Guanabenz Sensitizes Pancreatic β Cells to Lipotoxic Endoplasmic Reticulum Stress and Apoptosis

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Deficient as well as excessive/prolonged endoplasmic reticulum (ER) stress signaling can lead to pancreatic β cell failure and the development of diabetes. Saturated free fatty acids (FFAs) such as palmitate induce lipotoxic ER stress in pancreatic β cells. One of the main ER stress response pathways is under the control of the protein kinase *R*-like endoplasmic reticulum kinase (PERK), leading to phosphorylation of the eukaryotic translation initiation factor 2 (eIF2α). The antihypertensive drug guanabenz has been shown to inhibit eIF2α dephosphorylation and protect cells from ER stress. Here we examined whether guanabenz protects pancreatic β cells from lipotoxicity. Guanabenz induced β cell dysfunction *in vitro* and *in vivo* in rodents and led to impaired glucose tolerance. The drug significantly potentiated FFA-induced cell death in clonal rat β cells and in rat and human islets. Guanabenz enhanced FFA-induced eIF2α phosphorylation and expression of the downstream proapoptotic gene C/EBP homologous protein (CHOP), which mediated the sensitization to lipotoxicity. Thus, guanabenz does not protect β cells from ER stress; instead, it potentiates lipotoxic ER stress through PERK/eIF2α/CHOP signaling. These data demonstrate the crucial importance of the tight regulation of eIF2α phosphorylation for the normal function and survival of pancreatic β cells. (***Endocrinology* 158: 1659–1670, 2017)**

Accumulating evidence indicates that loss of functional pancreatic β cell mass in type 2 diabetes results from environmental insults causing cellular stress responses that activate specific transcription factor and gene networks. Lipotoxicity, a term referring to the deleterious effects of prolonged exposure to free fatty acids (FFAs), leads to the impairment of insulin secretion (1–3) and β cell death (4).

Perturbations in the endoplasmic reticulum (ER) environment lead to accumulation of unfolded proteins and activation of the ER stress response. Previously, it has been demonstrated that FFAs induce ER stress in β cells (5–8); the activation of this stress response mediates at least part of FFA-induced β cell apoptosis. ER stress markers are present in β cells from patients with type 2 diabetes (8–11).

One of the canonical ER stress pathways depends on activation of protein kinase *R*-like endoplasmic reticulum kinase (PERK) and its phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) leading to translation attenuation and reduced protein load on the ER. In parallel, eIF2α phosphorylation augments translation of the activating transcription factor 4 (ATF4), in turn leading to the transcription of the proapoptotic C/EBP homologous protein (CHOP). In a negative feedback loop, ATF4 and CHOP induce GADD34 expression, which targets protein phosphatase 1 (PP1) to dephosphorylate eIF2α (12). The other canonical ER stress pathways are

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in USA

Received 19 October 2016. Accepted 24 February 2017.
First Published Online 1 March 2017

Abbreviations: ATF4, activating transcription factor 4; BSA, bovine serum albumin; CHOP, C/EBP homologous protein; CPA, cyclopiazonic acid; CReP, constitutive repressor of eIF2α phosphorylation; eIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; FBS, fetal bovine serum; FFA, free fatty acid; HFD, high-fat diet; HOMA-IR, homeostasis model assessment–insulin resistance; IPGTT, intraperitoneal glucose tolerance test; IRE1α, inositol-requiring 1α; mRNA, messenger RNA; PCR, polymerase chain reaction; PERK, protein kinase *R*-like endoplasmic reticulum kinase; PP1, protein phosphatase 1; RD, regular diet; siRNA, small interfering RNA.

activated by inositol-requiring 1α (IRE1α; which activates XBP1) and ATF6, leading to RNA degradation and transcription of ER chaperones, such as BiP, and folding enzymes. This transcriptional response increases ER capacity (13).

Several studies have demonstrated that genetic dysregulation of the PERK pathway leads to β cell demise and diabetes. Loss-of-function mutations in *EIF2AK3*, encoding PERK, cause Wolcott-Rallison syndrome, a rare autosomal recessive disease characterized by early-onset nonautoimmune diabetes associated with skeletal dysplasia and growth retardation (14). We have recently shown that a loss-of-function mutation in *PPP1R15B*, encoding the constitutive repressor of eIF2α phosphorylation (CReP), is causal of a syndrome of young-onset diabetes, microcephaly, and growth retardation (15). As GADD34, CReP is a nonenzymatic cofactor for PP1. The CReP mutation greatly reduces PP1 binding and diminishes eIF2α dephosphorylation, causing β cell dysfunction and death. Mice carrying a homozygous mutation in the phosphorylation site of eIF2α (S51A) are insulin deficient and die shortly after birth (16). When challenged with a high-fat diet (HFD), mice heterozygous for this mutation develop β cell dysfunction and diabetes (17). These data suggest that both excessive and reduced eIF2α phosphorylation can lead to β cell dysfunction and apoptosis. Fine-tuning of this process therefore seems crucial for proper β cell function and survival.

Pharmacological approaches to modulate eIF2α phosphorylation therefore hold potential for the treatment of diabetes. Guanabenz, an α_2-adrenergic receptor agonist used for the treatment of hypertension, binds to GADD34 and inhibits eIF2α dephosphorylation. This compound has been shown to be protective in clonal β cells expressing insulin^{Akita} that cannot properly fold and causes severe ER stress, as well as in HeLa cells undergoing ER stress (18). This FDA-approved antihypertensive drug might therefore be β cell protective in type 2 diabetes. We examined here whether it holds that potential for β cells facing lipotoxic stress.

Materials and Methods

Cell culture

Clonal rat INS-1E cells (Research Resource Identifier: CVCL_0351; a gift from Claes Wollheim, Centre Médical Universitaire, Geneva, Switzerland) were cultured in RPMI medium as described (19). Male Wistar rats (Charles River Laboratories) were housed and handled following the rules of the Belgian Regulations for Animal Care. The experiments were approved by the Ethical Committee for Animal Experiments of the Université Libre de Bruxelles. Rat islets were handpicked under a stereomicroscope after isolation by collagenase digestion and dispersed as previously described (20). The islets were cultured in Ham's F10 (Invitrogen) containing 5% fetal bovine serum (FBS). Human islets from nondiabetic organ donors (five males and one female; age, 63 ± 5 years; body mass index, 25 ± 1 kg/m^2) were isolated and cultured as previously described (21). The collection and handling of human islets were approved by the Ethical Committee of the University of Pisa, Pisa, Italy.

