects, aged 64 ± 10 years, BMI 26 ± 1 kg/m^2) were isolated, and cultured as previously described (12). The human islets contained 58 ± 2% β-cells, assessed following islet dispersion (12).

Treatments. For FFA exposure, β-cells were cultured in medium with 1% BSA and without FBS; INS-1E cells were cultured in medium with 1% FBS and 1% BSA. Oleate and palmitate (Sigma-Aldrich, Schnelldorf, Germany) were dissolved in 90% ethanol and used at a final concentration of 0.5 mmol/l (10,33) and a FFA/BSA molar ratio of 3.4 (33). The sarcoendoplasmic-reticulum pump Ca^{2+}-ATPase (SERCA) blockers cyclopiazonic acid (CPA) and thapsigargin (Sigma) were dissolved in DMSO and used at concentrations of 25 and 1 μmol/l, respectively. Salubrinal (ChemBridge, San Diego, CA) was diluted in DMSO and used at 75 μmol/l. Tautomycin was diluted in ethanol and used at 30 nmol/l. Exendin-4 was diluted in RPMI-1640 and used at 10 nmol/l for primary cells and 50 nmol/l for INS-1E cells (29). Forskolin and the PKA inhibitor H89 were diluted in DMSO and used at concentrations of 20 and 15 μmol/l, respectively (Sigma).

Assessment of β-cell viability and apoptosis pathways. Apoptotic and necrotic β-cells and INS-1E cells were counted in fluorescence microscopy following staining with the DNA-binding dyes propidium iodide (5 μg/ml) and Hoechst 33342 (10 μg/ml) (11). The percentage human islet cell death was determined as described (12). The apoptosis index was calculated as [(% apoptosis in ER stressor–treated cells under indicated conditions transfected with negative or target siRNA − % apoptosis in ER stressor–treated negative siRNA-transfected cells)/(100% − % apoptosis in ER stressor–treated negative siRNA-transfected cells)] × 100. Apoptosis was also assessed by the Cell Death Detection ELISA^{plus} kit (Roche Diagnostics, Mannheim, Germany) (10). Forskolin did not modify INS-1E cell proliferation under the present experimental conditions (assessed by BrdU incorporation, data not shown).

To study cytochrome C release, cells were prepared as described in the online appendix supplementary Methods. After total protein quantification (Bio-Rad Laboratories, Munchen, Germany), equal amounts of protein were blotted using tubulin as cytoplasmic and apoptosis-inducing factor (AIF) as mitochondrial control.

Caspase 3 and 7 activation was measured with the Caspase-Glo 3/7 assay (Promega, Madison, WI) and caspase 12 activation with the CaspGlow Caspase-12 staining kit (Biovision, Mountain View, CA) (12).

Real-time PCR, Western blot, and RNA interference. Real-time PCR and Western blot were performed as described in the online appendix supplementary Methods.

Proteins were knocked down using small-interfering RNA (siRNA) against CHOP (Ambion, Austin, TX), XBP1, JunB (both from Invitrogen, Carlsbad, CA), GADD34, and BiP (both from Dharmacon, Chicago, IL). A negative control of 21-nucleotide duplex RNA with no known sequence homology was from Qiagen (Hilden, Germany). Lipid-RNA complexes were formed in Optimem1 (1 μl Dharmafect to 150 nmol/l siRNA; Dharmacon) and added at a final concentration of 30 nmol/l siRNA for transfection as described (12). Transfected cells were cultured for 1–4 days and subsequently treated.

Statistical analysis. Data are presented as means ± SE. Comparisons were performed by ANOVA followed by paired *t* test with the Bonferroni correction for multiple comparisons. A P value <0.05 was considered statistically significant.

RESULTS

Exendin-4 and forskolin protect β-cells from chemical and physiological ER stressors. In agreement with Yusta et al. (29), exendin-4 partially protected primary and clonal β-cells against the chemical SERCA2 blockers CPA and thapsigargin, and the adenylate cyclase stimulator forskolin had a similar effect (Fig. 1*A* and *E*).

We next examined whether GLP-1R activation protects β-cells against the physiologically relevant ER stressors oleate and palmitate (10–14). We also asked whether GLP-1 is antiapoptotic following selective activation of the PERK pathway. To this end, we used salubrinal, an inhibitor of eIF2α dephosphorylation (26) that, without causing ER stress, markedly upregulates ATF4 and CHOP and thereby triggers β-cell apoptosis (11). Exendin-4 and forskolin protected primary β- and INS-1E cells against oleate and palmitate after 14–24 h (Fig. 1*B* and *F*), in keeping with previous reports (34,35), but the protection against palmitate was lost following prolonged exposure (Fig. 1*C* and *G*). GLP-1R activation protected human islets against palmitate for 72 h (Fig. 1*D*). GLP-1 agonists also prevented salubrinal-induced β-cell death (Fig. 1*B* and *F*). The findings were confirmed using an enzyme-linked immunosorbent assay for DNA strand breaks (Fig. 1*H*).

Exendin-4 and forskolin do not protect β-cells through ATF4-CHOP-GADD34–mediated eIF2α dephosphorylation. In clonal β-cells exposed to synthetic ER stressors, exendin-4 induces ATF4 and CHOP, thereby upregulating GADD34 (29). The recruitment of PP1 by GADD34 results in eIF2α dephosphorylation, and this was proposed to accelerate recovery from ER stress

