Figure 1. Synergistic effects of glucose and palmitate on cell death but not JNK activation in MIN6 cells. MIN6 cells were treated with either control 0.5% BSA or 400 μM palmitate+0.5% BSA at a concentration of 5, 10, 15, 25 mM glucose for 24-h. (**A**) The percentage of cell death was then assessed by adding propidium iodide for the last hour of incubation as described under Methods. The bar graph depicts the averages of the data obtained from five individual experiments, and data are expressed as means ±S.E.M. ** $p<0.01$, *** $p<0.001$; (**B**) The cell lysates were subjected to Western blot analysis using anti-cleaved Caspase3, anti-phospho-JNK, anti-total JNK and anti-α-Tubulin antibodies. Protein level of phospho-JNK was normalized over total JNK. Cleaved Caspase3 levels were normalized over α-Tubulin. The representative result of three individual experiments is shown. The data obtained from three individual experiments are expressed as means ± S.E.M. * $p<0.05$, ** $p<0.01$.
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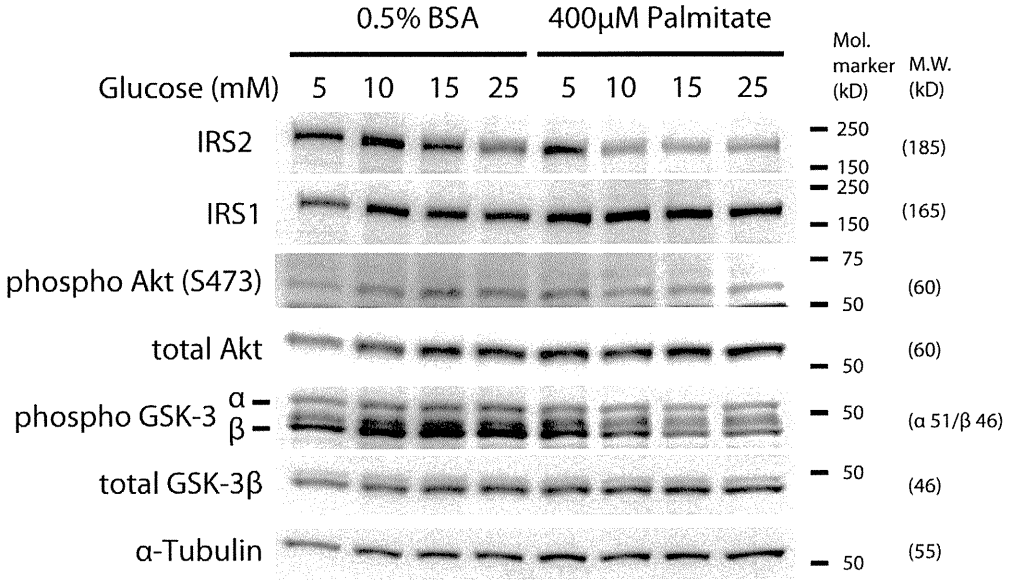
were initiated immediately following isolation at 500 multiplicity of infection (MOI). Infections were incubated for 15 hours and residual virus was removed prior to palmitate treatment. All procedures were performed in accordance with Washington University's Animal Studies Committee. The Principles of laboratory animal care (NIH publication no. 85–23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm) were followed.

Loss-of-function of ATF3 with shATF3

INS-r3 cells were seeded 24 hours prior to infection to achieve 70 percent confluence at time of infection. Control and ATF3



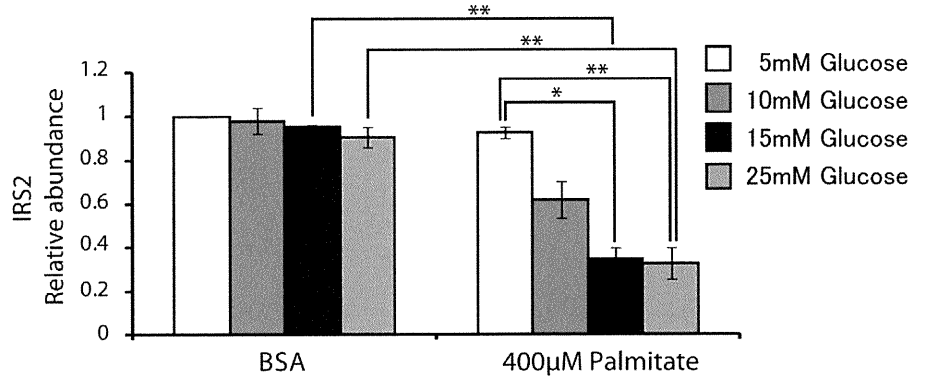


Figure 2. Glucose and palmitate potentiate to reduce insulin signaling. MIN6 cells were treated with either control 0.5% BSA (four lanes on left) or 400 μM palmitate+0.5% BSA (four lanes on right) at a concentration of 5, 10, 15, 25 mM glucose for 24-h. Total cell lysates were obtained and were subjected to Western blot analysis with antibodies to the indicated proteins. Protein level of IRS2 was normalized over α-Tubulin. The representative results of three individual experiments are shown. The results for IRS2 are graphically illustrated, data are expressed as means ±S.E.M. *$p<0.05$, **$p<0.01$.
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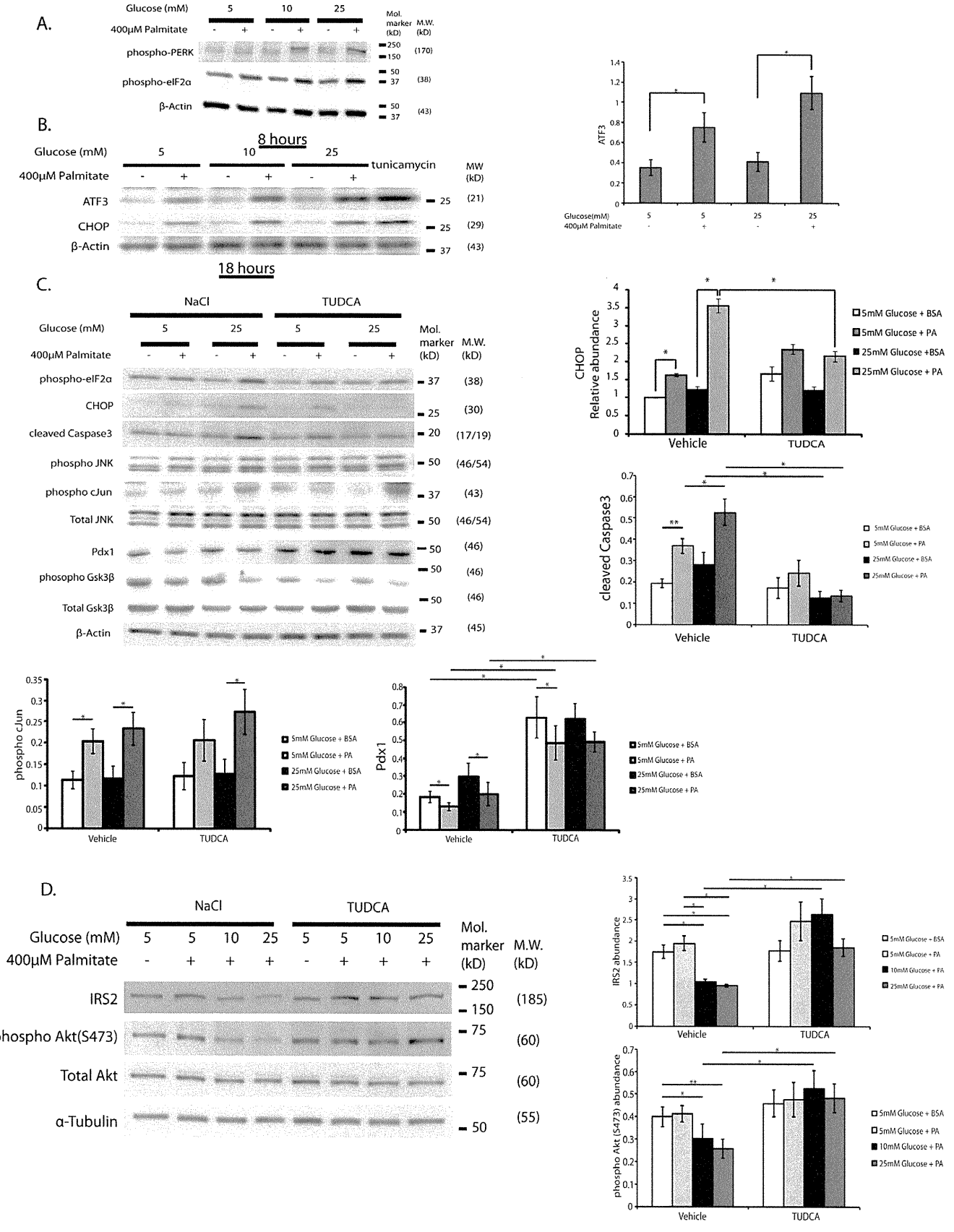

Figure 3. The synergistic effects of glucose and palmitate on ER stress and reduction of insulin signaling is attenuated by addition of a chemical chaperon. (**A**) MIN6 cells were treated with either control 0.5% BSA or 400 μM palmitate+0.5% BSA at a concentration of 5, 10, 25 mM glucose for 8 hours. Total cell lysates were extracted at indicated time points and were subjected to Western blot analysis using anti-

phospho-PERK (980Thr), anti-phopsho-eIF2α (51Ser), and (**B**) MIN6 cells were treated with either control 0.5% BSA or 400 μM palmitate+0.5% BSA at a concentration of 5, 10, 25 mM glucose for 18 hours and blotted with anti-ATF3 and anti-CHOP antibodies. β-Actin was detected for loading control. Tunicamycin treatment was control for ER stress. (**C**) Cells were treated with either 500 μg/ml NaCl (ionic control) or 500 μg/ml TUDCA 15-h prior to beginning of palmitate treatment and then were co-treated with either 0.5% BSA or 400 μM palmitate+0.5% BSA with either 5 mM or 25 mM glucose and NaCl or TUDCA for 24 h. Total cell lysates were subjected to Western blot analysis with antibodies to the indicated proteins. Densitometry of total CHOP and cleaved Caspase3 and Pdx1 were measured and normalized over α-Tubulin, respectively. Densitometry of phospho-cJun was measured and normalized over total JNK. The representative results of three individual experiments are shown. The effects on CHOP, cleaved Caspase3 and phospho-cJun and Pdx1 protein are graphically illustrated. *$p<0.05$. (**D**) Cells were treated with either 500 μg/ml NaCl (ionic control) or 500 μg/ml TUDCA 15-h prior to beginning of palmitate treatment and then were co-treated with either 0.5% BSA or 400 μM palmitate+0.5% BSA with either 5 mM, 10 mM or 25 mM glucose and NaCl or TUDCA for 24-h. Total cell lysates were subjected to Western blot analysis with antibodies to the indicated proteins. Densitometry of total IRS2 was measured and normalized over α-Tubulin and densitometry of phospho-Akt was measured and normalized over total Akt. The representative results of three individual experiments are shown. The effects on IRS2 protein are graphically illustrated, *$p<0.05$, **$p<0.01$.

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shRNA adenovirus constructs described previously [14] were incubated with the cells at an MOI of 30 for 4 hours in normal culture media. Palmitate and cytokine treatments were initiated 24 hours following removal of virus.

Inhibition of Gsk3β expression with a kinase dead adenovirus

Adenovirus expressing a catalytic inactive mutant of the human Gsk3β (Adv-Gsk3βKM) was prepared as previously described [21]. Control adenovirus-green fluorescent protein (AdV-GFP) was a gift from D. Kelly (Washington University, St. Louis, MO). Infection of the MIN6 cells was carried out at the indicated multiplicity of infection (MOI) for one hour in serum-free media. The MIN6 were then washed in PBS, maintained in the DMEM/15% FBS media, and then experiments were carried out 24 hours after infection.

Statistical analysis

The presented data were analyzed from at least 3 independent experiments and are shown as means ±S.E.M. The significance of the variations was analyzed using either a one- or two-way ANOVA with Bonferroni corrections with a significance level of 0.05 (95% confidence intervals).