Cell treatment and apoptosis assays

Guanabenz (Santa Cruz) or inactive guanabenz (a gift from Cecile Voisset, Université de Brest, Brest, France) was used at 50 μM unless otherwise indicated. INS-1E cells were exposed to FFAs in RPMI 1640 medium containing 0.75% FFA-free bovine serum albumin (BSA; Roche) and 1% FBS. Rat and human islets were exposed to FFAs in the presence of 1% charcoal-absorbed BSA without FBS (22). Oleate and palmitate (Sigma) were dissolved in 90% ethanol and diluted 1:100 to a final concentration of 0.5 mM (4, 22). Cyclopiazonic acid (CPA) was used at 25 μM, brefeldin A at 0.1 μg/mL, and tunicamycin at 5 μg/mL. Apoptotic cell death was detected by fluorescence microscopy after staining with DNA binding compounds Hoechst 33342 (5 μg/mL; Sigma) and propidium iodide (5 μg/mL) (5, 23) by two investigators, one of whom was blinded for the experimental conditions. Cleaved caspase 3 and insulin double immunostaining was performed in dispersed rat islet cells as previously described (24).

Mouse studies

The mouse studies were approved by the Ethical Committee for Animal Experiments of the Université Libre de Bruxelles. Male C57BL/6N mice (Janvier Laboratories) were fed regular diet (RD; 10% fat, Research Diets D12450B) or HFD (60% fat, Research Diets D12492). Guanabenz was administered by intraperitoneal injection (4 mg/kg guanabenz acetate, G110, Sigma-Aldrich, dissolved in 0.9% NaCl) every other day (25, 26). An intraperitoneal glucose tolerance test (IPGTT) was performed as described (27) after 1 week of diet and treatment. Blood glucose levels were measured using Accu-Chek Aviva Nano (Roche), and plasma insulin was measured using the Ultra Sensitive Mouse Insulin enzyme-linked immunosorbent assay (Crystal Chem). Homeostasis model assessment–insulin resistance (HOMA-IR) was calculated as: fasting glucose (mg/dL) × fasting insulin (μU/mL)/(18 × 22.5). The insulinogenic index was calculated as delta insulin/delta glucose between 0 and 15 minutes of the IPGTT. This measure of insulin secretion was divided by HOMA-IR to correct for the insulin sensitivity of the animal to obtain a measure of β cell function.

Protein translation

The SUnSET method was used to detect translation of nascent proteins (28). Just prior to collection in Laemmli buffer, cells were incubated for 30 min with 1 μM puromycin (Sigma). The rate of puromycin-labeled peptide formation reflects the overall protein synthesis rate (28).

Western blotting

Protein detection was done using primary antibodies listed in Supplemental Table 1, horseradish peroxidase–conjugated secondary antibodies, and SuperSignal West Femto chemiluminescence revealing reagent (Thermo Fisher Scientific). Immunoreactive bands were detected with a ChemiDoc XRS+ system and with Image Lab Software (BIO-RAD).