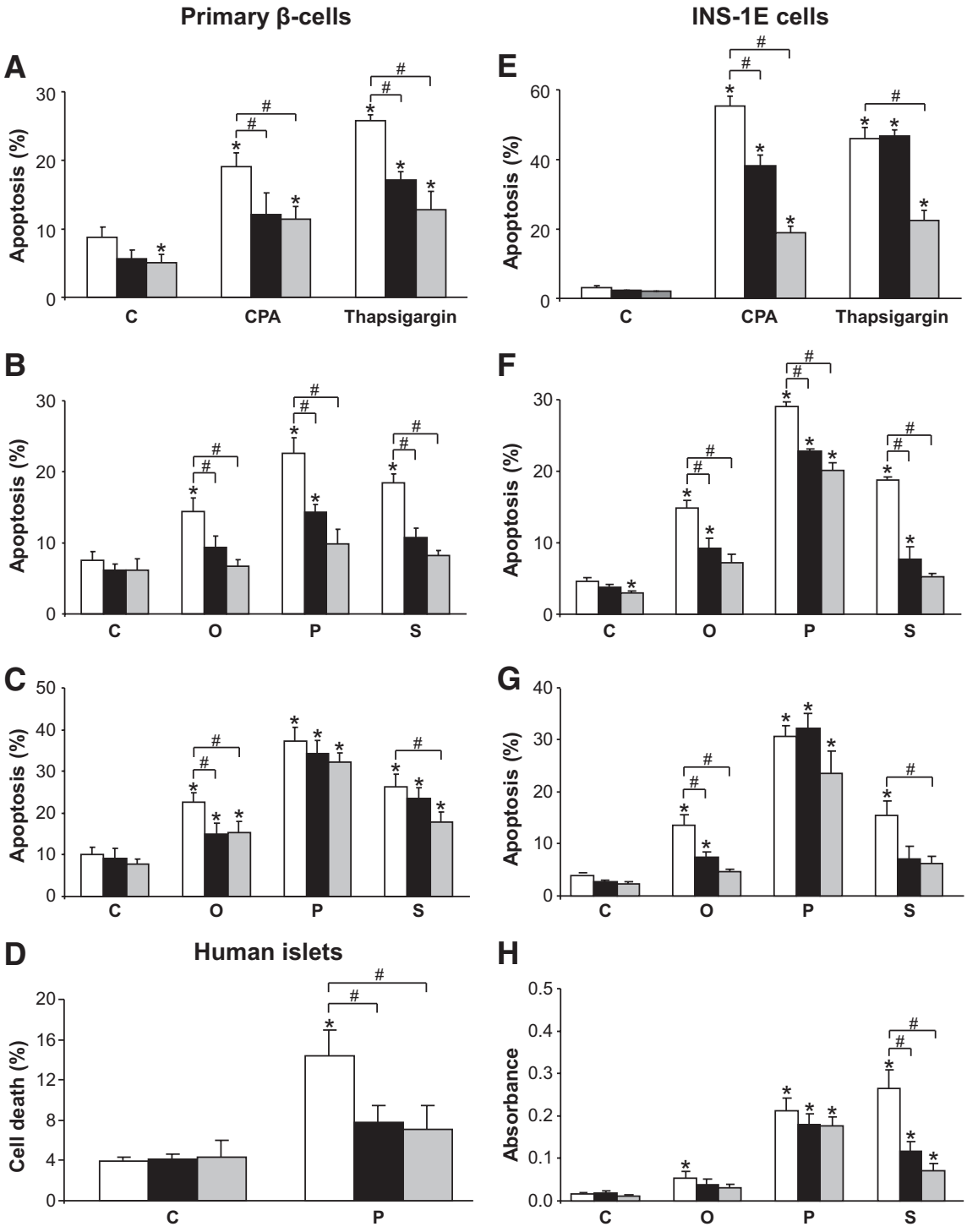

FIG. 1. Exendin-4 and forskolin protect β-cells against ER stress–mediated apoptosis. Primary rat β-cells (*A–C*), human islets (*D*), or INS-1E cells (*E–H*) were left untreated (*C*) or treated with 25 μmol/l CPA, 1 μmol/l thapsigargin, 0.5 mmol/l oleate (O) or palmitate (P), or 75 μmol/l salubrinal (S), alone (control, □) or in combination with exendin-4 (10 nmol/l for primary cells or 50 nmol/l for INS-1E cells, ■) or forskolin (20 μmol/l, ▨) for 14 h (*F*), 24 h (*A*, *B*, *E*, *G*, and *H*), and 3 days (*C* and *D*). Apoptosis was measured by fluorescence microscopy (*A–G*) or enzyme-linked immunosorbent assay (*H*). Results represent means ± SE of 3–11 independent experiments. **P* < 0.05 for the comparison against untreated cells; #*P* < 0.05.

(29). In FFA-treated INS-1E cells, exendin-4 and forskolin did not modify ATF4 and CHOP mRNA (Fig. 2*A* and supplementary Fig. S2*A*), but after 14 h, ATF4 and CHOP protein expression doubled in palmitate-treated cells (Fig. 2*B*), and this induced GADD34 mRNA (Fig. 2*A*). When combined with salubrinal, per se, a potent ATF4-CHOP inducer (11), forskolin further upregulated ATF4-CHOP-GADD34 RNA and protein (Fig. 2 and supplementary Fig. S2).

We next examined whether this GADD34 induction causes eIF2α dephosphorylation. Exendin-4 and forskolin effectively decreased salubrinal-induced eIF2α phosphorylation in INS-1E cells (Fig. 2*C* and supplementary Fig. S2*C*) and tended to decrease palmitate-induced eIF2α