Results

Glucose and palmitate synergize to induce apoptosis

Our previous study had shown a dose-dependent effect of FFA, both palmitate (50–400 μM) and oleate (50–400 μM), on ER stress and apoptosis in glucose-responsive insulinoma (MIN6) cells [16]. In the current study the dose-dependent effect of glucose (5–25 mM) was examined at 400 μM palmitate. As shown in Figure 1A, increasing glucose concentration had a clear synergistic effect on cell death characterized by propidium iodide incorporation normalized to DAPI staining. While 400 μM palmitate resulted in about 3% propidium iodide incorporation at 5 mM glucose, this was increased more than threefold when the glucose concentration was raised to 15 mM and 25 mM. While there was no effect of palmitate on cleaved Caspase3 at 5 mM glucose, consistent with the synergism observed on propidium iodide staining, glucose and palmitate also synergized on activation of the pro-apoptotic marker cleaved Caspase3 comparing that at 5 mM glucose/palmitate vs. 25 mM glucose/palmitate, $p<0.05$ (Figure 1B).

We next explored the underlying mechanisms for the glucose potentiation. FFA treatment has been shown to induce JNK activation that can contribute to FFA-induced apoptosis; we determined the effects of altering glucose concentrations on JNK activation. Interestingly, JNK activation by FFA was maximal at the lowest glucose concentration and did not increase further as glucose was increased (Figure 1B). Maximal FFA activation of JNK at 5 mM glucose with apparent increasing activation of cleaved Caspase3 with increasing glucose concentration suggested that further enhancement of JNK by glucose could not explain the synergistic effects on cell death.

Glucose and palmitate synergize to reduce insulin signaling associated with a decrease in IRS2 protein

Previous studies have shown that glucose treatment of insulinoma cells results in activation of the insulin receptor substrate-PI3-kinase-Akt pathway that serves to protect against β-cell death. In contrast, FFA treatment of insulinoma cells inhibits this pathway [8,16]. We examined whether the synergistic effect of glucose on FFA-induced apoptosis could be related to inhibition of this signaling pathway. Glucose alone (5–25 mM), as shown in the first four lanes of Figure 2, increased phospho-Akt (S473) and phospho-Gsk3 as expected, with no apparent change in IRS1 or IRS2 proteins, confirming previous observations [8,22]. However as glucose was raised in the presence of palmitate (Figure 2, lanes 5–8) a dose-dependent decrease in phospho-Akt and phospho-Gsk3β were observed and accompanied by a parallel decrease in IRS2, while IRS1 levels did not appear to change. This synergistic effect of increasing glucose in the presence of FFA on inhibition of IRS2 and PI-3 kinase-Akt signaling is a novel finding that might explain the synergistic effect on β-cell survival [23].

The effect of glucose and FFA is accompanied by a synergistic effect on ER stress that is reduced by addition of a chemical chaperone

FFA impair insulin signaling in β-cells in part via activation of ER stress [16,24]. Bachar et. al. [13] recently showed in insulinoma cells that palmitate at 22.2 mM glucose vs. 3.3 mM glucose increased activation of JNK, CHOP and the ER stress enzyme phospho-PERK. We examined the effects of increasing glucose from 5 mM to 25 mM at 8 and 18 hours of treatment to observe the different time course of development of ER stress markers. An enhancement of the ER stress markers phospho-PERK, phospho-eIF2α, CHOP, and ATF3 (Figure 3A and 3B) was observed, confirming and extending the results of Bachar et. al. [13]. The observation that glucose appeared to synergize with palmitate to increase ATF3 protein expression, although this was not statistically significant, was consistent with the previous finding by Hartman et. al. demonstrating that high glucose and FFA together increased ATF3 mRNA expression, and that this was associated with increased apoptosis of insulinoma cells [25]. These glucose-induced changes in the presence of palmitate on ER stress markers were again noted in the absence of phospho-c-Jun (Figure 3C).

We next examined whether reducing ER stress using TUDCA, a chemical chaperone that enhances ER functional capacity [26],

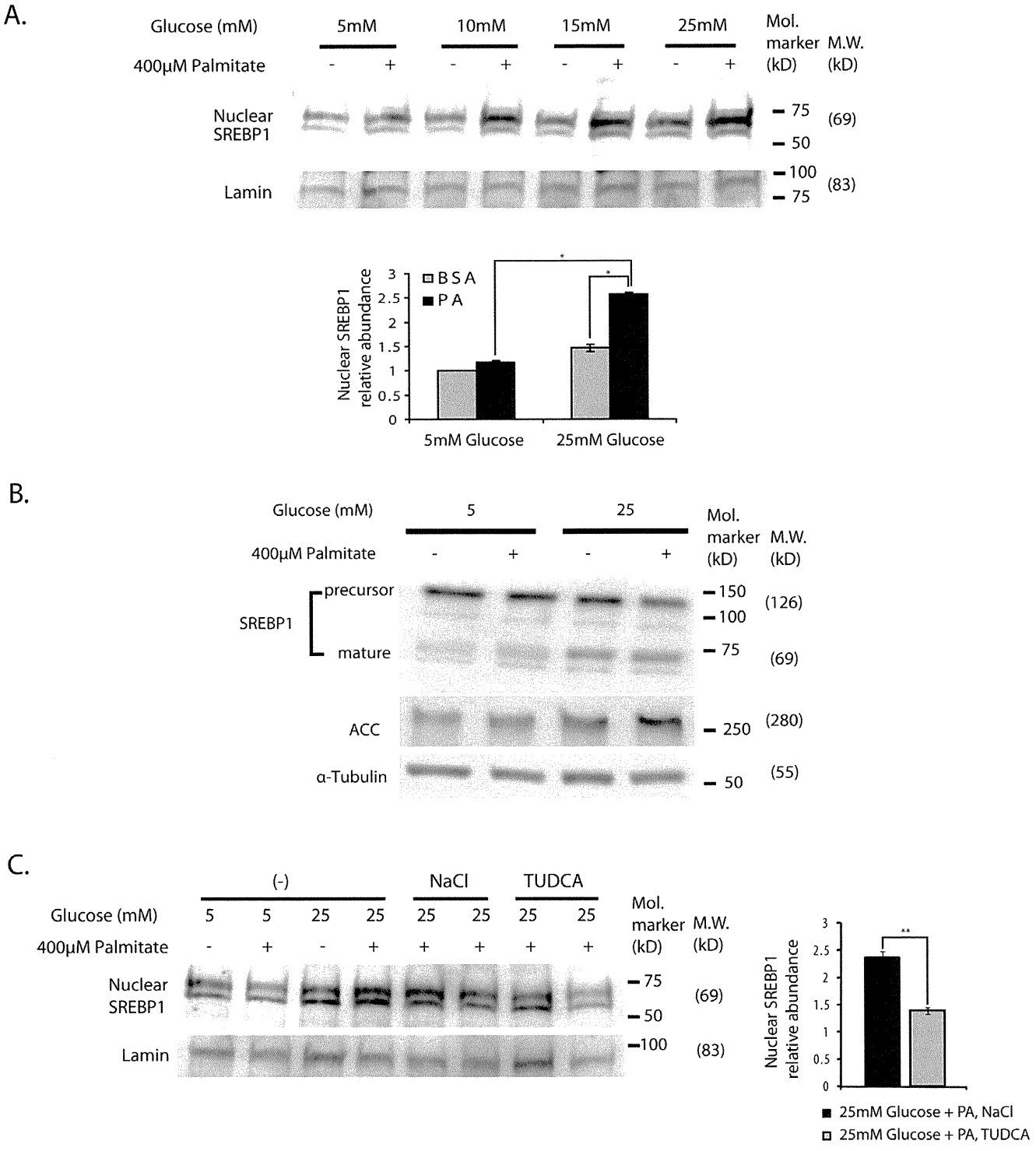

Figure 4. The synergistic effects of glucose and palmitate on ER stress results in concomitant effects on activation of SREBP1. (**A**) MIN6 cells were treated with either control 0.5% BSA or 400 μM palmitate+0.5% BSA at a concentration of 5, 10, 15, 25 mM glucose for 18-h. Nuclear fractions were extracted from the cells and were subjected to Western blot analyses using anti-SREBP1 and anti-Lamin antibodies. 25 μg of nuclear protein was loaded in each lane. The upper band normalized over Lamin was used to do the quantification (the lower band is nonspecific). The relative ratio of nuclear SREBP1 over Lamin calculated by densitometries was summarized as means ± S.E.M. in the graph respectively. The representative results of three experiments are shown, and graphically illustrated, * $p<0.05$. (**B**) MIN6 cells were treated with either control 0.5% BSA or 400 μM palmitate+0.5% BSA at a concentration of either 5 or 25 mM glucose for 24-h. Total cell lysates were subjected to Western blot analysis using anti-acetyl CoA carboxylase (ACC) and anti-α-Tubulin antibodies. The representative results of two individual experiments are shown. (**C**) Cells were treated with either 500 μg/ml NaCl or 500 μg/ml TUDCA 15-h prior to beginning of palmitate treatment. Cells were co-treated with either 0.5% BSA or 400 μM palmitate+0.5% BSA with 25 mM glucose and NaCl or TUDCA for 18-h. Nuclear fractions were extracted from the cells and were subjected to Western blot analyses using anti-SREBP1 and anti-Lamin antibodies. The upper band normalized over Lamin was used to do the quantification (the lower band is nonspecific). 25 μg of nuclear extracts were loaded in each lane. The representative results of three individual

experiments are shown. The relative ratio of nuclear SREBP1 over Lamin calculated by densitometries was summarized as means ± S.E.M. in the graph respectively **p<0.01.
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can reverse the synergistic effects of glucose and FFA on insulin receptor substrate signaling. As shown in Figure 3C, the effects of 24 hour treatment with 5 mM or 25 mM glucose in the presence and absence of palmitate (400 μM) on activation of JNK and other markers of ER stress are shown in the control condition with NaCl (first 4 lanes). The results of protein expression under the same conditions but in the presence of TUDCA are shown in the last 4 lanes of Figure 3C. JNK activation, which again was not augmented by the combination of high glucose and palmitate, was little affected by TUDCA treatment. In contrast the other ER stress markers phospho-eIF2α, CHOP and cleaved Caspase 3 were attenuated by co-treatment with TUDCA. Addition of TUDCA also increased Pdx1 (Figure 3C). The increase in phospho-Akt protein levels, an inhibitor of Gsk3 activity, and IRS2 protein levels were also observed with TUDCA treatment (Figure 3D). Together the results suggest that glucose potentiation of FFA induced apoptosis involves activation of ER stress with resultant inhibition of insulin signaling in a manner independent of further JNK activation.

Glucose and palmitate synergistically activate SREBP1

Sterol regulatory element-binding protein-1 (SREBP1) is a transcription factor that stimulates expression of lipid-regulatory genes [27]. SREBP1 is an ER membrane resident protein that in response to sterol depletion is cleaved to generate a transcriptionally active N-terminal fragment that translocates to the nucleus [28]. SREBP1 is increased in liver and islets of diabetic animals [29]. *In vivo* SREBP1 overexpression increased lipid accumulation in islets, reduced β-cell mass, and impaired insulin secretion [30]. Overexpression of the SREBP1 gene in insulinoma and islet β-cells also reduced IRS2 protein [31]. Furthermore, incubation of insulinoma cells and islets with high glucose (25 mM) was shown to activate SREBP1. The latter study examined only high glucose, and did not include FFA. As shown in Figure 4A, like the study of Wang et. al [31] we observed an apparent slight effect of increasing glucose on SREBP1 activation, when nuclear SREBP1 was corrected for nuclear Lamin. A synergistic effect was observed of glucose and FFA on activation of SREBP1 with a concomitant reduction of SREBP1 precursor and appearance of mature or nuclear SREBP1. SREBP1 activation was further confirmed by increased expression of its target acetyl-CoA carboxylase (ACC) (Figure 4B). SREBP1 activation was reduced by attenuation of ER stress with TUDCA pretreatment (Figure 4C) that suggested that the synergistic effects of glucose and palmitate on activation of SREBP1 were mediated by exacerbation of ER stress.