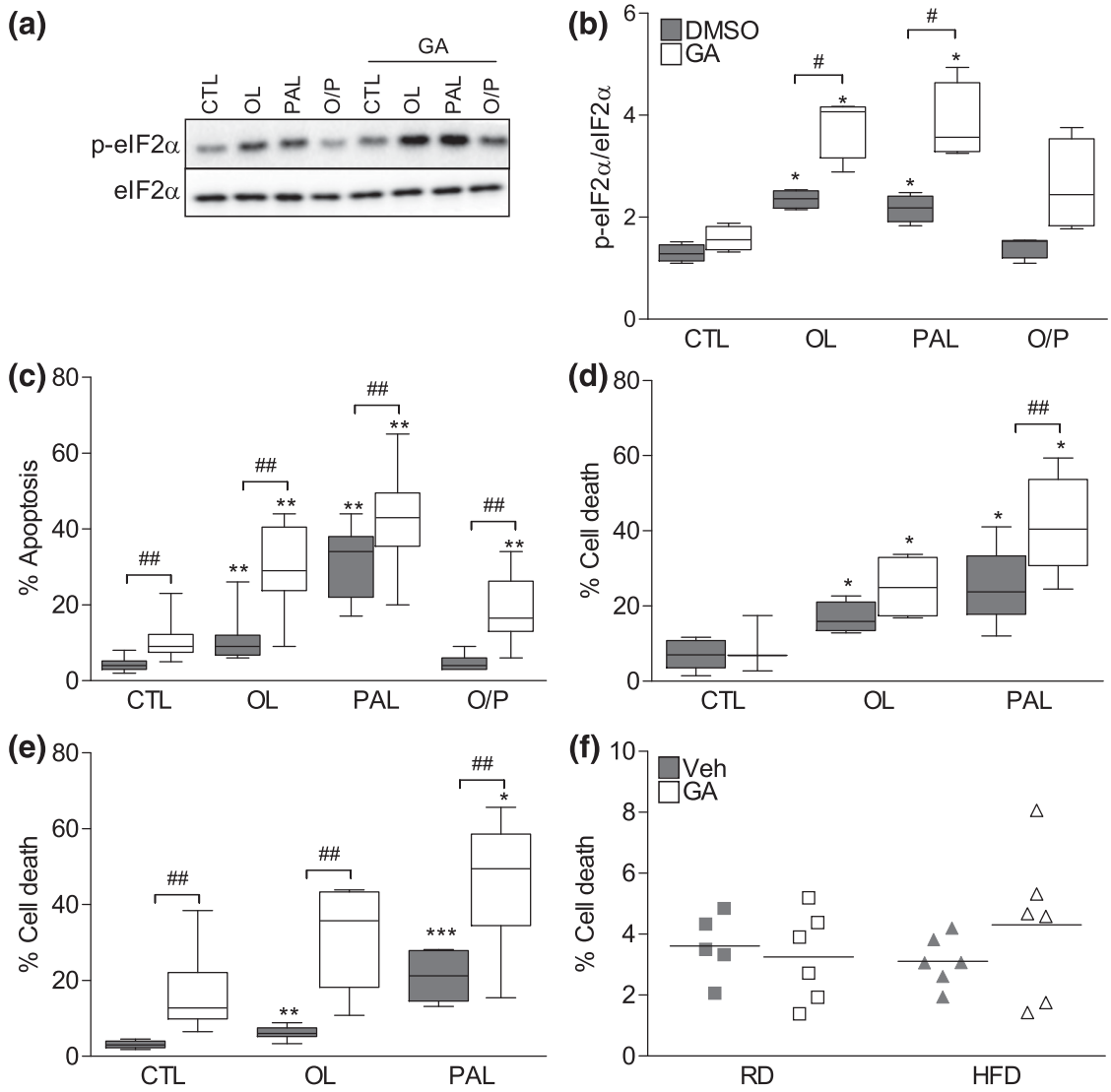

Figure 1. Guanabenz promotes eIF2α phosphorylation and sensitizes β cells to FFA-induced apoptosis. (a–c) INS-1E cells, (d) rat islets, and (e) human islets were exposed to 50 μM guanabenz (GA), alone or in combination with oleate (OL), palmitate (PAL), or a 1:1 mixture of oleate and palmitate (O/P) for (a, b) 16 hours, (c) 24 hours, (d) 48 hours, and (e) 72 hours. (f) Islets were isolated from C57BL/6N mice treated for 1 week with GA or vehicle (Veh) in combination with RD or HFD. (a, b) Western blots for phosphorylated eIF2α were quantified by densitometry and corrected for total eIF2α. (c–e) Apoptosis was measured by Hoechst 33342 and propidium iodide staining. (a–e) The boxes indicate lower quartile, median, and higher quartile; whiskers represent the range of remaining data points. (f) The dots represent individual animals, and the line indicates the mean. n = 4 to 14 independent experiments. *FFA vs control (CTL). #GA vs dimethyl sulfoxide (DMSO). $^{*/\#}P < 0.05$; $^{**/\#\#}P < 0.01$; $^{***}P < 0.001$.

Glucose-stimulated insulin secretion

Mouse islet glucose-stimulated insulin secretion studies were done as described (27). Rat islets were exposed for 24 hours to 10 μM guanabenz, alone or in combination with 0.5 μM oleate in the presence of 1% charcoal-absorbed BSA. The islets were incubated in modified Krebs-Ringer bicarbonate HEPES solution for 30 minutes, and insulin secretion was induced by 1-hour incubation in 1.67 or 16.7 mM glucose. Insulin was measured by enzyme-linked immunosorbent assay (15).

Messenger RNA extraction and real-time polymerase chain reaction

Poly(A)$^+$ messenger RNA (mRNA) was isolated and reverse transcribed as described (7, 29). Real-time polymerase chain reaction (PCR) was performed using Rotor-Gene SyBR Green on a Rotor-Gene Q cycler (Qiagen) or FastStart SYBR Green on a LightCycler (Roche) (7, 30). Primers were used in a conventional PCR for preparing the standard. Gene expression was calculated as copies per microliter (31). Expression levels were corrected for the reference genes GAPDH for rat and β-actin for human. These reference genes have been previously validated in β cells (22, 23, 32). Primer sequences are provided in Supplemental Table 2.

CHOP luciferase assay

INS-1E cells were transfected with a CHOP promoter construct (33). Twenty-four hours after transfection, the cells were treated for eight or 16 hours with palmitate alone or in combination with guanabenz. Luciferase activity was measured

using the dual luciferase reporter assay system (Promega) as described (34).

RNA interference

INS-1E cells were transfected overnight with 30 nM control small interfering RNA (siRNA) (Qiagen) or siRNA-targeting CHOP (*5*) using Lipofectamine RNAiMAX (Invitrogen) as described previously (35).

Statistical analysis

The data are presented as box plots or dot plots. Boxes indicate lower quartile, median, and higher quartile; whiskers represent the full range of data points. The dots represent each experimental animal, and the line indicates the mean. Comparisons were made by two-sided paired (or ratio) or unpaired *t* test, as appropriate. $P < 0.05$ was considered statistically significant.

Results

Guanabenz potentiates ER stress-induced apoptosis in β cells

Guanabenz was previously shown to inhibit eIF2α dephosphorylation (18). We confirmed that in clonal insulin-producing INS-1E cells, guanabenz *per se* tended to increase eIF2α phosphorylation, and it potentiated FFA-induced eIF2α phosphorylation [Fig. 1(a) and 1(b); Supplemental Fig. 1(A)]. We then examined whether guanabenz affects basal β cell survival using concentrations from 0.2 to 50 μM. At these concentrations, guanabenz induced low levels (6% to 8%) of apoptosis in INS-1E cells. The inactive form of guanabenz, inactivated by the replacement of one of the chlorines by fluorine (26), did not affect INS-1E cell survival (data not shown). Based on this dose-response study and previous reports (18), we selected 2 and 50 μM of guanabenz to test its cytoprotective effect in β cells undergoing ER stress. To this end, we exposed β cells to FFAs, which may elicit ER stress in type 2 diabetes, and to chemical ER stressors. As previously reported (*5*), the saturated FFA palmitate induced apoptosis in INS-1E cells; the unsaturated FFA oleate induced much less apoptosis and the equimolar mixture of oleate and palmitate was nontoxic [Fig. 1(c)]. Guanabenz (50 μM) did not protect INS-1E cells from lipotoxicity but instead sensitized the cells, in particular when exposed to oleate or the oleate/palmitate mixture [Fig. 1(c)] . A similar sensitization was seen with 2 μM guanabenz [Supplemental Fig. 1(B)], but not with the inactive form of guanabenz [Supplemental Fig. 1(C)]. Guanabenz also potentiated FFA-induced cell death in rat islets and even more so in human islets [Fig. 1(d) and 1(e)]. The sensitizing effect was also seen in INS-1E cells exposed to the chemical ER stressors CPA and tunicamycin, but not brefeldin A [Supplemental Fig. 1(D)]. This was not the case for the inactive form of guanabenz [Supplemental Fig. 1(E)]. The induction of apoptosis in INS-1E cells and rat islets was confirmed by increased caspase 3 cleavage, analyzed by Western blot [Fig. 2(a) and 2(b)]. We further confirmed the apoptosis to occur in β cells by insulin and cleaved caspase 3 immunostaining of dispersed rat islet cells treated with guanabenz alone or in combination with oleate [Fig. 2(c); Supplemental Fig. 2].