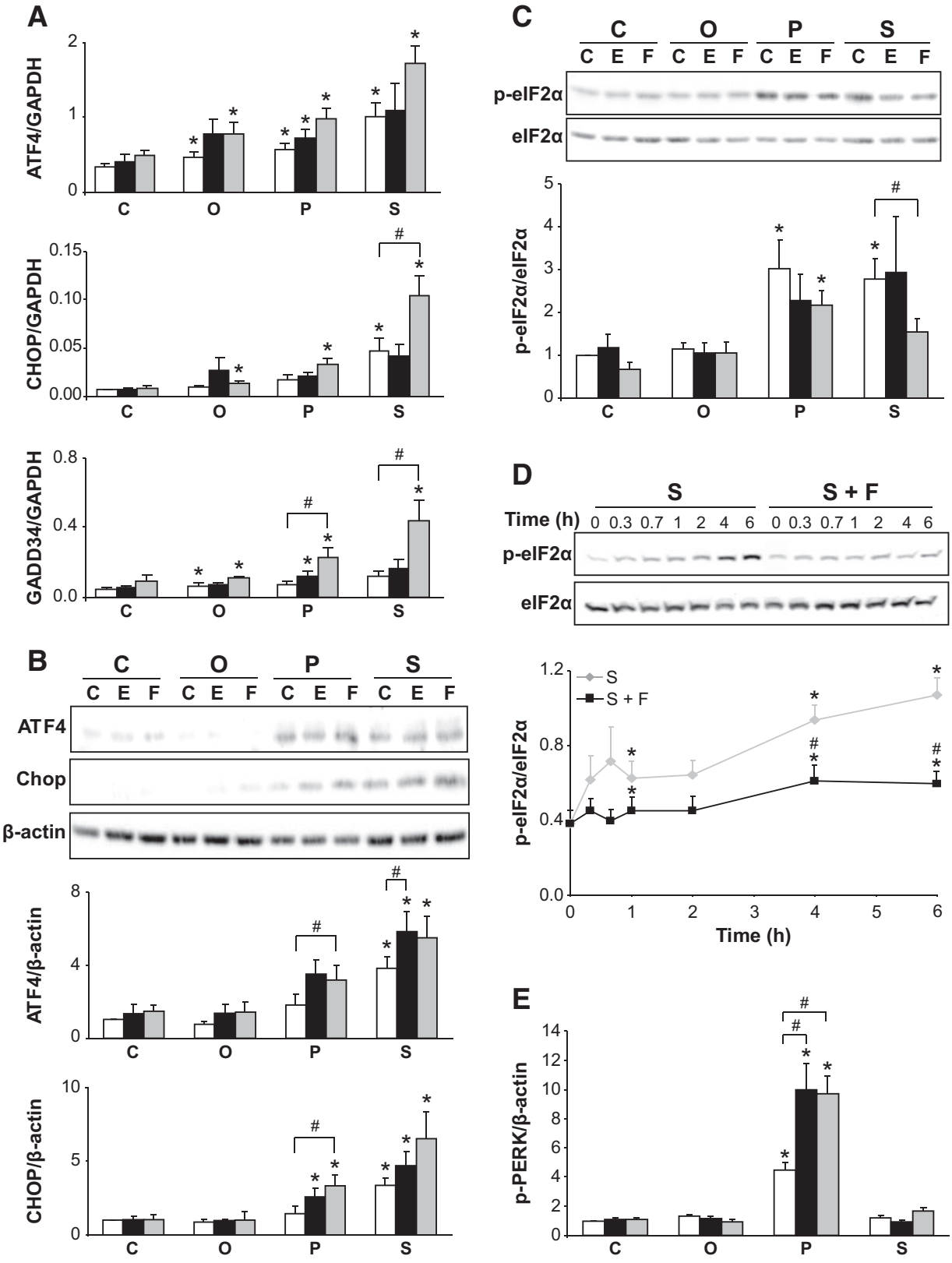

FIG. 2. Exendin-4 or forskolin increases signaling downstream of PERK. INS-1E cells were cultured in the presence or absence of 0.5 mmol/l oleate (O) or palmitate (P) or 75 μmol/l salubrinal (S), alone or in combination with 50 nmol/l exendin-4 (E) or 20 μmol/l forskolin (F) for the indicated times (*D*) or 14 h (*A–C* and *E*). □, control; ■, exendin-4; ▨, forskolin. ATF4, CHOP, and GADD34 mRNA expression was analyzed by real-time PCR, normalized for the expression level of the housekeeping gene GAPDH (*A*). ATF4 and CHOP (*B*) and phospho-PERK (*E*) protein expression was analyzed by Western blot, normalized for the expression level of β-actin, and expressed as fold induction of control. eIF2α phosphorylation (*C* and *D*) was normalized for total eIF2α. Results represent means ± SE of 3–5 independent experiments. *$P < 0.05$ against untreated cells; #$P < 0.05$.

phosphorylation after 14 h (Fig. 2*C*). GADD34 knockdown by siRNA (55% decrease in mRNA) increased eIF2α phosphorylation by 75% in salubrinal plus forskolin–treated INS-1E cells (14 h, $n = 3$). A detailed time-course analysis revealed that very early on (<1 h), forskolin diminished eIF2α phosphorylation by salubrinal (Fig. 2*D*), suggesting that forskolin immediately affects eIF2α phosphorylation, in addition to enhancing its dephosphorylation via ATF4-CHOP-GADD34. The effect of exendin-4 and forskolin on eIF2α was not PERK dependent, as phospho-PERK was unchanged or even increased (Fig. 2*E* and supplementary Fig. S2*D*). To examine whether GADD34-PP1 dephosphorylation of eIF2α mediates the antiapoptotic effect of forskolin, we used the PP1 inhibitor tautomycin (36). In the presence of tautomycin, forskolin did not prevent eIF2α phosphorylation by salubrinal (Fig. 3*A*). Despite the high phospho-eIF2α levels, forskolin still protected against salubrinal-induced apoptosis (Fig. 3*B*), suggesting that the eIF2α phosphorylation state does not determine forskolin-mediated β-cell survival. To further evaluate the participation of ATF4-CHOP-GADD34 in the forskolin protection, the feedback loop was interrupted by RNAi. CHOP knockdown prevented CHOP protein induction by palmitate/salubrinal plus forskolin (Fig. 3*C* and *F*) and decreased GADD34 mRNA (Fig. 3*D* and *G*), demonstrating efficient disruption of CHOP-GADD34 signaling. Decreasing CHOP expression partially prevented palmitate-induced (12) and salubrinal-induced apoptosis (Fig. 3*E* and *H*). The antiapoptotic effect of forskolin was unaffected by knockdown of CHOP (Fig. 3*E*, *H*, and *I*) or GADD34 (8 ± 1% apoptosis vs. 6 ± 1% with control siRNA following salubrinal plus forskolin exposure, compared with 18–22% after salubrinal exposure, 14 h, $n = 3$), strongly arguing against their participation in β-cell protection. CHOP knockdown did not abrogate the forskolin protection against CPA (supplemental Fig. S3), casting doubt onto its proposed importance for GLP-1 protection against ER stress (29).

Exendin-4 and forskolin-induced XBP1 splicing does not mediate protection from ER stress. We next examined the potential involvement of the other ER stress branches in the protective effects of GLP-1 (supplementary Fig. S1). Exendin-4 and forskolin upregulated XBP1s mRNA and protein in INS-1E cells basally (Fig. 4 and supplementary Fig. S4), suggesting that GLP-1R agonists activate IRE1, but they did not enhance the XBP1s induction by FFAs (Fig. 4). Similar changes were observed for ORP150 mRNA, a XBP1s transcriptional target (data not shown).

The role of XBP1s in β-cell death was studied using RNAi, resulting in 74% decreased XBP1 protein expression. XBP1s knockdown increased apoptosis by oleate (Fig. 4*C*) but not palmitate or salubrinal (Fig. 4*E* and *G*), illustrating that the signaling response engaged by different ER stressors has context-dependent cell survival outcomes (supplementary Fig. S8*A*). The antiapoptotic effect of forskolin in oleate-, palmitate-, and salubrinal-treated cells did not depend on XBP1s expression, as XBP1 knockdown did not abrogate its protective effect (Fig. 4*H*).

BiP induction by exendin-4 and forskolin contributes to the protection against palmitate. BiP is a key ATF6-dependent ER chaperone. Exendin-4 and forskolin doubled BiP protein levels in FFA-treated INS-1E cells; mRNA induction was observed for oleate only (Fig. 5*A–C* and supplementary Fig. S4). Conversely, forskolin induced BiP mRNA but not protein in salubrinal-exposed cells (Fig. 5 and supplementary Fig. S4), pointing to posttranscriptional regulation. Forskolin also increased basal BiP protein (Fig. 5*C* and supplementary Fig. S4*C*). BiP protein induction by forskolin was confirmed in primary β-cells (Fig. 5*D* and supplementary Fig. S4*D*). $p58^{IPK}$ mRNA levels, another ATF6 target, were comparable to BiP (data not shown).

The role of BiP induction in β-cell survival was examined by RNAi. The relatively modest 50% BiP knockdown (Fig. 5*E* and *G*) is probably due to the marked BiP stability in INS-1E cells (data not shown). BiP knockdown increased apoptosis induced by oleate and palmitate (Fig. 5*F* and *H* and supplementary Fig. S8A), which is in keeping with the reverse experiment showing palmitate resistance in BiP-overexpressing cells (13). The antiapoptotic action of forskolin was not affected by interference with BiP in oleate-exposed cells (Fig. 5*F*), but a marked loss of protection against palmitate was observed (Fig. 5*H* and *I*), showing that BiP induction is one of the mechanisms by which GLP-1 protects against ER stress.

To further elucidate the mechanisms of β-cell protection, we examined the apoptosis pathways downstream of ER stress that can be ER specific or employ the classic intrinsic mitochondrial cell death pathway.

GLP-1R activation induces antiapoptotic proteins and prevents mitochondrial caspase-mediated cell death. To assess the contribution of mitochondria in the apoptotic process, mitochondrial cytochrome C release and the antiapoptotic protein Bcl-2 were measured. Palmitate and salubrinal decreased Bcl-2 and stimulated cytochrome C release, as did CPA (Fig. 6 and supplementary Fig. S5) but not oleate (data not shown). Both exendin-4 and forskolin restored Bcl-2 protein to control levels in salubrinal-treated cells (Fig. 6*C* and supplementary Fig. S5*B*) and thereby largely prevented cytochrome C release (Fig. 6*B* and supplementary Fig. S5*A*). It has been suggested that CHOP mediates Bcl-2 downregulation (37), but we did not observe changes in Bcl-2 expression following CHOP knockdown (data not shown). In palmitate-treated β-cells, no restoration of Bcl-2 expression was observed, while forskolin increased Bcl-2 levels in control and oleate conditions (Fig. 6 and supplementary Fig. S5).