Glucose/palmitate activate ER stress and reduce insulin signaling in primary islets

To validate the relevance of synergistic effects of glucose and palmitate on pancreatic β-cells we treated isolated primary mouse islets with 11 mM or 30 mM glucose in either the absence or presence of 400 μM palmitate for 72 hours. This incubation time and different glucose concentrations were utilized as it was determined that primary islets are more resistant to FFA induced apoptosis than are insulinoma cells (data not shown). Treatment with high glucose (30 mM) and palmitate resulted in an apparent but not significant ($p = 0.14$) enhanced induction of cleaved Caspase3 (Figure 5A), and an apparent but not significant ($p = 0.07$) reduced IRS2 expression (Figure 5B). In addition, high glucose and palmitate synergistically enhanced the ER stress marker GRP78 ($p<0.05$) beyond JNK activation (Figure 5C), and caused an apparent increase in ATF3 ($p = 0.06$) (Figure 5D). Consistent parallel increases in nuclear SREBP1 and total ACC protein also appeared to be potentiated by high glucose and palmitate although the effects were not significant (Figure 5E). Treatment with high glucose and palmitate did however result in significant reduction of phospho-Akt, Pdx1, and phospho-Gsk3b with no change in phospho-cJun (Figure 5F). These data together support the relevance of findings in insulinoma cells to primary islet β-cells.

Loss-of-function of ATF3 and gain-of function of IRS2 reduce the effects of glucose and palmitate on apoptosis

To assess the effects of ER stress-induced ATF3 expression and subsequent suppression of IRS2 on glucolipotoxicity mediated apoptosis, loss- and gain-of-function studies were conducted. INS-r3 cells were utilized as the reagents for small hairpin RNA (shATF3) were controlled for rat ATF3 and not mouse. A similar role for ATF3 in MIN6 and INS1 insulinoma cell stress response has been observed (Zmuda and Hai, unpublished observations). INS-r3 cells were incubated with 400 μM palmitate/25 mM glucose for the indicated times in the presence of adenoviruses expressing either control or shATF3 to reduce ATF3 expression [14]. Note that phospho-Akt appeared to be elevated in cells expressing shATF3 at all times consistent with the known inhibitory effect of ATF3 on IRS2 transcription and subsequent insulin signaling. The IRS2 levels in the ATF3 knockdown cells appeared to be elevated relative to that in control cells at both 8 and 16 hours of glucose/palmitate treatment. Further cleaved Caspase3, following glucose/palmitate treatment, was suppressed in ATF3 knockdown cells ($p<0.002$). The conclusion that ER stress activates ATF3 that contributes to impaired insulin signaling and apoptosis is thus supported by these ATF3 knockdown experiments.

In another experiment using primary islets treated with glucose/palmitate for 72 hours, the effects of transfection with an adenovirus expressing IRS2 was compared to that of islets with control adenovirus. As shown in Figure 6B, where Immunodetection was set to assess high levels of IRS2 in IRS2 transfected cells, there was a marked increase in IRS2 protein with IRS2 overexpression. Glucose/palmitate treatment resulted in increased ATF3 and cleaved Caspase3 with control adenovirus, while overexpression of IRS2 appeared to reduce the degree of apoptosis as measured by reduction of cleaved Caspase3 (Figure 6B). These results are consistent with the previously demonstrated role of ATF3 on ER stress induced apoptosis in islets [14,25].

Loss-of-function of Gsk3β on glucose and palmitate induced β-cell apoptosis

The progressive decline in insulin receptor substrate signaling observed with decreasing IRS2 expression and phospho-Akt was associated with decreased phosphorylation of Gsk3β (Figure 2) and thus activation of the pro-apoptotic form of Gsk3β [32]. To examine the contribution of activation of Gsk3β, the effects of palmitate treatment with increasing amounts of an adenovirus expressing a catalytic inactive mutant of the human Gsk3β (Adv-Gsk-3βKM) were analyzed by Western blot (Figure 7A). A control sample was placed on either end of the blot to facilitate

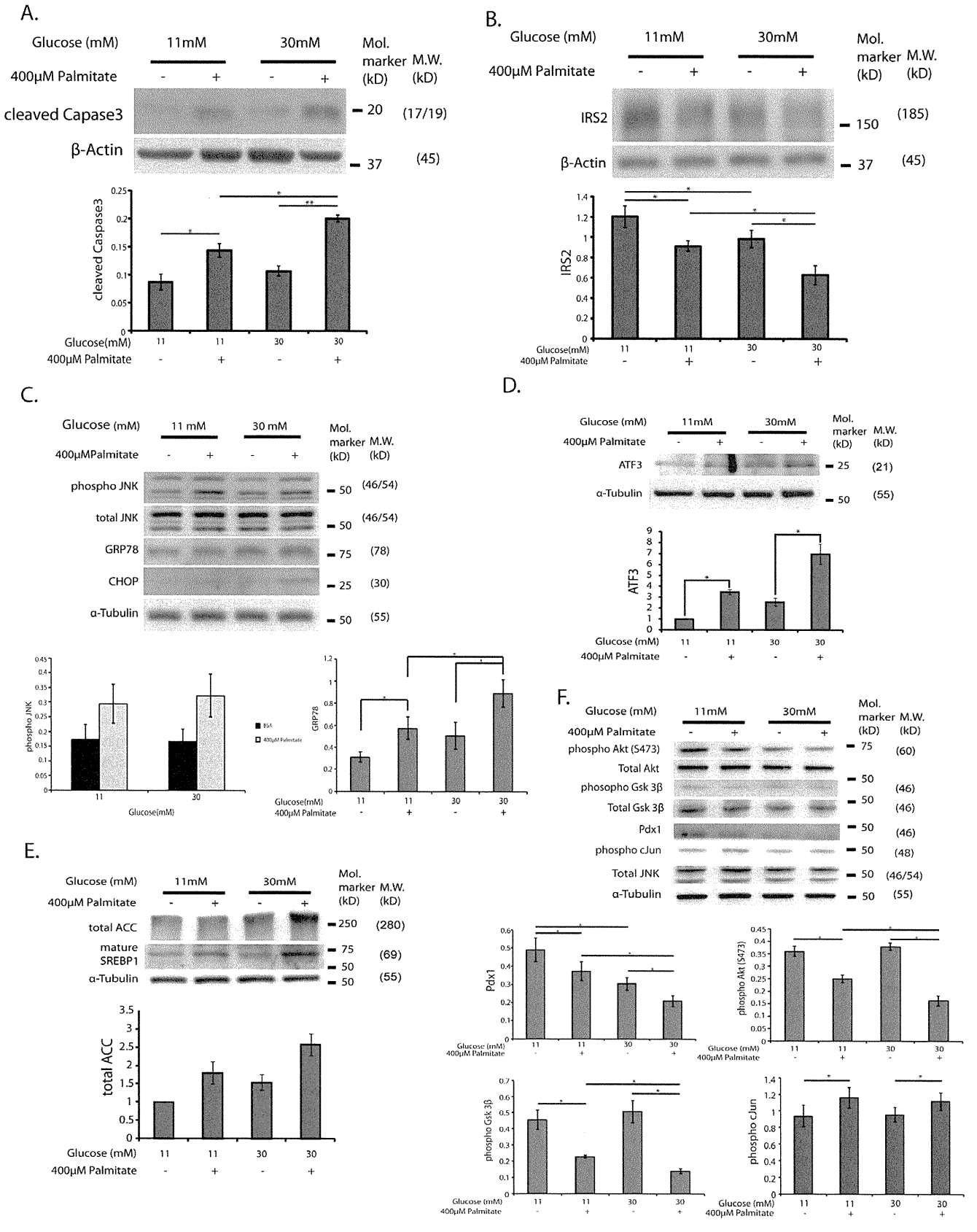

Figure 5. Synergistic effects of glucose and palmitate on ER stress and suppression of IRS2 expression levels in primary mouse islets. Islets from 14 weeks of age C57BL/6 male mice were isolated as described in Methods and were treated with either control 0.5% BSA or 400 μM palmitate+0.5% BSA in RPMI medium containing either 11 mM or 30 mM glucose, 10% FBS for 72-h. Total cell lysates were extracted from

the islets and subjected to Western blot analysis using (**A**) anti-cleaved Caspase3 and anti-β-Actin, (**B**) anti-IRS2 antibodies, (**C**) anti-phospho-JNK, anti-total JNK, anti-GRP78, anti-CHOP, anti-α-Tubulin antibodies, (**D**) anti-ATF3 antibodies, (**E**) anti-Acetyl CoA Carboxylase (ACC), anti-SREBP1, anti-α-Tubulin antibodies, (**F**) anti-Pdx1, anti-phospho-Gsk3β, anti-total Gsk3β, anti-phospho-Akt (S473), anti-phospho-cJun, anti-α-Tubulin antibodies. The blots shown are representative of 3 individual islet experiments. The relative ratio of indicated protein over β-Actin or α-Tubulin as a loading control calculated by densitometries was summarized as means ± S.E.M. in the graph respectively *$p<0.05$, **$p<0.01$.
doi:10.1371/journal.pone.0018146.g005

comparisons. For instance, observe the increase in cleaved Caspase3 and phopho-JNK between the two controls on either end of the blot. Increasing doses of the virus correlated with increased levels of total Gsk-3β, and reduced levels of the Gsk-3β substrate phospho-GS. Increased kinase dead Gsk-3β virus also resulted in increased expression of Pdx1, reduced apoptosis as suggested by cleaved Caspase 3 levels, and cell death measured by propidium iodide incorporation (Figure 7B). Interestingly the protective effects of the Adv-Gsk-3βKM occurred in spite of apparent comparable activation of phospho-JNK with palmitate treatment.

Discussion

The combination of hyperglycemia and hyperlipidemia that is associated with insulin resistance may contribute to reducing β-cell mass and promoting the transition to full blown Type 2 diabetes, but the underlying mechanisms are only partially understood. This study examined the sequence of molecular events that may be involved in this process, and resulted in several novel observations: 1) While early induction of JNK plays a role in FFA-induced apoptosis [13,16], it does not appear involved in the high glucose potentiation of FFA effects; 2) the potentiating effects of glucose and FFA on ER stress result in activation of ER-associated SREBP1 and ATF3 leading to reduced IRS2 expression further impairing insulin-receptor substrate-PI-3K-Akt signaling, and 3) treatment with an adenovirus expressing a kinase dead Gsk3β significantly restored Pdx1 levels, and reduced the apoptosis induced by high glucose and FFA. Together these findings provide a molecular model for the synergistic effects of glucose and FFA on islet cell death and identify potentially useful therapeutic targets.

The role of JNK activation in FFA-induction of apoptosis in β-cells has been documented [13,16,33]. In the current study, JNK activation by palmitate was significant as expected, but was maximal at low glucose, and glucose potentiation of FFA-induced apoptosis was JNK independent (Figure 1B). How FFA treatment of β-cells leads to JNK activation is not completely known, but FFA induce ER stress, which activates JNK activation, and JNK activation itself can induce ER stress [13,34]. Interestingly, TUDCA appeared to reduce cleaved Caspase3 and CHOP induction, while not appearing to reduce JNK activation (Figure 3) suggesting that in this case JNK activation is upstream of ER stress. On the other hand the results of inhibition of ATF3 by shRNA in Figure 6A suggest that JNK activation is upstream of ER stress. Our results do not therefore settle this issue of the relative position of JNK activation and ER stress. Regardless of whether JNK activation is upstream or downstream of ER stress, the findings in the current study show that the combination of high glucose and FFA does not associate with further activation of JNK as compared to FFA in low glucose. This result is to some extent in conflict with that of Bachar et. al. [13] who showed that incubating islet cells in low vs. high glucose resulted in both increased ER stress and JNK activation. A difference in the two experiments is that Bachar et.al. evaluated islet cells in 3.3 mM vs. 22 mM glucose, and our experiments assessed cells in 5.5 mM vs. 25 mM glucose. Our results do not rule out the contributory effect of JNK on β-cell apoptosis as previously shown [13,16,33] but they emphasize the importance of additional mechanisms contributed by high glucose. Additionally, since glucose and palmitate have been shown to evoke oxidative stress, impairing nuclear translocation of Pdx1 and triggering β-cell failure [35], it is conceivable that oxidative stress interacts with JNK, ATF3 and ER stress to contribute to glucose/palmitate induced apoptosis, although this hypothesis remains to be tested.

The role of SREBP1 in high glucose induced apoptosis in islet β-cells has been reported [31]. In the absence of FFA, high glucose alone for 48 hours was shown to activate SREBP1 and to repress IRS2 and Pdx1 levels. Expression of a dominant negative SREBP1 reversed these transcriptional effects. In the current study, glucose alone at high concentration slightly activated SREBP1 as shown in Figure 4, an event that did not correlate with ER stress or apoptosis. A much more significant activation of SREBP1 was observed with glucose and palmitate together and this was shown to be a function of induced ER stress since it was attenuated by TUDCA (Figure 4C). These results only show an association of SREBP1 nuclear translocation, and do not document its causal role. It is likely however that SREBP1 nuclear translocation participates in glucose/palmitate induced apoptosis, as previous studies documented the causal role of SREBP1 in ER stress induced apoptosis in insulinoma cells [31]. SREBP1 resides in the ER membrane, where it is anchored by the labile protein INSIG1 [36]. The link between ER stress and SREBP1 activation has been little studied. Lee et. al. using CHO cells [36] showed that thapsigargin, a chemical that induces ER stress, activates SREBP1 due to rapid degradation of INSIG1 [36]. We observed that glucose and palmitate together appeared to reduce INSIG1 protein (data not shown), which likely contributed to augmentation of nuclear SREBP1 under these conditions.