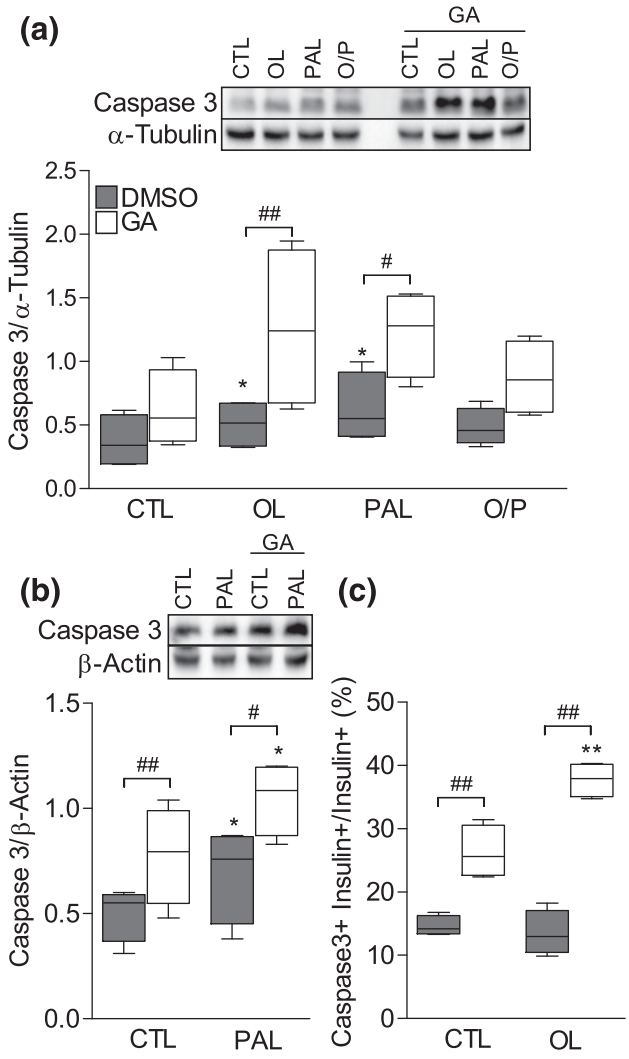

Figure 2. Guanabenz induces caspase 3 cleavage in β cells. (a) INS-1E cells and (b) whole or (c) dispersed rat islets were exposed to 50 μM guanabenz (GA), alone or in combination with oleate (OL), palmitate (PAL), or a 1:1 mixture of oleate and palmitate (O/P), for (a) 16 hours (b) 48 hours, and (c) 24 hours. (a, b) Western blot for cleaved caspase 3 and α-tubulin or β-actin, used as controls for protein loading. (c) After immunostaining for insulin and cleaved caspase 3, double-positive rat islet cells were counted and expressed as percentage of insulin-positive cells. The boxes indicate lower quartile, median, and higher quartile; whiskers represent the range of remaining data points (n = 4 to 6 independent experiments). *FFA vs control (CTL). #GA vs dimethyl sulfoxide (DMSO). $*/\#P < 0.05$; $**/\#\#P < 0.01$.

Guanabenz induces β cell dysfunction in mice

To investigate the impact of guanabenz *in vivo*, mice were treated with guanabenz for 1 week in combination

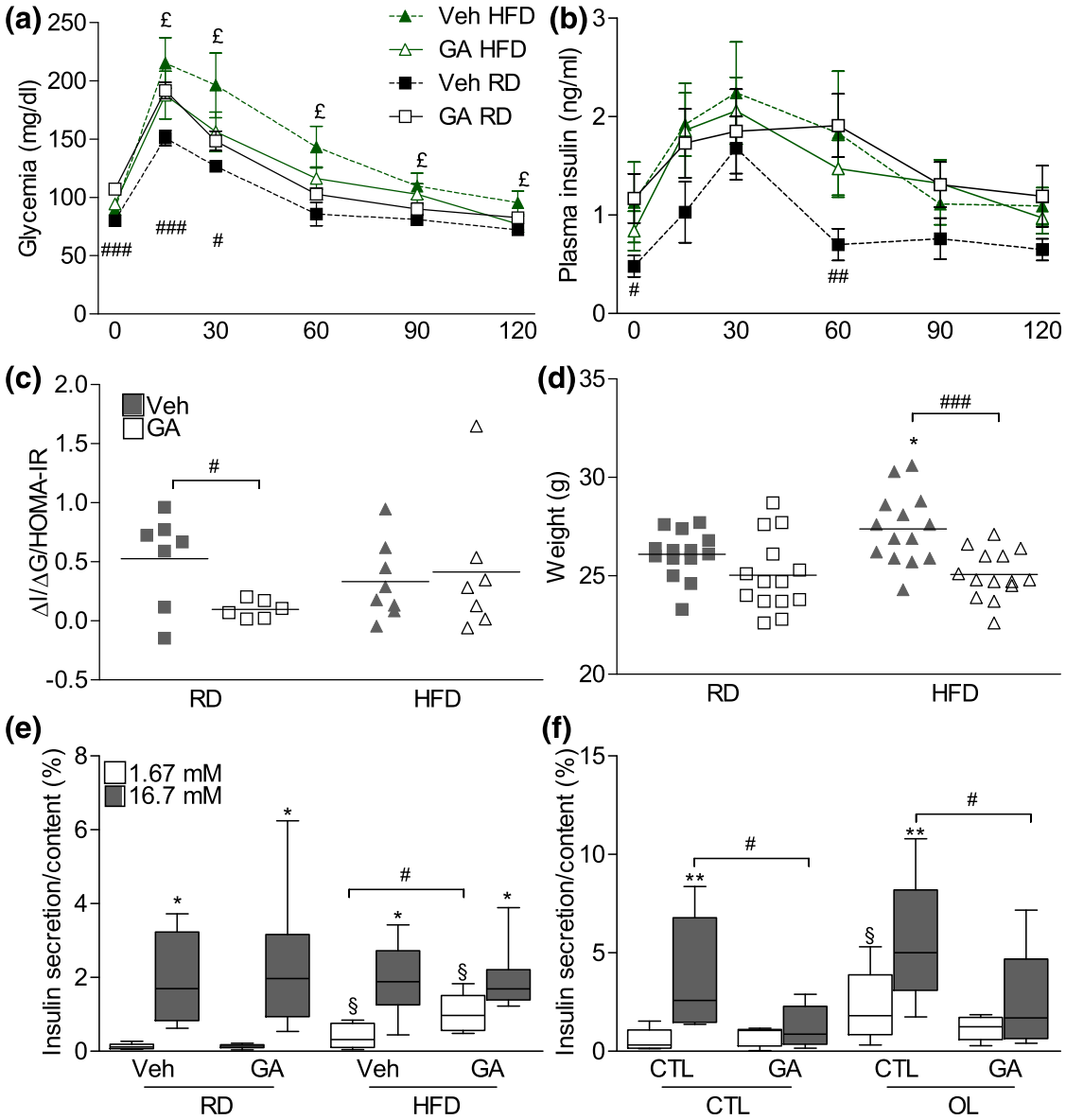

Figure 3. Guanabenz induces β cell dysfunction and impairs glucose tolerance. (a) Blood glucose and (b) plasma insulin levels measured during an IPGTT of mice treated for 1 week with guanabenz (GA) or vehicle (Veh) while on RD (black lines) or HFD (green lines). (c) β cell function was calculated as Δinsulin/Δglucose between 0 and 15 minutes of the IPGTT and corrected for HOMA-IR. (d) Body weight at the end of treatment. (e) *Ex vivo* islet insulin secretion at 1.67 or 16.7 mM glucose (n = 9). (f) Rat islets were exposed to GA alone or in combination with oleate for 24 hours (n = 5). (a, b) Data are mean ± standard error. (c, d) The dots represent individual animals, and the line indicates the mean. (e, f) The boxes indicate lower quartile, median, and higher quartile; whiskers represent the range of data points. CTL, control; OL, oleate. $^{£}$HFD vs RD; $^{\#}$GA vs Veh; *16.7 mM vs 1.67mM; §HFD/OL vs RD/CTL; $^{*/\#/§/£}P < 0.05$; $^{**/\#\#}P < 0.01$; $^{\#\#\#}P < 0.01$.