Cytochrome C released from the mitochondria binds to the proapoptotic protease-activation factor 1 (APAF1) and caspase 9, constituting the apoptosome that activates the effector caspase 3 (38). This is counteracted by X-chromosome–linked inhibitor of apoptosis (XIAP), a potent caspase inhibitor that binds to caspase 9 or 3 (39). XIAP mRNA was not modified (data not shown), but as with Bcl-2 XIAP protein expression was reduced by palmitate, salubrinal, and CPA (Fig. 6*C* and supplemental Fig. S5*C*). Again, exendin-4 and forskolin restored XIAP levels in salubrinal- and CPA-treated cells but not in palmitate-treated cells (supplemental Fig. S5*C*).

In keeping with the restoration of Bcl-2 and XIAP and the inhibition of cytochrome C release, caspase 3 activation was nearly entirely prevented by exendin-4 and forskolin in salubrinal- or CPA-treated β-cells (Fig. 6*D*). Exendin-4 and forskolin partially reduced caspase 3 activation by palmitate, but residual activity remained three- to fourfold above control (Fig. 6*D*).

During ER stress, caspase 3 can also be activated by caspase 12 (40). FFAs and CPA activate ER membrane–localized caspase 12 in β-cells (12). Exendin-4 or forskolin did not modify caspase 12 activation in FFA-treated cells but abolished its activation by CPA (Fig. 6*E*). This represents an additional mechanism through which GLP-1 can

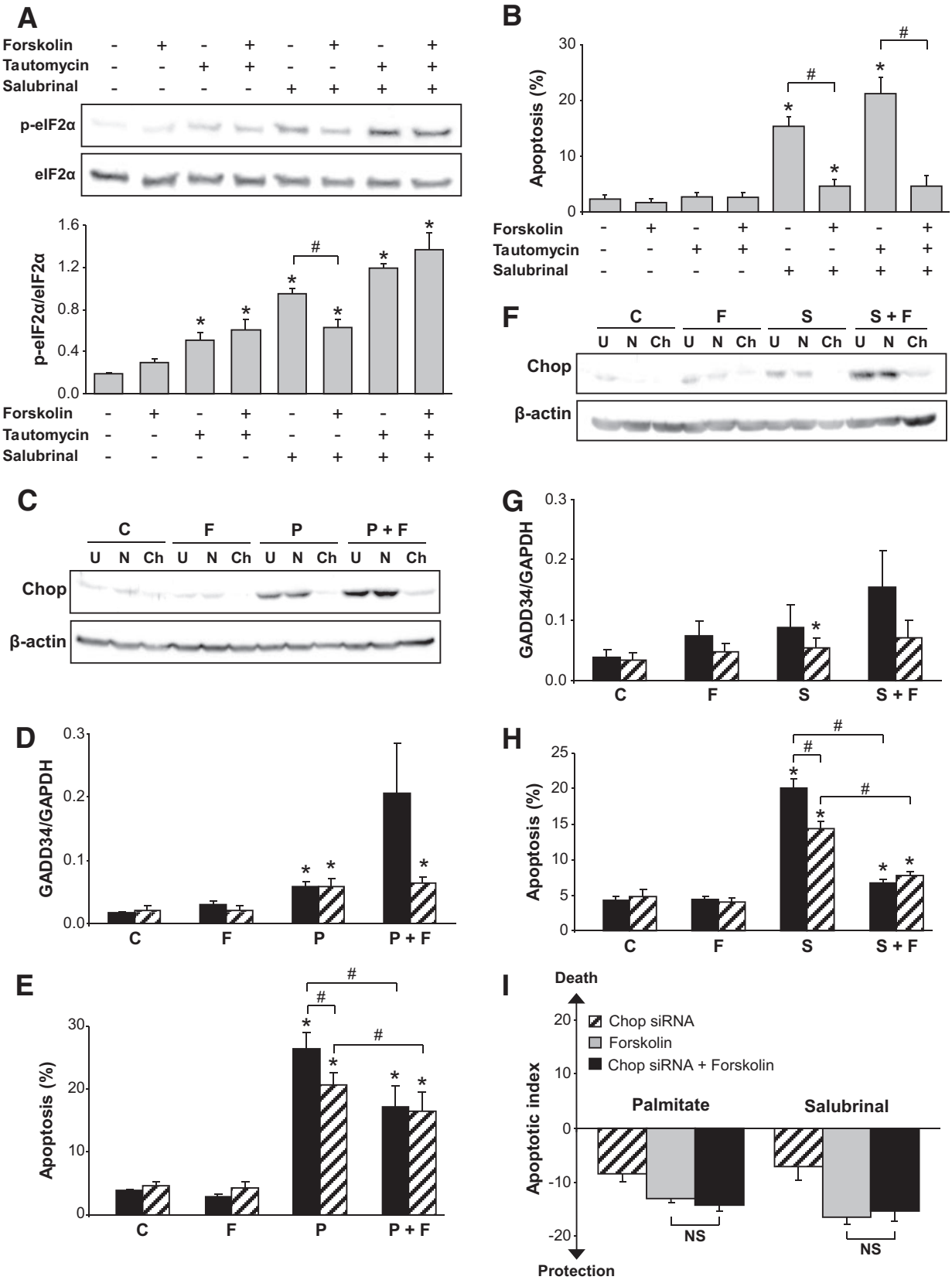

FIG. 3. ATF4-CHOP feedback does not mediate antiapoptotic effects of exendin-4 and forskolin. *A*: Forskolin (20 μmol/l) decreases eIF2α phosphorylation induced by salubrinal (75 μmol/l) but not by the PP1 inhibitor tautomycin (30 nmol/l) after 14 h. The blot is representative of three independent experiments; densitometric quantification of eIF2α phosphorylation expressed per total eIF2α is shown below. *B*: Apoptotic INS-1E cell death following 14 h exposure to salubrinal, tautomycin, and/or forskolin. Data are means ± SE of four independent experiments. Representative blot of CHOP protein (*C* and *F*) and GADD34 mRNA expression (*D* and *G*) following CHOP knockdown and 14-h incubation with palmitate (P) or salubrinal (S), alone or in combination with forskolin (F). *C–H*: Cells were left untransfected (U) or transfected with negative siRNA (N, ■) or CHOP siRNA (Ch, ▨) 2 days before treatment. *E* and *H*: Apoptosis following CHOP knockdown and exposure for 14 h to palmitate