Similarly a synergistic effect of glucose and FFA was observed on expression of the ER-associated stress marker ATF3. ATF3 expression paralleled suppression of IRS2 protein levels, and induction of apoptosis measured by CHOP (Figure 3A–B), and Caspase3 activation (Figure 3A). The association of ATF3 with ER stress and cell death has been well documented [37] but there is relatively little known related to ATF3 targets [14]. Recently, ATF3 was shown to suppress the IRS2 protein by binding to the IRS2 promoter [14] and implicated this mechanism in apoptosis induced by agents such as γ-interferon, TNF-α, or thapsigargin. Our findings are consistent with the involvement of ATF3 in the apoptotic effects of nutrient induced ER stress in islet cells. In this context, we note that Cunha et. al. [33] did not find a pro-apoptotic role of ATF3 in the context of palmitate treatment. Potential explanations for this apparent difference include the difference in glucose concentration (25 mM glucose in our study and 11 mM glucose in theirs), the cells used (INS-r3 in our study and INS-1E in theirs), and assays (activated Caspase3 in our study and propidium iodide plus Hoechst stain). An interesting question is whether ATF3 is a direct repressor of Pdx1 expression. Insulin signaling alters Gsk3β and FoxO activity [16,32,38,39] and these proteins are known regulators of Pdx1 expression. As ATF3 represses IRS2 and insulin signaling, at least part of ER stress and ATF3 induced Pdx1 suppression is due to decreased insulin signaling, and perhaps also due to direct suppression of Pdx1 expression, but this latter question remains to be determined by future experiments. Interestingly

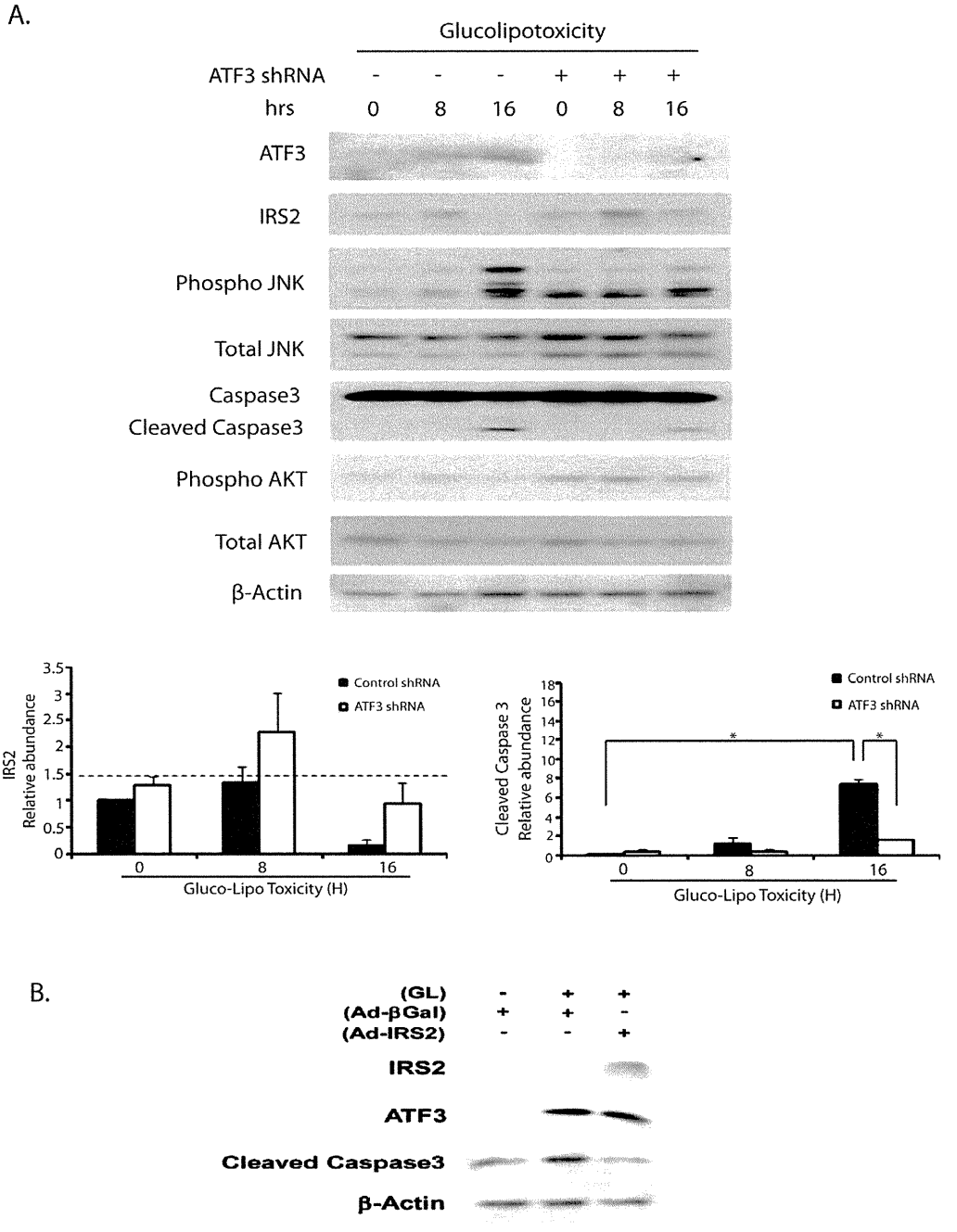

Figure 6. Loss-of-function of ATF3 and gain-of function of IRS2 reduce the effects of glucose and palmitate on apoptosis. (**A**) INS-r3 cells were infected with either control or ATF3 shRNA adenovirus 24-h prior to treatment with 400 μM palmitate+0.5% BSA and 25 mM glucose for the indicated times. Total cell lysates were obtained and subjected to Western blot analysis with antibodies to the indicated proteins. The relative ratio of IRS2 and cleaved Caspase3 expression over β-Actin was quantified by densitometry. The data obtained from three individual experiments are expressed as means ± S.E.M. * $p<0.02$, ** $p<0.012$. (**B**) In a single experiment, primary mouse islets were infected with adenovirus expressing βgal or IRS2 expressing adenovirus prior to treatment with either control 0.5% BSA and 5.5 mM glucose or 400 μM palmitate+0.5% BSA and 25 mM glucose for 72-h. "GL" refers to incubation in 25 mM glucose and 400 μM palmitate. Total cell lysates were subjected to Western blot using antibodies to indicated proteins.
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ATF3 induction is dependent on the P38 kinase pathway [15] which is part of signaling transduced by the membrane fatty acid translocase CD36 and a role of CD36, which is induced by glucose, has been proposed in mediating palmitate induced apoptosis of kidney tubular epithelial cell [40]. The role of this pathway in islet cells will need to be explored in future studies.

In the current studies we have shown a correlation among suppression of IRS2 protein levels (Figure 2), ATF3 expression (Figure 3), and resultant induction of apoptosis. The causal relationships among these events were demonstrated by Li. et. al. [14] when insulinoma cells and/or mouse islets were transfected with adenoviruses expressing inducible gain- or loss-of-function of ATF3

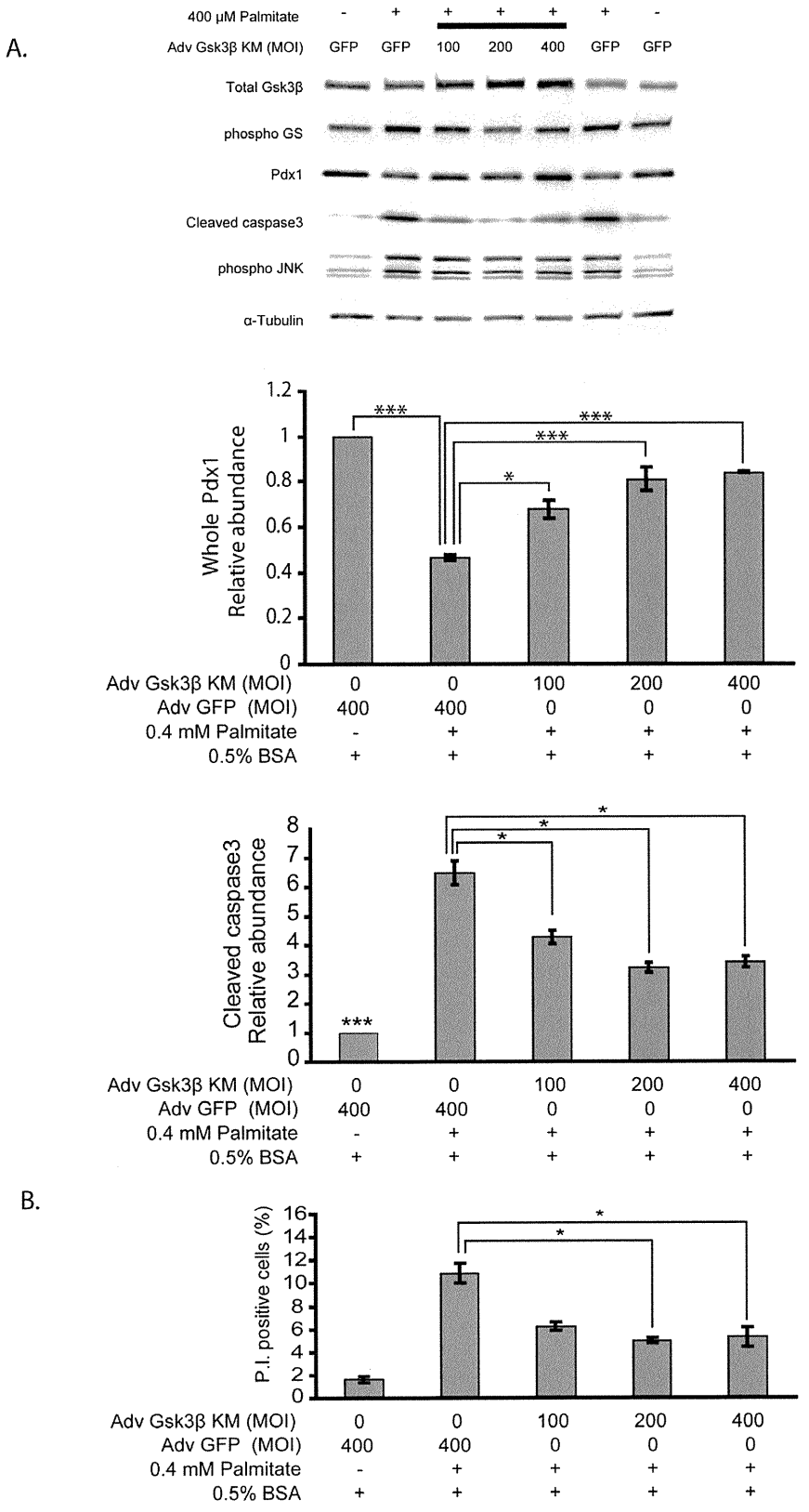

Figure 7. Inhibition of Gsk3β protects against glucose and palmitate-induced apoptosis in MIN6 cells. MIN6 cells were infected with 100, 200, or 400 MOI of adenovirus expressing a catalytically inactive mutant of the human Gsk3β (Adv-Gsk3βKM) or adenovirus expressing GFP (Adv-GFP) 24 hours prior to palmitate treatment. Cells were treated with 25 mM glucose and with either 0.5% BSA or 400 μM PA+0.5% BSA for 24 hours. A GFP control was placed on either end of the blot to facilitate comparison of control and Adv-Gsk3βKM. (**A**) Western blots using the indicated antibodies, with relative expression of Pdx1 normalized over α-Tubulin, and expression levels of cleaved Caspase3 normalized over α-tubulin. (**B**) Percentage of Propidium Iodide incorporation (n = 3, means ± S.E.M., *$p<0.05$, *** $p<0.001$).
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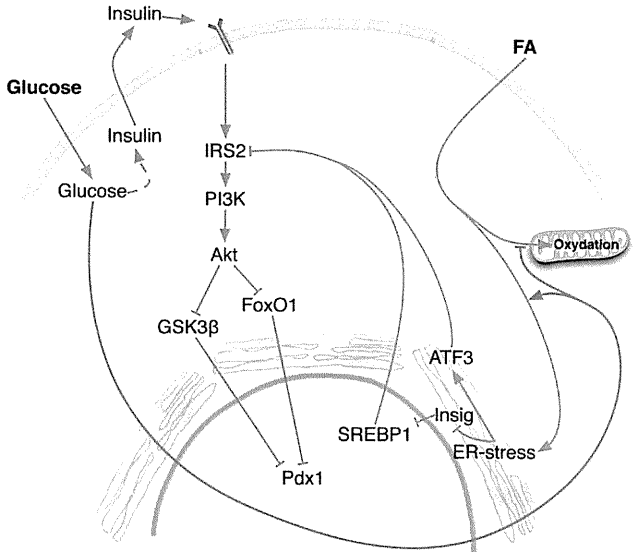