with RD or HFD. After this short-term guanabenz treatment, we noticed a trend for increased cell death in HFD-fed mouse islets *ex vivo*, but the drug did not affect islet cell survival in the RD-fed group [Fig. 1(f)]. The low rate of cell death detected *ex vivo* is probably due to the efficient clearance of apoptotic cells *in vivo* (36, 37).

Guanabenz treatment of RD-fed mice increased fasting blood glucose and insulin levels and HOMA-IR, indicating the development of insulin resistance [Supplemental Fig. 3(A–C)]. To investigate the impact of guanabenz on β cell function, we performed an IPGTT and measured glucose and insulin levels [Supplemental Fig. 3(A–E)]. Guanabenz impaired glucose tolerance in RD-fed mice [Fig. 3(a)]. In a compensatory response to the insulin resistance, the guanabenz-treated mice were hyperinsulinemic [Fig. 3(b)], but this increase failed to fully normalize glycemia [Fig. 3(a)]. In keeping with this, β cell function, calculated as the insulinogenic index corrected for HOMA-IR, was decreased by guanabenz [Fig. 3(c)].

As expected, HFD feeding increased body weight, and it impaired glucose tolerance and induced hyperinsulinemia during the IPGTT [Fig. 3(a), 3(b), and 3(d)].

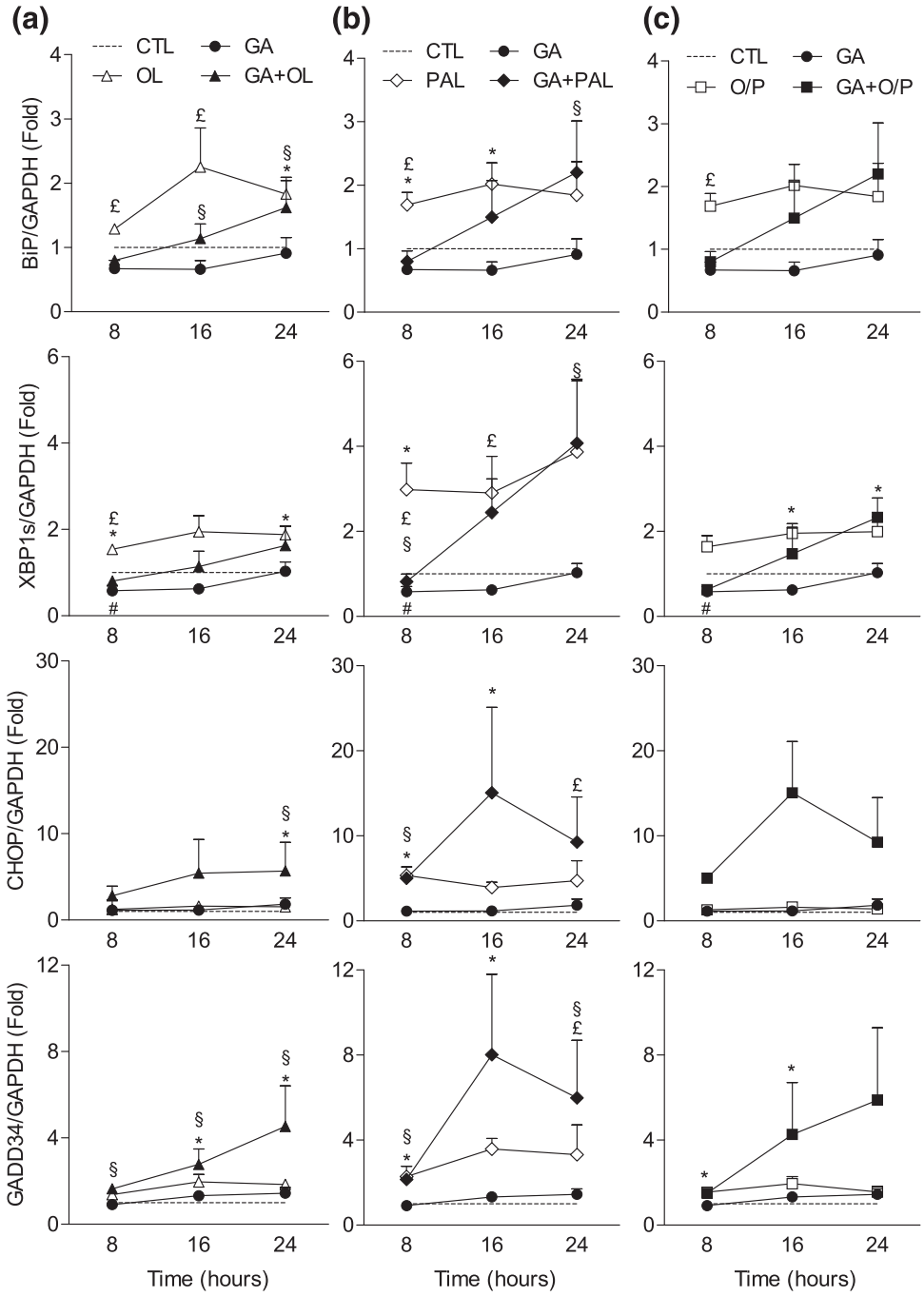

Figure 4. Guanabenz potentiates FFA-induced CHOP and GADD34 expression. INS-1E cells were treated with 50 μM guanabenz (GA), alone or in combination with (a) oleate (OL), (b) palmitate (PAL), or (c) a 1:1 mixture of oleate and palmitate (O/P), for the indicated times. mRNA expression of BiP, XBP1s, CHOP, and GADD34 was examined by real-time PCR and corrected for the expression of the reference gene GAPDH. Data are presented as fold change of control, indicated by the dashed line. Results are mean ± standard error of four independent experiments. CTL, control. *FFA vs CTL; §FFA + GA vs GA; $^{\#}$GA vs dimethyl sulfoxide (DMSO); $^{£}$FFA vs FFA + GA; $^{*/§/\#/£}P < 0.05$.