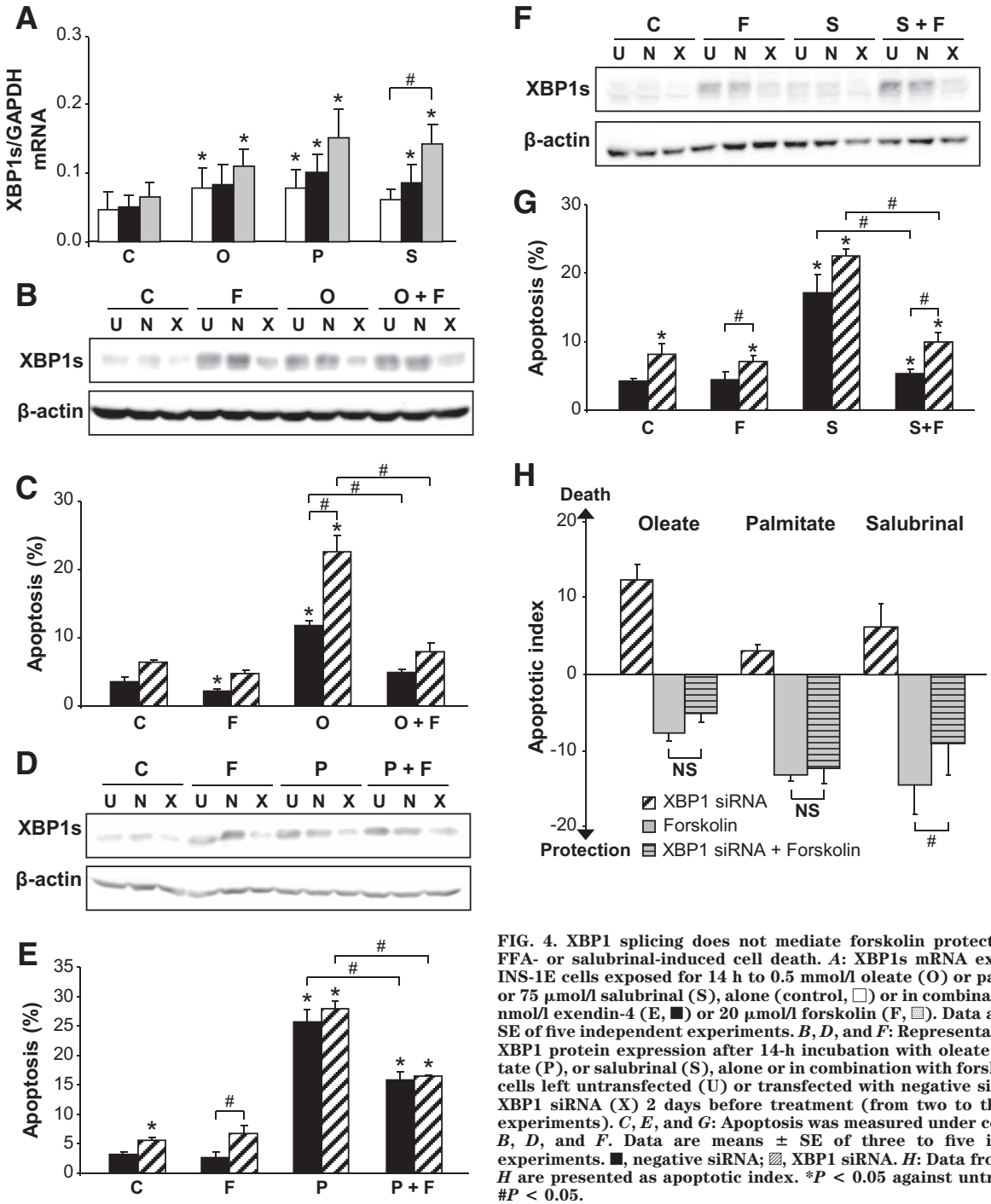

FIG. 4. XBP1 splicing does not mediate forskolin protection against FFA- or salubrinal-induced cell death. *A*: XBP1s mRNA expression in INS-1E cells exposed for 14 h to 0.5 mmol/l oleate (O) or palmitate (P) or 75 μmol/l salubrinal (S), alone (control, □) or in combination with 50 nmol/l exendin-4 (E, ■) or 20 μmol/l forskolin (F, ▨). Data are means ± SE of five independent experiments. *B*, *D*, and *F*: Representative blots of XBP1 protein expression after 14-h incubation with oleate (O), palmitate (P), or salubrinal (S), alone or in combination with forskolin (F), in cells left untransfected (U) or transfected with negative siRNA (N) or XBP1 siRNA (X) 2 days before treatment (from two to three similar experiments). *C*, *E*, and *G*: Apoptosis was measured under conditions of *B*, *D*, and *F*. Data are means ± SE of three to five independent experiments. ■, negative siRNA; ▨, XBP1 siRNA. *H*: Data from *D*, *F*, and *H* are presented as apoptotic index. *$P < 0.05$ against untreated cells; #$P < 0.05$.

protect against synthetic ER stress, but it does not play a role in physiological ER stress.

JunB protects β-cells against FFAs and plays a major role in the antiapoptotic effects of GLP-1R activation. The transcription factor JunB was recently shown to be antiapoptotic in β-cells exposed to chemical ER stressors (41). JunB protein expression was downregulated by CPA, palmitate, and salubrinal but not by oleate (Fig. 7*A* and supplemental Fig. S6). Exendin-4 restored JunB protein to control levels, and forskolin further induced JunB by two- to fivefold (Fig. 7*A* and supplemental Fig. S6). This marked induction was not observed at the mRNA level (data not shown). Forskolin also induced JunB protein in primary β-cells by threefold (Fig. 7*B* and supplemental Fig. S6B).

An 85% JunB knockdown by siRNA (Fig. 7*C*, *E*, and *G*) sensitized INS-1E cells to oleate (Fig. 7*D*). The protection by forskolin against oleate was abolished in JunB-depleted cells demonstrating that cAMP-stimulated JunB induction is necessary and sufficient to prevent cell death (Fig. 7*D*

(P) or salubrinal (S), alone or in combination with forskolin (F). Data are means ± SE of four independent experiments. *I*: Data from *E* and *H* are presented as apoptotic index. Negative index values indicate protection against apoptosis by CHOP siRNA (▨), forskolin (▨), or the combination of both (■). The lack of difference between CHOP siRNA + forskolin and forskolin illustrates that CHOP does not contribute to the protective effect of forskolin against palmitate or salubrinal. *$P < 0.05$ against untreated cells; #$P < 0.05$.