Figure 8. Working diagram illustrating some of the key steps involved in "glucolipotoxicity" of β-cells. High glucose and FFA together result in a vicious negative cycle that ultimately promotes β-cell death. As suggested by our findings, high glucose addition to FFA treated β-cells results in much more activation of SREBP1 than glucose alone. SREBP1 enhances ACC expression with generation of malonyl-CoA which impairs FFA oxidation. This in turn leads to augmented ER stress with further activation of ER-localized SREBP1 as a result of degradation of the anchoring protein Insig1. The excess non-metabolized FFA due to more impairment of FFA oxidation would partition in ER membranes compounding ER stress. In addition to SREBP1, ER stress activates ATF3. Both nuclear SREBP1 and ATF3 result in inhibition of IRS2, with concomitant impairment of insulin signaling, activation of Gsk3β and reduction of Pdx1 leading to apoptosis.
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and IRS2. Treatments that induced ATF3 activation and IRS2 suppression included induction of apoptosis by combined treatment of insulinoma cells with γ-interferon, TNF-α, or the ER stress activator thapsigargin. These studies demonstrated that ATF3, like the transcription factor CREB, alters IRS2 expression by binding to the IRS2 promoter. In the current study, we have examined the role of combined glucose and palmitate on this pathway (Figure 6). Transduction with an adenovirus expressing shATF3 significantly reduced this effect, while transduction with AdV-IRS2 ameliorated the apoptotic effect, thus mechanistically linking this pathway.

In this study co-incubation of insulinoma cells with an adenovirus expressing a kinase dead Gsk3β (Adv-Gsk3βKM, Figure 7) along with high glucose and palmitate for 24 hours significantly reduced cleaved Caspase3 and cell death. A previous study in IRS2 null mice had demonstrated that the severe diabetes associated with markedly increased apoptosis and reduced proliferation of islet β-cells was reversed when crossed with Gsk3β haploinsufficient mice [32]. The double knockout was also associated with enhanced expression of Pdx1 in islet β-cells. Like the IRS2 null mice with severe insulin resistance, glucose and FFA treatment of insulinoma cells and primary islets induces a state of insulin resistance. Also like IRS2 null mice on the Gsk3β genetic deficient background, reducing Gsk3β activity with a kinase dead Gsk3β adenovirus restored Pdx1 levels and reduced apoptosis and cell death. These findings emphasize the contribution of impaired insulin receptor substrate signaling in the apoptosis of β-cells treated with glucose and FFA, and the contribution of Gsk3β activity to this process.

A schematic diagram highlights the key concepts implied by our findings (Figure 8). In the insulin resistant subject abnormal metabolism of FFA and glucose result in chronically high levels of both nutrients in the circulation [41]. Under such conditions, the combination of hyperglycemia and FFA has been suggested to be particularly harmful for β-cells leading to so-called glucolipotoxicity [6,42,43]. Our findings suggest that addition of high glucose to FFA treated β-cells results in an escalating negative cycle. Under such conditions activation of the transcription factor SREBP1 leads to enhanced ACC expression with generation of malonyl-CoA (MCC), which impairs FFA oxidation. These in turn lead to ER stress with further activation of SREBP1, ATF3, and impairment of FFA oxidation. The excess unmetabolized FFA would partition in ER membranes compounding ER stress. Additionally nuclear SREBP1 and ATF3 result in inhibition of IRS2, with concomitant impairment of insulin receptor substrate signaling, increase of Gsk3β activity and reduction of Pdx1 leading to apoptosis. Several steps in the cycle shown in Figure 8 may be amenable to therapeutic intervention. These include the impairment of FFA oxidation by glucose, the synergistic effects of glucose and FFA on ER stress, the activation of SREBP1 and its negative effects on insulin signaling, and the downstream mediators of apoptosis that are activated by reduced IRS-signaling involving both Gsk-3β and Fox01.

The results of the current studies illustrate some of the potential mechanisms whereby a combination of high glucose and FFA, as occurs in insulin resistant subjects, may result in eventual destruction of β-cells. However, the ultimate contribution of these mechanisms to the etiology of β-cell failure and diabetes remains unknown and will need to be validated *in vivo*.

Author Contributions

Conceived and designed the experiments: MAP YT YL SCM CCM EBM EZ TH NAA. Performed the experiments: KT YL CMW EZ. Analyzed the data: KT YL SDH SCM CCM EBM YT CJR EZ TH NAA MAP. Contributed reagents/materials/analysis tools: SCM CMW CJR EZ. Wrote the paper: KT YL SDH CCM EBM YT TH NAA MAP.

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LETTER TO THE EDITOR

Activation of the unfolded protein response in primary acute myeloid leukemia cells

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The unfolded protein response (UPR), which plays an important role in maintaining homeostasis of the endoplasmic reticulum (ER), is known to be activated in various solid tumors [1]. The role of the UPR in different forms of cancer or metastasis remains poorly characterized, and it is unclear whether UPR activation in cancer is due solely to microenvironmental stress, or to other mechanisms. The influence of the UPR on leukemogenesis remains largely uninvestigated. We previously reported that the UPR is activated in Philadelphia (Ph)-positive leukemia cells using cell lines and primary cells from Ph-positive acute lymphoid leukemia (ALL) patients [2]. In the present study, we investigated whether the UPR is activated in another type of acute leukemia, primary acute myeloid leukemia (AML).

We performed real-time reverse transcription-polymerase chain reaction (RT-PCR) focusing on four UPR-related genes to validate the upregulation of the UPR in AML cells: the spliced form of X-box-binding protein 1 (XBP1s), which is a key transcription factor of the UPR; ER-degradation enhancing α-mannosidase-like protein 1 (EDEM1), which is involved in ER-associated degradation; glucose-regulated protein 78 (GRP78), which is an ER chaperone; and C/EBP-homologous protein-10 (CHOP), which is also a transcription factor. We analyzed samples from AML patients [26 bone marrow (BM) and four peripheral blood (PB) samples] and healthy controls (16 BM and 18 PB samples). The collection of mononuclear cells and total RNA extraction was described previously [2]. Total RNA was subjected to reverse transcription with a high-capacity RNA-to-cDNA kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative RT-PCR of the indicated genes was performed using a Power SYBR® PCR Master Mix (Applied Biosystems) with a StepOnePlus™ Real Time PCR System (Applied Biosystems). The primer sequences for each of the human UPR-related genes are as follows: XBP1s, 5′-ggagttaagacagcgcttggg-3′ and 5′-acctgctgcggactcagc-3′; GRP78/BiP, 5′-gagttcttcaatggcaagg-3′ and 5′- ggggacatacatcaagcag-3′; EDEM1, 5′-cagctccaactgcaatcgtgta-3′ and 5′-atctggtcaatctgtcgcatgta-3′; CHOP/GADD153, 5′-gagagagtgttcaagaaggaagtgtatc-3′ and 5′-ccgaaggagaaaggcaatga-3′; β-actin, 5′-tggcaccacaccttctacaatg-3′ and 5′-tctcaaacatgatctgggtcatc-3′. Calculated concentrations were normalized to the expression level of β-actin mRNA. Statistical analyses were performed using Mann–Whitney's U test.

The UPR involves the cleavage of XBP1 mRNA to generate the spliced form XBP1s, which has been shown to be a sensitive marker for the activation of the UPR [3]. We found that the expression of XBP1s mRNA was significantly higher in the AML patients than in the healthy controls in both the BM and PB samples ($P < 0.05$). Furthermore, the expression of GRP78, EDEM1, and CHOP mRNAs was significantly higher in the AML patients than in the healthy controls ($P < 0.05$) (Fig. 1a). The patients' data classified according to the French-American-British classification are shown in Fig. 1b. In the acute promyelocytic leukemia (APL) patients, the expression of GRP78 mRNA was especially higher than in the other AML samples.

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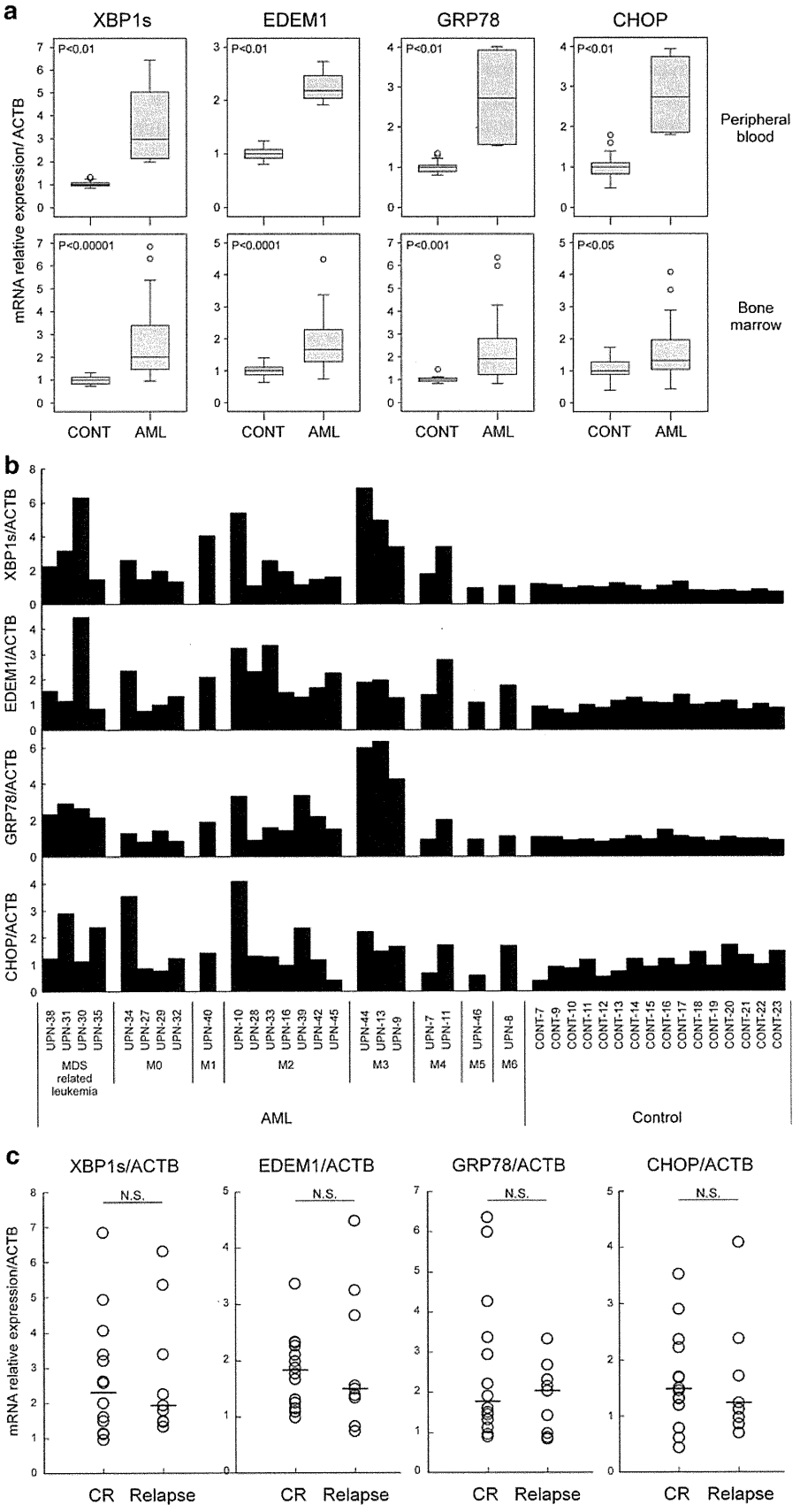