There was little additional impact of guanabenz on measures of insulin sensitivity and insulin secretion, possibly because the treatment decreased food intake and body weight [Supplemental Fig. 3(F); Fig. 3(d)]. The decreased food intake could be due to the drug's side effects, including drowsiness and nausea in humans (38) and decreased rotarod performance in mice (39).

To directly assess β cell function, islets were isolated from the mice and *ex vivo* insulin secretion was measured. Glucose, 16.7 mM, induced a 15-fold increase in insulin secretion in control islets [Fig. 3(e)]. HFD-fed mouse islets had increased basal insulin secretion and a lesser, fivefold, response to high glucose. Guanabenz induced even higher basal insulin secretion and no absolute difference in response to high glucose (twofold

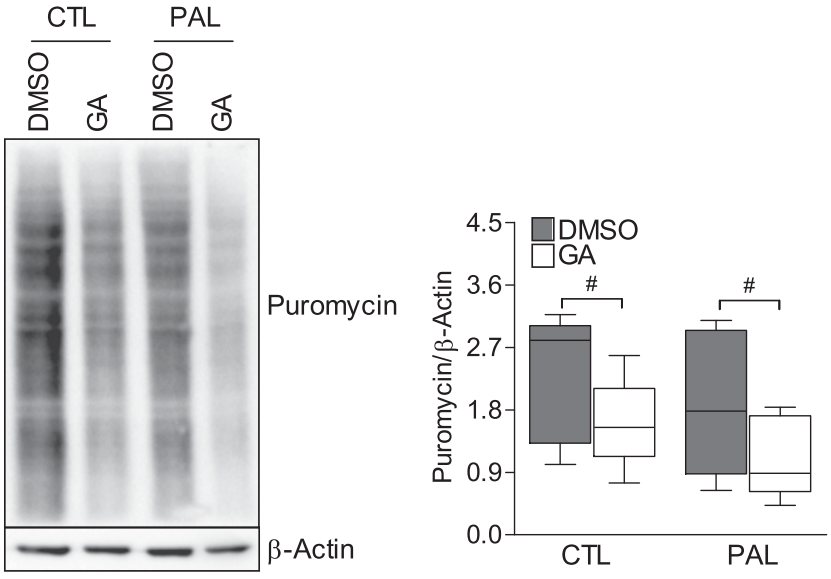

Figure 5. Guanabenz decreases protein translation. INS-1E cells were treated with 50 μM guanabenz (GA), alone or in combination with palmitate (PAL), for 16 hours and then incubated for 30 minutes with puromycin. The Western blot for puromycin is representative of five independent experiments, quantified by densitometry and corrected for β-actin in the graph. The boxes indicate lower quartile, median, and higher quartile; whiskers represent the range of remaining data points. CTL, control. #GA vs dimethyl sulfoxide (DMSO); $P < 0.05$.

increase compared with basal insulin secretion) [Fig. 3(e)]. We also examined the impact of guanabenz on β cell function *in vitro*. Guanabenz *per se* impaired rat islet glucose-stimulated insulin secretion [Fig. 3(f)]. In keeping with the *ex vivo* data, oleate exposure increased basal insulin secretion and reduced the glucose-stimulated response; the addition of guanabenz significantly worsened β cell dysfunction [Fig. 3(f)]. The decrease in glucose-stimulated insulin secretion was not due to insulin depletion, as guanabenz did not reduce the insulin content of mouse or rat islets [Supplemental Fig. 3(G) and 3(H)].

Guanabenz enhances FFA-induced PERK signaling

FFAs induce ER stress signaling in β cells (5). This was confirmed in a time course analysis of expression of the ER stress markers BiP, XBP1s, CHOP, and GADD34 in FFA-exposed INS-1E cells (Fig. 4). As previously reported, palmitate induces stronger signaling in the PERK and IRE1 branches of the ER stress response, whereas saturated and unsaturated FFAs similarly induce BiP expression (5).

Guanabenz *per se* did not modify BiP, CHOP, or GADD34 mRNA expression and slightly decreased XBP1s levels at the earliest time point (Fig. 4). Guanabenz reduced BiP and XBP1s expression in FFA-exposed INS-1E cells at early time points. However, guanabenz markedly potentiated the FFA-induced expression of genes in the PERK pathway, namely CHOP and GADD34, and this was true for both saturated and unsaturated FFAs (Fig. 4).

Guanabenz inhibits eIF2α dephosphorylation (18) and is thereby expected to attenuate protein translation. By measuring puromycin incorporation into elongating peptide chains, we observed that guanabenz decreases protein translation under basal and FFA conditions (Fig. 5). Also downstream of eIF2α phosphorylation [Fig. 1(a) and 1(b); Supplemental Fig. 1(A)], and in keeping with our mRNA expression studies, guanabenz potentiated FFA-induced CHOP protein expression [Fig. 6(a)]. CHOP induction was further confirmed using a CHOP promoter luciferase construct. Guanabenz *per se* induced CHOP promoter activity (8 hours) [Supplemental Fig. 4(A)] and potentiated palmitate-induced promoter activation (16 hours) [Fig. 6(b)]. Signaling in the other branches of the ER stress response, assessed by measuring BiP and XBP1s protein expression, was not induced by guanabenz. The transient decrease in mRNA levels (Fig. 4) resulted in lower XBP1s protein [Supplemental Fig. 4(B) and 4(D)] but did not affect BiP protein [Supplemental Fig. 4(C) and 4(E)], probably due to the 46-hour-long half-life of BiP protein (40).

Signal transduction was also assessed in human islets. Guanabenz did not affect XBP1s mRNA [Supplemental Fig. 4(F)], suggesting that the IRE1 pathway does not mediate the sensitization of islet cells to apoptosis. The drug significantly increased CHOP mRNA expression by twofold under basal condition and three- to fourfold after FFA exposure [Fig. 6(c)].

The *in vivo* treatment of mice with guanabenz did not result in detectable changes in islet XBP1s protein expression or eIF2α phosphorylation [Supplemental Fig. 4(G) and 4(H)], but it increased CHOP protein expression in the HFD-fed group [Fig. 6(d)]. The discrepancy between eIF2α phosphorylation and CHOP expression may be due to dynamic regulation, by several kinases and phosphatases (41), of eIF2α phosphorylation. Detailed time course experiments are more difficult to perform *in vivo* compared with the *in vitro* models.