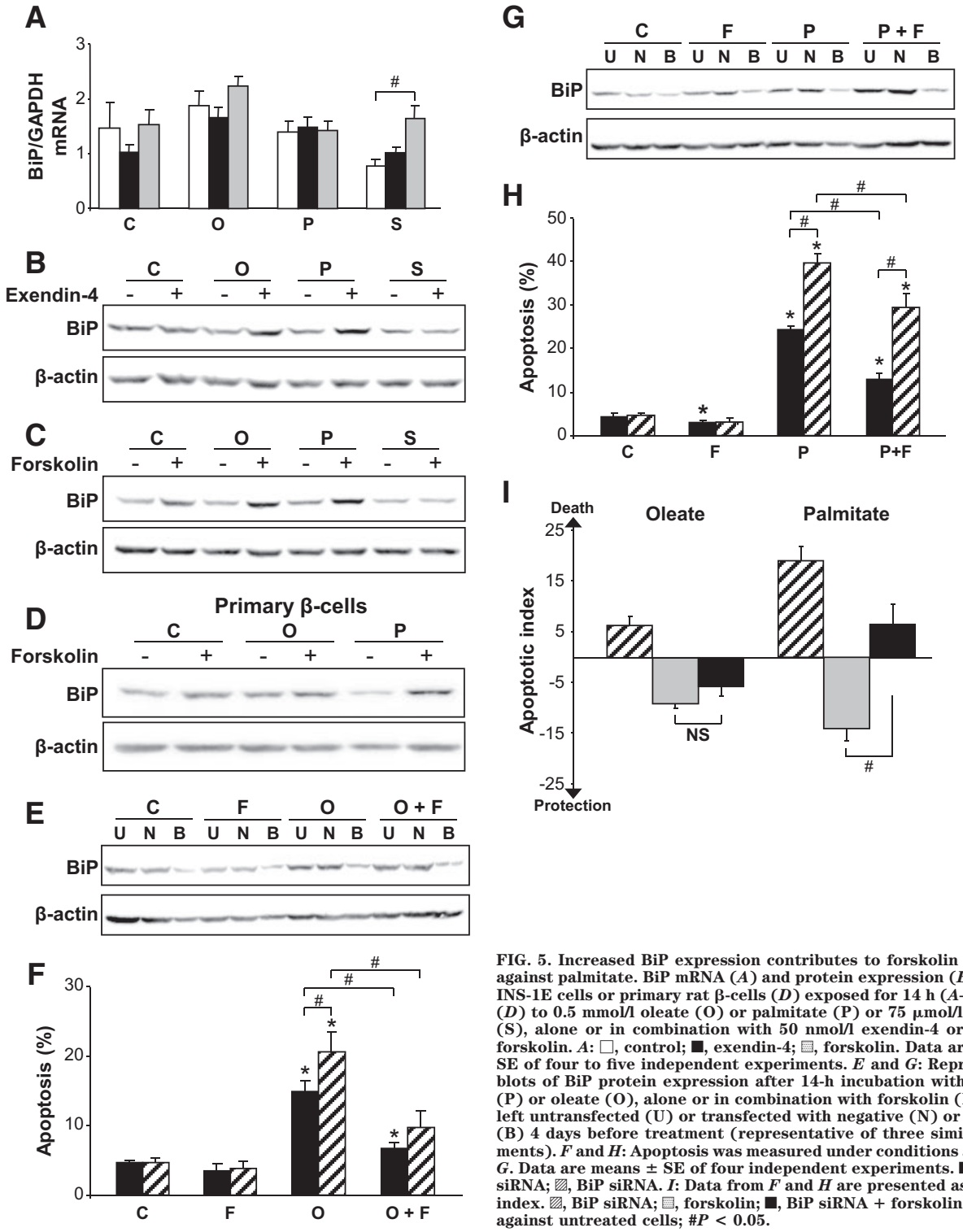

FIG. 5. Increased BiP expression contributes to forskolin protection against palmitate. BiP mRNA (*A*) and protein expression (*B* and *C*) in INS-1E cells or primary rat β-cells (*D*) exposed for 14 h (*A–C*) or 24 h (*D*) to 0.5 mmol/l oleate (O) or palmitate (P) or 75 μmol/l salubrinal (S), alone or in combination with 50 nmol/l exendin-4 or 20 μmol/l forskolin. *A*: □, control; ■, exendin-4; ▨, forskolin. Data are means ± SE of four to five independent experiments. *E* and *G*: Representative blots of BiP protein expression after 14-h incubation with palmitate (P) or oleate (O), alone or in combination with forskolin (F), in cells left untransfected (U) or transfected with negative (N) or BiP siRNA (B) 4 days before treatment (representative of three similar experiments). *F* and *H*: Apoptosis was measured under conditions as in *E* and *G*. Data are means ± SE of four independent experiments. ■, negative siRNA; ▨, BiP siRNA. *I*: Data from *F* and *H* are presented as apoptotic index. ▨, BiP siRNA; ▤, forskolin; ■, BiP siRNA + forskolin. *$P < 0.05$ against untreated cells; #$P < 0.05$.

and *I*). Loss of protection against palmitate was also observed with JunB knockdown, though it was partial only (Fig. 7*F*). Salubrinal-induced apoptosis was not modified by JunB knockdown, and neither was the antiapoptotic effect of forskolin (Fig. 7*H* and *I*).

Finally, we examined whether the upregulation of these antiapoptotic proteins by exendin-4 and forskolin was PKA dependent. The PKA inhibitor H89 prevented BiP, JunB, and Bcl-2 protein induction basally and following palmitate treatment (supplemental Fig. S7*A*) and abrogated the protective effect of forskolin (supplemental Fig. S7B).

DISCUSSION

Lipotoxic ER stress–mediated β-cell dysfunction and apoptosis may be relevant in the development of type 2 diabetes (9). Therapies that increase β-cell resistance to FFAs and ER stress could have considerable clinical impact, as they might prevent type 2 diabetes or attenuate