Fig. 1 UPR-related genes are upregulated in bone marrow (BM) and peripheral blood (PB) from acute myeloid leukemia (AML) patients. **a** The expression of XBP1s, EDEM1, GRP78, and CHOP mRNA is significantly upregulated in primary PB (*upper*) and BM (*lower*) AML samples compared to healthy control samples. **b** The expression of the 4 types of mRNA from primary BM samples individually with their diagnosis and the transcript levels after normalization against β-actin (ACTB) in healthy individuals and leukemia patients are shown. **c** Comparison of the expression of the 4 types of mRNA from BM AML samples at the initial visit between the patients with sustained complete response (*CR*) and those who experienced relapse (*Relapse*) (*NS* not significant, *P* values > 0.1)

The UPR is initially a self-protective response aimed at restoring normal ER function and maintaining cell survival; however, when ER stress is severe or prolonged, it overrides the salvage mechanisms of the initial UPR, leading to apoptosis [4]. Schardt et al. [5] reported UPR activation in AML patients using the expression of XBP1s by gel electrophoresis analysis; AML patients with an activated UPR presented a favorable course of the disease. This was unexpected, as increasing evidence suggests that the UPR is closely associated with cancer cell survival and resistance to anti-cancer treatments. Bagratuni et al. [6] reported that myeloma patients with high XBP1s levels have a poor clinical outcome. Ph-positive ALL patients, a representative leukemia with a poor prognosis, showed especially high expression levels of XBP1s and EDEM1 mRNA compared to Ph-negative ALL patients and control samples in our study (data not shown). In our previous study, we found that the Bcr-Abl fusion protein activated the UPR, which plays an anti-apoptotic role in Ph-positive leukemia cells [2]. We evaluated the treatment response and the expression of the four mRNAs in AML patients at the initial visit, but did not find any correlation ($P > 0.1$) (Fig. 1c). UPR activation did not seem to be a prognostic factor in the present study. Kahn et al. reported that the promyelocytic leukemia (PML)-retinoic acid receptor (RAR) α fusion protein activated the UPR in APL cells. They suggested that PML-RARα induces the accumulation of the nuclear hormone receptor corepressor in the ER, leading to the induction of ER stress and ATF6 processing [7]. We found a relatively higher expression of UPR-related genes in APL patients, indicating that UPR activation is not only a common characteristic in primary AML cells, but a specific translocation product or genetic alteration might also activate the UPR in leukemic cells. AML cells with prognostically favorable or unfavorable karyotypes and gene mutations might show significant alterations on UPR activation. Larger and more intensive studies are required to determine the influence of the UPR on clinical characteristics and leukemogenesis. It would also be interesting to investigate this mechanism for targeting the UPR in leukemic cells from a therapeutic perspective.

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Efficacy and safety of patient-directed titration of once-daily pre-dinner premixed biphasic insulin aspart 70/30 injection in Japanese type 2 diabetic patients with oral antidiabetic drug failure: STEP-AKITA study

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ABSTRACT

Aims/Introduction: To clarify clinical characteristics related to optimal glycemic control achieved after adding once-daily pre-dinner biphasic insulin aspart 70/30 (BIAsp 30) in Japanese type 2 diabetic (T2D) patients with oral antidiabetic drug (OAD) failure.

Materials and Methods: Under this regimen, we evaluated changes in HbA_{1c} levels and daily self-monitoring blood glucose (BG) profiles, as well as the incidences of hypoglycemia and retinopathy progression. The patients adjusted BIAsp 30 dosages themselves every 3–4 days according to a pre-determined algorithm to achieve fasting BG levels of 101–120 mg/dL. HbA_{1c} levels were expressed as Japan Diabetes Society values.

Results: Of 29 enrolled patients, 22 (HbA_{1c} levels, 8.5 ± 1.5% [mean ± SD]) and 20 patients completed the 16- and 24-week follow-up, respectively. At 16 weeks 68.2 and 45.5%, and at 24 weeks 80.0 and 35% of patients had achieved HbA_{1c} levels of <7.0 and <6.5%, respectively. The patients who had achieved optimal glycemic control, including daytime postprandial BG profiles after treatment, had lower post-breakfast BG excursions at baseline, shorter diabetes durations and younger age. No severe hypoglycemic episodes were recorded. Progression of retinopathy was observed in 3 of the 29 enrolled patients.

Conclusions: Lower post-breakfast BG excursions, shorter diabetes duration and younger age in Japanese T2D patients with OAD failure might warrant optimal glycemic control with safety after adding once-daily pre-dinner BIAsp 30 initiating regimen. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2010.00062.x, 2010)

KEY WORDS: Biphasic insulin aspart 70/30, Insulin initiation, Self-adjusted treatment algorithm

INTRODUCTION

To reduce the risk of diabetic chronic complications, it is crucial to achieve ideal glycemic control as early as possible in diabetic patients[1–4]. In clinical practice, under recent guidelines, a HbA_{1c} level of <6.5–7.0% is recommended as the target for glycemic control in diabetic patients[5,6].

The progressive decline in β-cell function in type 2 diabetic (T2D) patients has been reported, despite lifestyle modifications and pharmacological interventions using oral antidiabetic agents (OAD)[7,8]. Therefore, at present, the initiation of insulin therapy is generally thought to be inevitable in a certain proportion of T2D patients. However, how and when to initiate insulin therapy in T2D patients remains controversial.

In recent reports, once or twice daily use of basal insulin analogues as an add-on therapy to OAD (so-called basal-supported oral therapy [BOT]) in insulin-naïve T2D patients has shown that glargine or detemir can achieve clinically important improvements in glycemic control, similar to those achievable with neutral protamine Hagedorn insulin, but with less risk of hypoglycemia[9–14]. On the basis of these results, the recent guidelines from Western countries have recommended that insulin should be initiated with basal insulin[6]. However, in a report of Japanese T2D patients, approximately half of the patients could not achieve a HbA_{1c} level of <7.0%[15]. In that study, no clear explanatory characteristics distinguished patients who could achieve good glycemic control from those who did not.

In contrast to basal analogue insulin, premixed analogue insulin, such as biphasic insulin aspart 70/30 (BIAsp 30), can improve postprandial glucose levels and provide basal insulin

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coverage by one injection at mealtime. The ability of BIAsp 30 to improve postprandial glucose levels is superior to human premixed insulin 70/30[16,17]. In the recent 'The 1-2-3 study', insulin therapy was initiated with once-daily pre-dinner BIAsp 30, titrated to a target fasting blood glucose (FBG) level of 80–110 mg/dL by add-on OAD therapies (phase 1), followed by the addition of pre-breakfast BIAsp 30 (phase 2) and pre-lunch BIAsp 30 (phase 3), with OAD withdrawal in patients showing a HbA_{1c} level ≥6.5% at the end of phase 1 or 2. Just after phase 1, 2 and 3 of this regimen 41, 70 and 77%, respectively, of patients had a HbA_{1c} level of <7.0% [18]. A recent study that used a similar step-up regimen with BIAsp 30 in Japanese T2D patients[19] confirmed the efficacy and usefulness of the method.

Quite recently, the regimen of adding a once-daily pre-dinner injection of BIAsp 30 has been reported to have equal or a slightly superior efficacy in improving glycemic control than those of basal insulin analogues as add-on OAD therapies[20]. However, the efficacy and safety of this method in Japanese T2D patients has not yet been sufficiently tested. In particular, the differences in the clinical characteristics of patients who can or cannot achieve optimal glycemic control, including postprandial glycemic control, after this regimen need to be elucidated.

In an outpatient setting, patient-directed insulin dosage titration, according to a predetermined dosage-escalation algorithm, reportedly shows the same levels of improvement in glycemic control and safety as physician-led dosage titration[21,22].

We, therefore, investigated the extent to which a regimen with once-daily pre-dinner BIAsp 30 as an add-on OAD therapy would improve HbA_{1c} levels and daily profiles of blood glucose (BG) in Japanese T2D patients under OAD failure. In the present study, we used patient-directed insulin titration method on the basis of self-monitoring BG (SMBG) levels according to a predetermined algorithm. Furthermore, we attempted to identify differences in clinical characteristics between patients with and without optimal glycemic control after undergoing this regimen.

MATERIALS AND METHODS

Patients

Eligible patients were as follows: diagnosed with T2D for ≥1 year, ≥20 years-of-age, not pregnant, insulin naïve and HbA_{1c} levels of >7.0% or fasting plasma glucose levels of ≥140 mg/dL on a standard regimen[5] for ≥3 months with OAD. Sulfonylurea (SU) dosages were to be at least equivalent to a daily dose of 5 mg of glibenclamide, 80 mg of gliclazide or 3 mg of glimepiride with or without metformin (MET), thiazolidinedione (TZD) and/or alpha-glucosidase inhibitors (AGI). In addition, SMBG was introduced to eligible patients during the 4 weeks before initiation of BIAsp 30. Patients were instructed to monitor their FBG levels every day. Patients with FBG of ≥140 mg/dL (mean of the last 3 days during the 4-week SMBG period) were confirmed as final eligible patients. Enrolled patients did not have hepatic insufficiency (alanine transaminase or aspirate transaminase is ≥2-fold of the upper reference limit of each institute), renal insufficiency (serum creatinine ≥1.4 mg/dL), severe diabetic complications (overt proteinuria with renal failure, unstable proliferative retinopathy or symptomatic orthostatic hypotension), malignant tumors or dementia. We carried out this 24-week, open-label, interventional, multicenter (five hospitals in Akita Prefecture, Japan: Akita University Hospital, Akita Red Cross Hospital, Akita City Hospital, Akita Kumiai General Hospital and Yokote Municipal Hospital) study in accordance with the Declaration of Helsinki. All the patients provided written informed consent.

Medication and BIAsp 30 Titration

Eligible patients were instructed to add a once-daily BIAsp 30 injection within 15 min pre-dinner to their OAD regimens. Dosages of SU were reduced to the allowed minimum dosages for Japan (2.5 mg of glibenclamide, 40 mg of gliclazide or 1 mg of glimepiride) to avoid hypoglycemia, particularly during the night. MET, TZD and AGI were taken as baseline treatment in each patient. The initial dosage of BIAsp 30 was 3 U, followed by self-adjustment of the pre-dinner BIAsp 30 dosage every 3–4 days on the basis of an average of 3–4 previous FBG values. The dosage-titration algorithm was as follows:

Mean fasting SMBG (mg/dL)	≤80	81–100	101–120	121–140	141–160	160<
Adjustment of insulin dosage (U)	−3	−1	0	+1	+2	+3

On the basis of this method, the present study was named the 'STEP-AKITA study', abbreviated from SMBG based management of type 2 diabetes under oral antidiabetic drugs failure with evening premixed biphasic insulin aspart 70/30 injection in AKITA.

Patients were encouraged to visit an outpatient clinic every 2–4 weeks. If necessary, patients were allowed to consult their physicians about the adjustment of insulin dosages by phone or fax. BIAsp 30 was given with the prefilled (3 mL, 100 U/mL) Novorapid 30 mix FlexPen delivery system (Novo Nordisk, Bagsvaerd, Denmark).

Assessments

Blood samples for the assessment of HbA_{1c} levels were obtained every 4 weeks along with a routine examination of hepatic and renal function. HbA_{1c} levels and the proportion of patients achieving those of <7.0% or <6.5% at 16 and 24 weeks after the initiation of BIAsp 30 were assessed, as it is unclear how long an observation period needs to be for sufficient evaluation of the effect on HbA_{1c} levels in insulin therapies added to OAD. Patients were encouraged to record 8-point SMBG profiles (including pre- and post-breakfast, -lunch and -dinner profiles; bedtime and 03.00 h, where 'post' times were 2 h later) at least once a week. Because sufficient 8-point SMBG profiles were obtained in more patients around the time BIAsp 30 titration finished than at 16 or 24 weeks, 8-point SMBG profiles

recorded just before the initiation of BIAsp 30 (baseline) and just after BIAsp 30 titration was judged to be finished were used for evaluation. Bodyweight (BW) was recorded in the outpatient clinic once a month.