Guanabenz potentiates FFA-induced β cell demise through CHOP

Based on the converging findings in these different models, we examined whether CHOP mediates guanabenz-induced apoptosis using RNA interference.

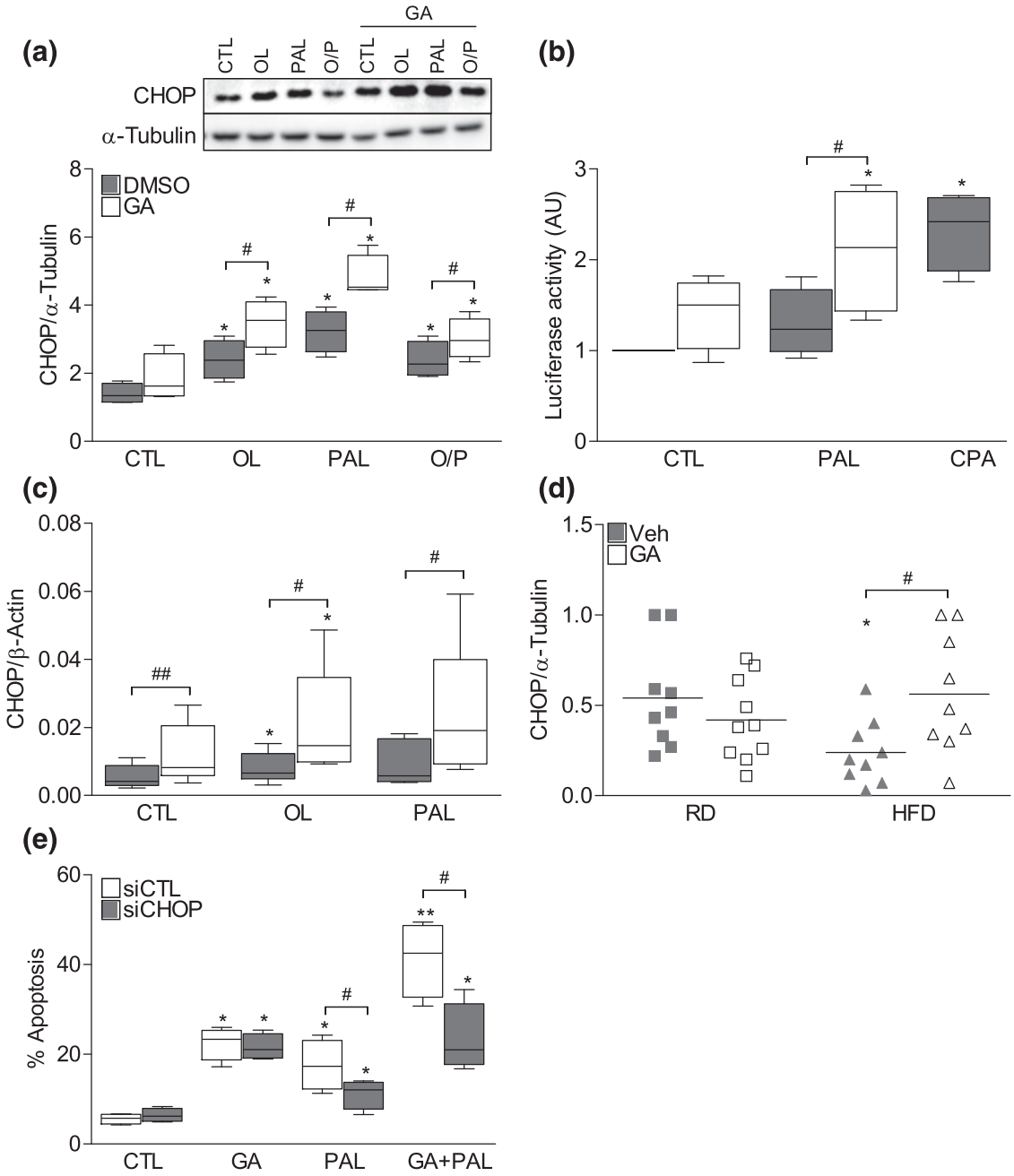

Figure 6. Guanabenz potentiates FFA-induced CHOP expression leading to apoptosis. (a, b) INS-1E cells were treated with 50 μM guanabenz (GA), alone or in combination with oleate (OL), palmitate (PAL), or a 1:1 mixture of oleate and palmitate (O/P), for 16 hours. (a) Western blots for CHOP were quantified by densitometry and corrected for α-tubulin. Blots are representative of four independent experiments. (b) INS-1E cells transfected with a CHOP luciferase reporter construct were treated with GA, alone or in combination with PAL. CPA (25 μM) was used as a positive control (CTL). (c) Human islets were treated with 50 μM GA, alone or in combination with OL or PAL, for 72 hours. CHOP mRNA expression was examined by real-time PCR and corrected for the expression of the reference gene β-actin. (d) Islets were isolated from C57BL/6N mice treated for 1 week with GA or vehicle while on RD or HFD. CHOP protein levels were corrected by α-tubulin and presented as fold of highest value. (e) INS-1E cells transfected with a control siRNA (siCTL) or siRNA targeting CHOP (siCHOP) were treated with GA, alone or in combination with PAL, for 16 hours. The boxes indicate lower quartile, median, and higher quartile; whiskers represent the range of remaining data points (a–c and e: n = 4 to 5 independent experiments). (d) The dots represent individual animals, and the line indicates the mean. *FFA vs control (CTL); #GA vs dimethyl sulfoxide (DMSO); $^{*/\#}P < 0.05$; $^{**/\#\#}P < 0.01$.

An efficient CHOP knockdown of around 65% was achieved [Supplemental Fig. 4(I)]. CHOP silencing protected INS-1E cells from palmitate-induced apoptosis, as previously described (5), and significantly reduced the potentiating effect of guanabenz [Fig. 6(e)]. Taken together, these data show that guanabenz sensitizes β cells to lipotoxic apoptosis through the eIF2α/CHOP pathway.