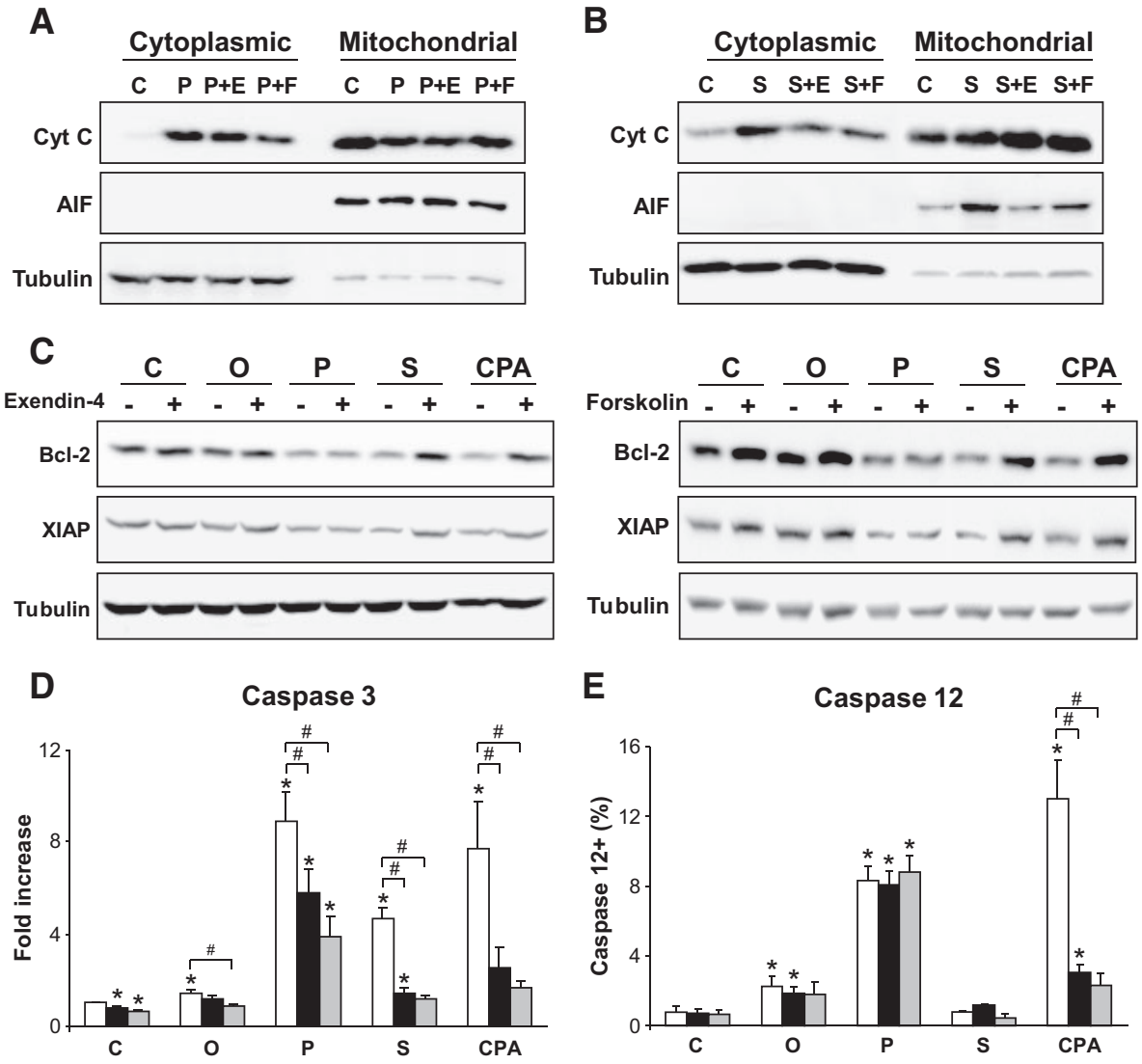

FIG. 6. Exendin-4 and forskolin modulate ER and mitochondria apoptosis pathways. *A* and *B*: Cytochrome C release in INS-1E cells treated for 14 h in the presence or absence of 0.5 mmol/l palmitate (P) or 75 μmol/l salubrinal (S) alone or in combination with 50 nmol/l exendin-4 (E) or 20 μmol/l forskolin (F). Representative blots of cytochrome C (Cyt C), AIF, and tubulin are shown (for four similar experiments). *C*: Representative blots of Bcl-2 and XIAP protein in INS-1E cells incubated for 14 h with 0.5 mmol/l oleate (O), palmitate, salubrinal, or 25 μmol/l CPA alone or in combination with exendin-4 or forskolin. *D* and *E*: Caspase 3 activity and percentage of caspase 12–positive INS-1E cells after 14-h treatment. Data are means ± SE of three to seven independent experiments. □, control; ■, exendin-4; ▨, forskolin. *$P < 0.05$ against untreated cells; #$P < 0.05$.

the progression of the disease. Activation of the GLP-1R by agonists such as exendin-4 induce cAMP-PKA, extracellular signal–regulated kinase 1/2 mitogen-activated protein kinase, and phosphoinositide 3 kinase-Akt signaling, leading to improved β-cell function through increased insulin biosynthesis and secretion, β-cell proliferation (at least in rodents), and decreased β-cell death (28,42). It has recently been shown that GLP-1R activation also stimulates β-cell survival during ER stress. In rodent models, exendin-4 treatment decreased in vivo pancreatic expression of CHOP, XBP1s, and BiP (29,43), but detailed in vitro studies demonstrated that GLP-1R activation leads to a cAMP-PKA–dependent enhancement of ATF4-CHOP-GADD34 signaling, followed by PP1-mediated eIF2α dephosphorylation and accelerated recovery of translational repression (29). Since ER stress signaling and outcomes are stimulus dependent, we examined whether these mechanisms mediate GLP-1R protection from the more pathophysiologically relevant lipotoxic ER stress. We observed that the protective mechanisms previously described for synthetic ER stress (29) are not operative in this ER stress condition and that exendin-4 and forskolin reduce FFA-induced apoptosis by increasing β-cell defense mechanisms.

The PERK-regulated ER stress branch that modulates global protein translation is necessary for β-cell survival. Humans and/or mice with a PERK deletion or Ser51Ala substitution in eIF2α (precluding its phosphorylation by PERK) cannot attenuate insulin translation and have progressive β-cell loss (24,44,45), suggesting that PERK-dependent eIF2α phosphorylation is beneficial to β-cells. Excessive or prolonged eIF2α phosphorylation and downstream signaling is, however, poorly tolerated by β-cells and specifically exacerbates FFA-induced apoptosis (11). We therefore asked whether increased signaling in the PERK pathway by exendin-4 during lipotoxic ER stress would be pro- or antiapoptotic. The present and previous (29) data support the idea that exendin-4 and forskolin