Patients were re-taught how to recognize the symptoms of hypoglycemia and were instructed to obtain and record their SMBG level whenever a hypoglycemic event was suspected. Hypoglycemic episodes were classified as mild when SMBG levels were <70 mg/dL, regardless of hypoglycemic symptoms, and when patients could manage themselves. Events were classified as severe when BG levels were <70 mg/dL and when patients were unable to manage themselves.

To detect any worsening of retinopathy, all patients were required to consult ophthalmologists before initiation of BIAsp 30 and after titration.

Laboratory Procedures

SMBG was carried out with provided BG (capillary) meters (One Touch Ultra, LifeScan, Milpitas, CA, USA). HbA_{1c} level was measured by high performance liquid chromatography using an automated analyzer at each hospital and values were calibrated with standard substances recommended by Japan Diabetes Society (JDS Lot 2). The reference range of HbA_{1c} levels is 4.3–5.8%. HbA_{1c} values were expressed as JDS values.

Statistical Analysis

Values are expressed as mean ± SD. The Friedman test was used to identify global differences in values throughout the study period. Furthermore, values at each time after the initiation of BIAsp 30 were compared with the value at baseline using Dunn's multiple comparison test (non-parametric). The Wilcoxon signed-ranks test was used for paired comparisons between values before and after treatment. The Mann–Whitney *U*-test and Pearson's χ^2-test were used to calculate the significance of differences in values between groups. Pearson's correlation analysis was carried out to explore relationships between corresponding values. All calculations were made using StatFlex Version 5.0 (Artec, Osaka, Japan). Two tailed *P*-values of <0.05 were considered statistically significant.

RESULTS

Participants

A total of 29 patients, with a mean fasting SMBG ≥140 mg/dL, even in the last 3 days during the 4-week SMBG period, initiated BIAsp 30 according to the protocol. Of those, seven patients did not complete the study. Reasons for withdrawal were adverse events in two patients, (progression of diabetic retinopathy and skin allergy at injection site) and non-compliance of insulin injection or SMBG recording in five patients. Finally, 22 and 20 patients completed 16- and 24-week follow up of HbA_{1c} levels, respectively. The clinical characteristics of 22 patients are shown in Table 1. No patient had been treated with SU monotherapy. More than 80% of patients had been treated with MET or TZD.

Table 1 | Clinical characteristics of type 2 diabetic patients

Male/female (*n*)	10/12
Age (years)	62.1 ± 10.8
Body mass index (kg/m^2)	25.1 ± 5.0
Duration of diabetes (years)	13 ± 9.4
Retinopathy (nil/simple/proliferative)	(15/7/0)
Albuminuria (normo/micro/macro)	(13/3/6)
HbA_{1c} at baseline (%)	8.5 ± 1.5
No. OAD (%)	
Monotherapy	0 (0)
Combination therapy	
SU + MET	3 (13.6)
SU + TZD	3 (13.6)
SU + MET + TZD	11 (50.0)
SU + TZD + AGI	1 (4.5)
SU + MET + TZD + AGI	4 (18.2)
Use of MET	18 (81.8)
Use of TZD	19 (86.4)

Data are expressed as mean ± SD or otherwise indicated.
AGI, alpha-glucosidase inhibitor; MET, metformin; OAD, oral anti-diabetic agents; SU, sulfonylurea; TZD, thiazolidinedione.

Glycemic Control

Efficacy Achieved as Per HbA_{1c} Levels

Achieved HbA_{1c} levels are shown in Figure 1a. At 8 weeks after the initiation of BIAsp 30, mean HbA_{1c} level was significantly decreased (7.4 ± 1.2, *vs* 8.5 ± 1.5% at baseline; $P < 0.05$). At 16 weeks, mean HbA_{1c} level had stabilized to 6.8 ± 1.0% ($P < 0.01$ *vs* baseline) and showed a tendency toward a slight decrease until 24 weeks (at 24 weeks, 6.6 ± 0.7%, $P < 0.01$ *vs* baseline). At 16 weeks, the rates of patients who achieved HbA_{1c} levels of <7.0 and <6.5% were 68.2% (15/22) and 45.5% (10/22), respectively. At 24 weeks, 16 of 20 (80.0%) and 7 of 20 (35.0%) patients had achieved HbA_{1c} levels of <7.0 and <6.5%, respectively. Reduction of the rate of patients achieving a HbA_{1c} level of <6.5% at 24 weeks was a result of worsening of HbA_{1c} level to ≥6.5% in two patients and a patient dropping out before 24-week follow up, among 10 patients who achieved a HbA_{1c} level of <6.5% at 16 weeks. When the patients were stratified according to baseline HbA_{1c} levels of <8.0 or ≥8.0%, 87.5% (7/8) and 75.0% (9/12) of patients, respectively, had achieved HbA_{1c} levels of <7.0% at 24 weeks. Further, 37.5% (3/8) and 33.3% (4/12) of patients with respective baseline HbA_{1c} levels of <8.0 and ≥8.0% had achieved HbA_{1c} levels of <6.5% at 24 weeks. The rates of patients who had achieved HbA_{1c} levels of <7.0 and <6.5% as the final evaluations at 24 weeks were not influenced by baseline HbA_{1c} levels. Figure 2b shows a strong linear correlation between baseline HbA_{1c} levels and change in HbA_{1c} levels from the baseline (ΔHbA_{1c}) at 24 weeks in 20 patients (Figure 2b), showing that an even higher HbA_{1c} level at baseline can be lowered sufficiently by the regimen of the present study.

Efficacy Achieved as Per Daily Profiles of SMBG

Figure 1b shows the improvement in 8-point SMBG profiles in 21 patients whose daily 8-point SMBG profiles at baseline and

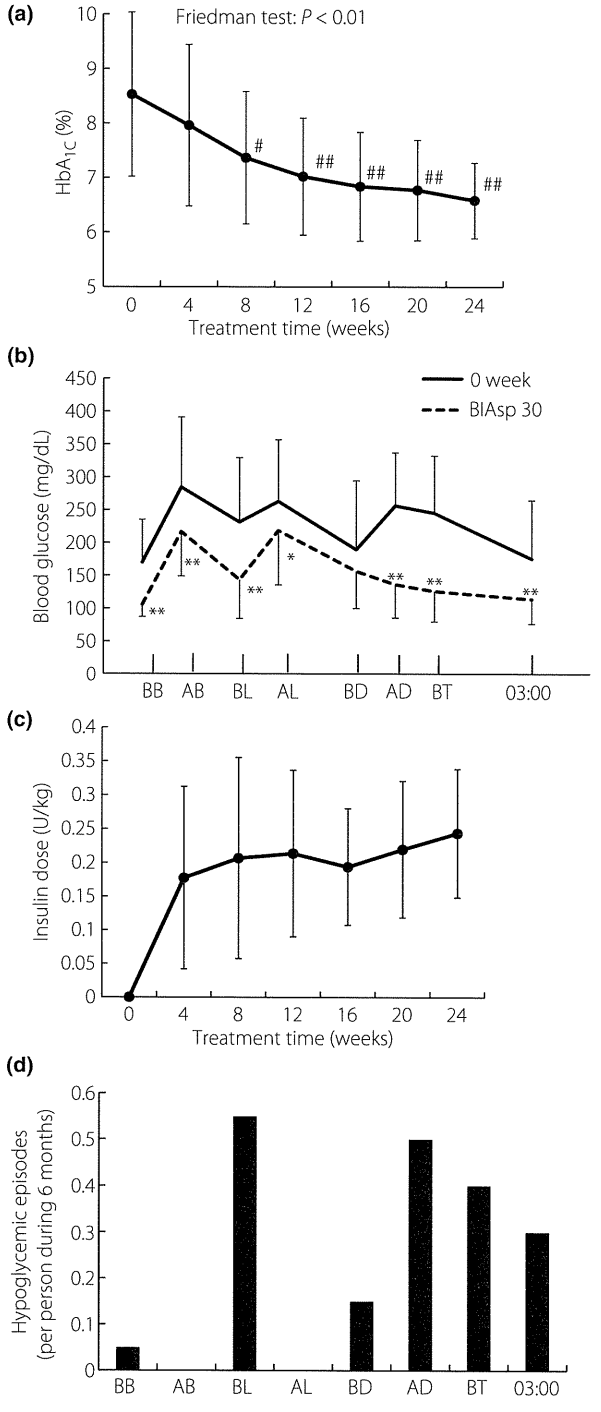

Figure 1 | Clinical parameters during the treatment with pre-dinner biphasic insulin aspart 70/30. (a) Changes in HbA_{1c}. (b) Mean 8-point self-monitored blood glucose profiles at baseline and just after BIAsp 30 titration. (c) Changes in self-titrated insulin dose. (d) Incidences of hypoglycemic episodes. Data are expressed as mean ± SD in a, b and c. AB, 2 h post-breakfast; AD, 2 h post-dinner; AL, 2 h post-lunch; BB, pre-breakfast; BD, pre-dinner; BL, pre-lunch; BT, bed time. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ versus values at 0 week revealed by Dunn's multiple comparison test. $^{*}P < 0.05$; $^{**}P < 0.01$ versus values at 0 week revealed by the Wilcoxon signed-ranks test.

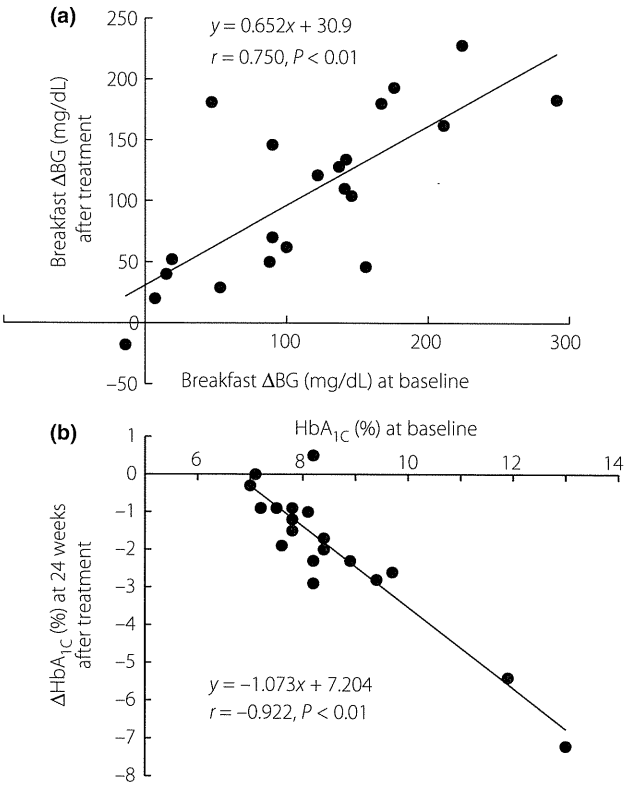

Figure 2 | Relationships (a) between post-breakfast blood glucose excursion (ΔBG) at baseline and that just after titration of pre-dinner biphasic insulin aspart 70/30 and (b) between baseline HbA_{1c} levels (%) and changes in HbA_{1c} levels (ΔHbA_{1c}, %) at 24 weeks from baseline.

just after BIAsp 30 titration (after titration) were sufficiently obtained. All the points of BG measured were significantly decreased, except for pre-dinner BG. In particular, the decreases in BG at pre- and post-breakfast, pre-lunch, post-dinner, at bedtime and at 03.00 h were remarkable ($P < 0.01$). As shown in Figure 1b, the BG profiles from post-dinner to pre-breakfast were well stabilized without post-dinner BG excursion (ΔBG) after titration (67.1 ± 85.4 mg/dL at baseline and −23.6 ± 67.4 mg/dL after titration; $P < 0.01$), whereas post-breakfast ΔBG after BIAsp 30 titration remained comparable to that at baseline (114.7 ± 77.9 mg/dL at baseline and 105.8 ± 67.7 mg/dL after titration).