Appendix. Antibody Table

Peptide/Protein Target	Name of Antibody	Manufacturer, Catalog #, and/or Name of Individual Providing the Antibody	Species Raised in; Monoclonal or Polyclonal	Dilution Used	RRID
p-eIF2α	Phospho-eIF2α (Ser51)	Cell Signaling, cat. #3597	Rabbit; monoclonal	1/1000	AB_390740
eIF2α	eIF2α (D7D3) XP	Cell Signaling, cat. #5324	Rabbit; monoclonal	1/1000	AB_10692650
Cleaved caspase 3	Cleaved caspase-3 (Asp175)	Cell Signaling, cat. #9661	Rabbit; polyclonal	1/1000	AB_2341188
BiP	BiP antibody	Cell Signaling, cat. #3183	Rabbit; polyclonal	1/2000	AB_10695864
α-Tubulin	Monoclonal anti–α-Tubulin antibody	Sigma-Aldrich, cat. #T9026	Mouse; monoclonal	1/5000	AB_477593
CHOP	GADD 153 antibody (B-3)	Santa Cruz, cat. #SC-7351	Mouse; monoclonal	1/1000	AB_627411
XBP1s	XBP-1 antibody (M-186)	Santa Cruz, cat. #Sc-7160	Rabbit; polyclonal	1/1000	AB_794171
β-Actin	β-Actin antibody	Cell Signaling, cat. #4967	Rabbit; polyclonal	1/5000	AB_330288
Puromycin	Puromycin (3RH11)	Kerafast, cat. #EQ0001	Mouse; monoclonal	1/1000	AB_2620162
Insulin	Insulin antibody	Dako, cat. #A0564	Guinea Pig; polyclonal	1/200	AB_10013624
Anti-rabbit IgG	Peroxidase AffiniPure F(ab')2 fragment donkey anti-rabbit IgG (H+L)	Lucron Bioproducts, cat. #711-036-152	Donkey; polyclonal	1/5000	AB_2340590
Anti-mouse IgG	Peroxidase AffiniPure F(ab')2 fragment donkey anti-mouse IgG (H+L)	Lucron Bioproducts, cat. #715-036-150	Donkey; polyclonal	1/5000	AB_2340773
Anti-rabbit IgG	Goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 568	ThermoFisher, cat. #A-11036	Goat; polyclonal	1/500	AB_2534094
Anti–guinea pig IgG	Goat anti–guinea pig IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 488	ThermoFisher, cat. #A-11073	Goat; polyclonal	1/500	AB_2534117

Abbreviations: H+L, antibody that reacts with the heavy and light chains of the immunoglobulin; IgG, immunoglobulin G; RRID, Research Resource Identifier.

Discussion

Accumulating evidence suggests that ER stress contributes to β cell demise in type 2 diabetes (5–8). Saturated FFAs, the most common in humans being palmitate, markedly activate the PERK branch of the ER stress response, leading to eIF2α phosphorylation and attenuation of translation (5). Translational attenuation is cytoprotective, as this reduces the protein load in the ER and lessens ER stress. Prolonged or intense PERK signaling, however, triggers apoptosis. Interest in finding drugs intervening in this pathway is high. Guanabenz, an α_2-adrenergic agonist, was proposed to protect cells against ER stress–induced apoptosis (18). In stark contrast with the previous report, and with findings in cardiac myocytes (42) and retinal cells (43), we show here that guanabenz does not protect, but rather potentiates FFA-induced ER stress and β cell demise.

The PERK pathway plays a major role in β cell survival and function. As in other cell types, the initial PERK response is aimed at reducing protein translation and relieving the stressed ER (44, 45). Intense or prolonged eIF2α phosphorylation causes β cell dysfunction and apoptosis (5, 46, 47). Guanabenz was shown to bind GADD34 and inhibit its binding to PP1 (18). We confirmed that guanabenz enhances eIF2α phosphorylation in FFA-treated β cells. In *in vivo* and *in vitro* models, guanabenz treatment impairs β cell function, leading to hyperglycemia in mice. The latter is in keeping with an earlier report (48). Guanabenz upregulates expression of CHOP, a proapoptotic transcription factor (49, 50) downstream of eIF2α phosphorylation, and potentiates FFA-induced β cell apoptosis. Using RNA interference, we showed that CHOP mediates this β cell sensitization.

The induction of insulin resistance [Supplemental Fig. 3(C)] may contribute to the loss of glucose tolerance in guanabenz-treated animals. Previous studies have shown that eIF2α phosphorylation leads to insulin resistance in liver (51, 52). Conversely, deletion of CHOP results in obesity but preserved insulin sensitivity (53). Insulin resistance is normally compensated for by increased insulin secretion in both humans and mice (54, 55). Although guanabenz-treated mice were hyperinsulinemic, this was not enough to reduce glycemia to normal levels [Fig. 3(a)].

These findings are consistent with our previous work on salubrinal (56). Salubrinal was identified in a high throughput screen for small molecules that protect cells from tunicamycin-induced ER stress and shown to inhibit formation of GADD34/PP1 and CReP/PP1 complexes (57). The synergistic activation of PERK-eIF2α

signaling by salubrinal and FFAs led to inhibition of protein synthesis and insulin release, increased ATF4 and CHOP expression and apoptosis, both in rodent β cells (56) and human islets (30). The guanabenz and salubrinal data concur with the phenotype of human *PPP1R15B* loss-of-function mutations (15): Driving eIF2α phosphorylation either by pharmacological approaches or genetic causes results in β cell dysfunction and death.

Few studies have investigated whether guanabenz therapy affects insulin secretion and glucose tolerance in humans. One small study did not find changes in insulinemia, but it did not consider patients' glycemia and insulin sensitivity (58). In another study of guanabenz-treated diabetic patients, no changes were seen in glucose control or antidiabetic treatment needs over a mean follow-up of 7 months (59). Ideally, a potential link between guanabenz use and diabetes development/progression should be examined in large studies that couple prescription medicine registers with glycemic data in patients.

In conclusion, we have demonstrated that guanabenz potentiates FFA-induced β cell dysfunction and apoptosis through enhanced signaling downstream of PERK. We caution that interventions aimed at modulating eIF2α phosphorylation in β cells should consider the sensitivity of these cells to any imbalance in this pathway.

Acknowledgments

We thank Isabelle Millard, Michael Pangerl, Anyishai Musuaya, and Nathalie Pachera at the Université Libre de Bruxelles Center for Diabetes Research for excellent technical support, and Decio L. Eizirik for his valuable input on the experimental design of this study and in data discussions.

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This work was supported by the European Union's Horizon 2020 research and innovation programme, project T2DSystems, under Grant Agreement 667191, the Fonds National de la Recherche Scientifique, the Actions de Recherche Concertées de la Communauté Française, Belgium (all to M.C.), a Fonds National de la Recherche Scientifique-Fund for Research Training in Industry and Agriculture fellowship (to B.A.), and the Fonds David et Alice Van Buuren and Fondation Jaumotte-Demoulin (to B.A.).

Disclosure Summary: The authors have nothing to disclose.

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