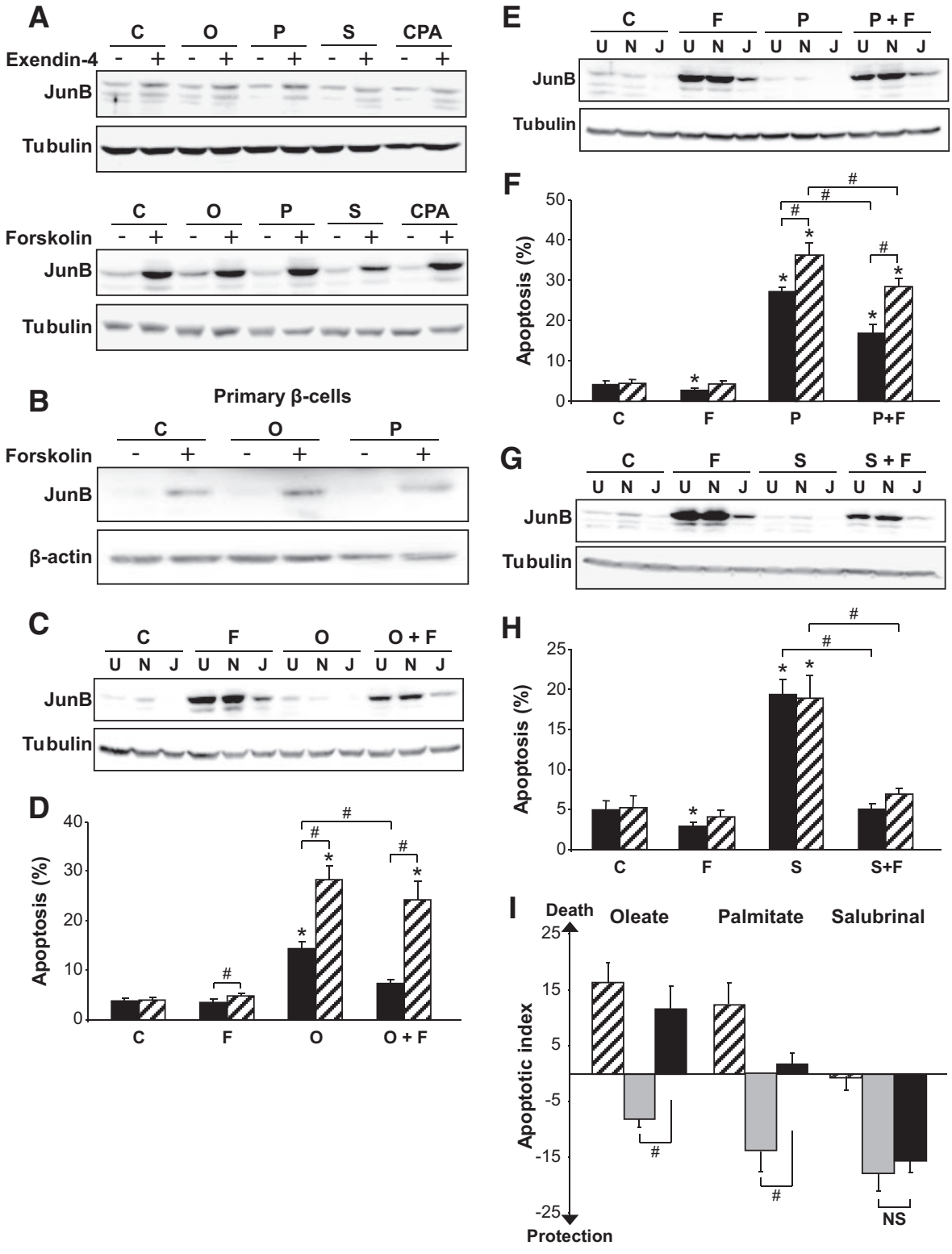

FIG. 7. Increased JunB expression by exendin-4 and forskolin mediates protection against lipotoxic ER stress. JunB protein expression in INS-1E cells cultured for 14 h (*A*) or primary rat β-cells cultured for 24 h (*B*) with 0.5 mmol/l oleate (O) or palmitate (P), 75 μmol/l salubrinal (S) or 25 μmol/l CPA, alone or in combination with 50 nmol/l exendin-4 or 20 μmol/l forskolin. *C*, *E*, and *G*: Representative blots of JunB protein expression after 14-h exposure to oleate, palmitate, or salubrinal, alone or in combination with forskolin (F), in cells left untransfected (U) or transfected with negative (N) or JunB siRNA (J) 2 days before treatment (from two to three similar experiments). *D*, *F*, and *H*: Apoptosis following RNAi under conditions as in *C*, *E*, and *G*. Data are means ± SE of three to four independent experiments. ■, negative siRNA; ▨, JunB siRNA. *I*: Data from *D*, *F*, and *H* are presented as apoptotic index. ▨, JunB siRNA; ▤, forskolin; ■, JunB siRNA + forskolin. **P* < 0.05 against untreated cells; #*P* < 0.05.

enhance ATF4-CHOP-GADD34 expression. This does not, however, explain the antiapoptotic outcome because CHOP or GADD34 knockdown, or chemical PP1 inhibition, do not abrogate the protective effect of forskolin in lipotoxic or synthetic ER stress conditions (Fig. 3 and supplementary Fig. S3). We therefore undertook a search

for alternative mechanisms mediating β-cell survival by GLP-1R activation in lipotoxic ER stress.

Activation of IRE1 leads to the expression of XBP1s that induces ER chaperones and ER expansion; for the latter lipid synthesis genes are upregulated. The induction of XBP1s by exendin-4 and forskolin suggests that these agents activate IRE1, either directly or by triggering a mild UPR (through enhanced protein synthesis) with activation of IRE1 but not PERK. GLP-1 and high glucose mildly activate IRE1 (46), and chronic exposure to high glucose causes XBP1s induction in β-cells that is not accompanied by ATF4 or CHOP expression (46,47). Knockdown of XBP1s leads to a small decrease in β-cell viability (present data). XBP1 RNAi potentiated oleate- but not palmitate-induced β-cell apoptosis (Fig. 4*H* and supplementary Fig. S8A), suggesting differential activation of pro- and anti-apoptotic signals by the FFAs downstream of IRE1; moreover, XBP1s might play a role in oleate handling. The prosurvival effect of forskolin was not altered by XBP1 knockdown, excluding it plays part in the observed protection.

IRE1 activation can directly cleave caspase 12. GLP-1 agonists did not modify caspase 12 cleavage by FFAs but nearly completely prevented its activation by CPA. In parallel, forskolin increased CPA-mediated PERK phosphorylation, suggesting that ER stress is not improved in this condition but that UPR transducers can be differentially regulated by GLP-1. ER stress can trigger apoptosis via the mitochondrial pathway of cytochrome C release, formation of the apoptosome, and caspase 3 activation. eIF2α phosphorylation is essential to proteasome degradation of antiapoptotic Bcl-2 family members during chemical ER stress (48). We currently observed that palmitate, but not oleate, induced degradation of Bcl-2 and cytochrome C release. eIF2α phosphorylation by salubrinal and CPA also decreased Bcl-2 with consequent cytochrome C translocation. The repression of eIF2α phosphorylation by exendin-4 and forskolin may restore Bcl-2 expression and thus prevent cytochrome C release and caspase 3 activation by salubrinal and CPA, but they have little effect on palmitate-induced apoptosis. GLP-1R activation also restored the antiapoptotic protein XIAP in cells exposed to salubrinal or CPA but not to palmitate, delineating context-dependent mechanisms of GLP-1R–mediated antiapoptotic effects (supplementary Fig. S8*B*).

BiP is an ATF6-dependent ER chaperone and a master regulator of UPR activation. GLP-1 agonists doubled BiP expression in a PKA-dependent manner, while a 50% reduction in BiP expression sensitized β-cells to FFAs, confirming the role of ER stress in lipotoxic β-cell apoptosis. The upregulation of BiP by forskolin protected β-cells against palmitate, as the protection was partially lost following BiP knockdown (Fig. 5*I*). Conclusive demonstration for the role of BiP will come from studies examining whether GLP-1 agonists are protective over and above adenovirus-mediated BiP overexpression. GLP-1R activation thus increases β-cell protein folding capacity and defense mechanisms against lipotoxic ER stress, prompting us to examine the potential contribution of other antiapoptotic proteins.

GLP-1 was previously shown to induce several early response genes in INS-1 cells, among which was JunB (49). JunB was recently shown to play a protective role in ER-stressed β-cells (41). Palmitate, salubrinal, and CPA decreased JunB expression, which was restored and further increased by exendin-4 and forskolin (supplementary Fig. S6). This PKA-dependent JunB induction is an important promoter of β-cell survival since JunB RNAi sensitized cells to FFAs and it abrogated the antiapoptotic effect of GLP-1R activation.

GLP-1 analogs provide hope that the natural history of type 2 diabetes can be modified by the preservation of functional β-cell mass (28). We demonstrate here that exendin-4 and cAMP production protect β-cells against ER stress in a context-dependent manner. Lipotoxic β-cell apoptosis is prevented by increasing cellular defense mechanisms through the induction of the ER chaperone BiP and the antiapoptotic protein JunB, whereas protection from synthetic ER stressors occurs via inactivation of caspase 12 and upregulation of Bcl-2 and XIAP to block mitochondrial apoptosis (supplementary Fig. S8). The present identification of the proapoptotic ER stress pathways and downstream signaling modulated by GLP-1 is an important milestone for the development of targeted approaches to alleviate β-cell ER stress in diabetes.

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