Clinical Characteristics of Patients With or Without Optimal Post-breakfast ΔBG Pattern After Treatment

The patients in the present study showed widely divergent post-breakfast ΔBG (from −18 to 228 mg/dL) even after nearly ideal FBG levels had been established by BIAsp 30 titration (Fig. 2a). Therefore, clinical characteristics with or without optimal post-breakfast ΔBG after titration were explored. Individual post-breakfast ΔBG at baseline and after BIAsp 30 titration showed a strong correlation (Fig. 2a), indicating that the treatment might scarcely influence the individual post-breakfast ΔBG pattern. Therefore, we divided the patients into two groups according to

Table 2 | Clinical characteristics of type 2 diabetic patients divided according to 2-h post-breakfast blood glucose levels after biphasic insulin aspart 70/30 titration

	Blood glucose at 2 h after breakfast		
	<200 mg/dL $n = 10$ (Group A)	≥200 mg/dL $n = 11$ (Group B)	*P*-value
Male/female (*n*)	8/2	4/7	<0.05
Age (years)	55.3 ± 5.7	66.8 ± 10.5	<0.05
Body mass index (kg/m^2)	25.3 ± 4.1	24.6 ± 6.4	NS
Duration of diabetes (years)	8.7 ± 7.0	18.9 ± 11.7	<0.05
AGI use (*n*, %)	3, 30.0	2, 18.1	NS
HbA_{1c} levels at 0 week (%)	8.9 ± 1.8	8.7 ± 2.0	NS
HbA_{1c} levels at 16 weeks (%)	6.7 ± 0.62	6.8 ± 0.96	NS
ΔHbA_{1c} 0–16 weeks (%)	−2.2 ± 1.9	−1.9 ± 1.4	NS
FBG at 0 week (mg/dL)	173.8 ± 80.0	166.3 ± 52.8	NS
2h-BG-M at 0 week (mg/dL)	239.3 ± 119.3	325.6 ± 71.6	NS
ΔBG-M at 0 week (mg/dL)	65.5 ± 58.5	159.4 ± 66.6	<0.01
FBG after BIAsp 30 titration (mg/dL)	106.7 ± 14.5	109.9 ± 19.8	NS
2h-BG-M after BIAsp 30 titration (mg/dL)	152.8 ± 29.5	269.9 ± 36.9	<0.01
ΔBG-M after BIAsp 30 titration (mg/dL)	46.2 ± 33.5	160.0 ± 36.9	<0.01
Insulin dosage after BIAsp 30 titration (U/kg)	0.21 ± 0.14	0.23 ± 0.15	NS

Data are expressed as mean ± SD or otherwise indicated. The Mann–Whitney *U*-test and Pearson's χ^2-test was used to calculate statistically significant differences of the values between groups. 2h-BG-M, blood glucose 2 h after breakfast; ΔHbA_{1c} 0–16 weeks, changes in HbA_{1c} levels from baseline to 16 weeks; AGI, alpha-glucosidase inhibitor; FBG, fasting blood glucose; ΔBG-M, blood glucose excursion for 2 h after breakfast; NS, not significant.

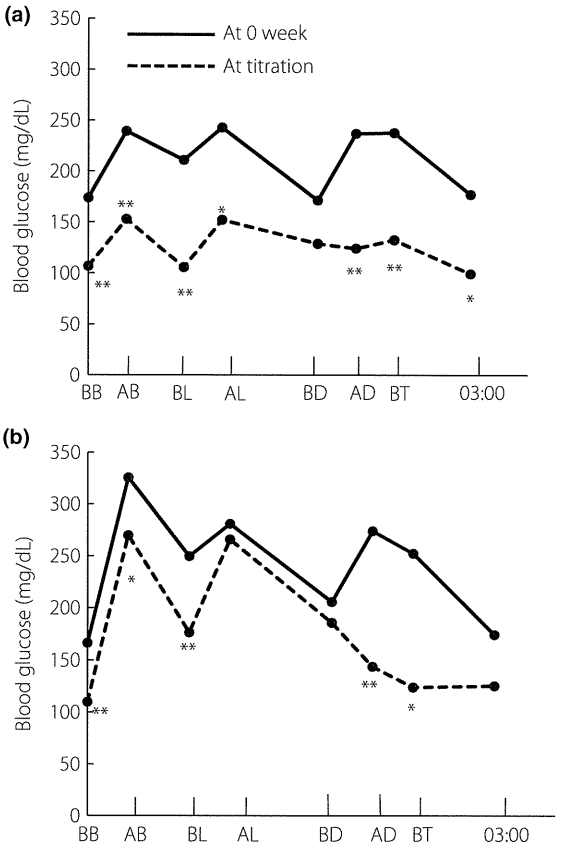

Figure 3 | Mean 8-point self-monitored blood glucose (BG) profiles at 0 week and just after pre-dinner biphasic insulin aspart 70/30 (BIASp 30) titration (a) in patients with 2 h post-breakfast BG after BIAsp 30 titration <200 mg/dL and (b) in patients with 2 h post-breakfast BG after BIAsp 30 titration ≥200 mg/dL. AB, 2 hours post-breakfast; AD, 2 h post-dinner; AL, 2 h post-lunch; BB, pre-breakfast; BD, pre-dinner; BL, pre-lunch; BT, bed time. *$P < 0.05$; **$P < 0.01$ versus values at 0 week revealed by the Wilcoxon signed-ranks test.

post-breakfast SMBG levels after BIAsp 30 titration: Group A, <200 mg/dL; and Group B, ≥200 mg/dL (Table 2). Figure 3 shows the daily 8-point SMBG profiles at baseline and after BIAsp 30 titration, indicating relatively flat BG profiles in Group A and ruggedness in Group B. Table 2 shows the clinical characteristics of both groups. Patients in Group B had a greater proportion of women, older age, longer duration of diabetes and higher post-breakfast ΔBG both before and after BIAsp 30 titration. Despite the rugged daytime BG profiles, even after BIAsp 30 titration, HbA_{1c} levels after BIAsp 30 treatment in Group B were not significantly higher compared with that in Group A (Table 2).

Insulin Dosages

Insulin dosage (U/kg) was titrated to 0.18 ± 0.14 at 4 weeks, 0.19 ± 0.09 at 16 weeks, 0.22 ± 0.10 at 20 weeks and 0.24 ± 0.09 at 24 weeks (Figure 1c).

BW Changes

BW changes from 0 week (kg) were −0.12 ± 1.0, 0.23 ± 1.8, 0.59 ± 2.3, 1.1 ± 2.2, 1.9 ± 2.8 and 2.5 ± 2.7 at 4, 8, 12, 16, 20 and 24 weeks, respectively. The Friedman test showed significant BW changes throughout the study period ($P < 0.01$). BW at 20 and 24 weeks were significantly increased compared with baseline ($P < 0.05$ by Dunn's multiple comparison test).

Hypoglycemic Episodes and Adverse Events

No severe hypoglycemic episodes were reported. Mild hypoglycemic episodes were reported by 13 of 29 patients (including 7 patients who withdrew from the study) during the study at a low rate of 1.95 events per patient over the course of 6 months (Figure 1d). Principally, patients had experienced hypoglycemic episodes pre-lunch, post-dinner, at bedtime and at 03.00 h.

One woman withdrew because of a progression of diabetic retinopathy (progression from no clinical lesions at baseline to proliferative retinopathy requiring photocoagulation and surgical procedure by an ophthalmologist 6 weeks after initiation of

BIAsp 30). Another woman withdrew because of skin allergy at the injection site. The patient with a marked retinopathy progression mentioned previously had a long estimated duration of diabetes (23 years) and marked poor glycemic control (HbA_{1c} level at baseline of 13.8%, ΔHbA_{1c} at 4, 8 and 12 weeks of −2.7, −3.8 and −4.5%, respectively). Retinopathy progression was reported in another two patients who were able to complete the study. In one woman, simple retinopathy before the initiation of BIAsp 30 had progressed to proliferative retinopathy 1 month after the end of this study (HbA_{1c} levels at baseline and at 24 weeks were 7.8, 6.9%, respectively. ΔHbA_{1c} at 4, 8 and 12 weeks were −0.5, −0.6 and −0.7%, respectively). In another patient, a man without retinopathy at baseline, simple retinopathy was found 24 months after the end of the present study (HbA_{1c} levels at baseline and at 24 weeks were 7.2 and 6.3%, respectively. ΔHbA_{1c} at 4, 8 and 12 weeks were −0.3, −1.1 and −1.6%, respectively).

DISCUSSION

This study showed that in Japanese T2D patients with failed OAD therapies, patients who had achieved optimal glycemic control, including daytime postprandial BG profiles after an addition of appropriate dosage of once-daily pre-dinner BIAsp 30 (Group A), had lower post-breakfast BG excursions at baseline, shorter diabetes durations and younger age. The other patients with opposite clinical characteristics (Group B) had still shown higher daytime postprandial BG excursions, even after achievement of ideal BG profiles from post-dinner to pre-breakfast, by the regimen used in the present study.

In Group A patients, the ranges of daily BG profiles before and after BIAsp 30 titration were relatively flat (Figure 3) and resembled those of T2D patients from Western countries, who used BOT or pre-dinner BIAsp 30 as an add-on to OAD[9–14,18,20]. Additionally, daily BG profiles after titration were ideal and didn't seem to require additional insulin supplementation at breakfast or lunch. In contrast, Group B patients had higher daytime postprandial glucose excursions both before and after BIAsp 30 titration and higher baseline post-breakfast ΔBG levels were preserved even after treatment (Figure 3), requiring bolus dosages at breakfast and/or lunch time to achieve ideal daily BG profiles. Collectively, higher post-breakfast ΔBG levels with OAD failure might predict a need for two or more bolus supplements of insulin after the establishment of ideal BG profiles from post-dinner to pre-breakfast by pre-dinner BIAsp 30 titration.

Group B patients had relatively higher age and longer durations of diabetes (Table 2), indicating that they had the characteristics of more declined β-cell function reported in long-standing T2D patients[7,8]. The main characteristics of Asian T2D patients have been thought to include declined β-cell function[23–25] and a loss of early-phase insulin response[26]. Taking these findings together, Japanese T2D patients with OAD failure can be divided into two groups: one characterized with relatively declined β-cell function with higher postprandial glucose excursions requiring appropriate basal and multiple bolus insulin supplements; and the other with relatively preserved β-cell function with lower postprandial glucose excursions requiring only appropriate FBG correction that might be achieved with the addition of once-daily injections to OAD therapies, such as pre-dinner BIAsp 30 or basal insulin analogues.

Table 2 shows that there is no difference in HbA_{1c} levels at 16 weeks between Group A and B, despite the markedly different patterns of daytime BG excursion (Figure 3). It is difficult to explain this phenomenon. Daily SMBG profiles had been well preserved during the study (the post-breakfast ΔBG last recorded during the study in Group A and B were 57.6 ± 55.5 and 141.7 ± 55.5 mg/dL [$P < 0.01$], respectively). Postprandial glucose spikes of relatively short duration might have minor effects on HbA_{1c} levels. Because glycated albumin (GA) is reported to be a more sensitive marker than HbA_{1c} for glucose excursions[27], we would have measured GA levels in the present study.

The results that 80 and 35% of participants were able to achieve HbA_{1c} levels of <7.0 and <6.5%, respectively, at 24 weeks confirmed the satisfactory results of phase 1 in 'The 1-2-3 study' (in which HbA_{1c} level was <7.0% in 41% and <6.5% in 21% of patients)[18]. Recently, it was reported that HbA_{1c} levels of 7.0 and 6.5% in the USA are equivalent to those of 6.6 and 6.1% in Japan (JDS values)[28]. Considering the methodological differences used to determine HbA_{1c} levels between Japan and the USA, the result that 35% of the patients in the present study achieved HbA_{1c} levels of <6.5% (equivalent to 6.9% in the USA) is comparable to the result of 'The 1-2-3 study'. Recent guidelines from Western countries recommend that insulin should be initiated with basal insulin analogues as add-on OAD therapies[6]. However, the satisfactory results of 'The 1-2-3 study', the study of Strojek *et al.*[20] and the present study show that adding a once-daily pre-dinner injection of BIAsp 30 in T2D patients with OAD failure might represent an alternative regimen at the initiation of insulin therapy.

The present study used an insulin titration method managed by patients themselves on the basis of SMBG levels according to a predetermined algorithm[21,22]. The satisfactory effects of the present study on HbA_{1c} levels with a minimal incidence of hypoglycemia also confirmed the usefulness of this insulin titration method in Japanese T2D patients. Dosage-adjustment levels at each correction were designed to be approximately one-third of those applied in Western countries[18,21,22], where the body mass index (BMI) of patients is approximately 30. The BMI of our patients was approximately 25, suggesting a less insulin-resistant feature of Japanese T2D patients and the need for a finer insulin titration regimen.

In the present study, no severe hypoglycemic episodes were recorded and the frequency of mild hypoglycemic episodes was considered acceptable. Recording SMBG, especially pre-breakfast everyday and self-adjustment of pre-dinner BIAsp 30 dosage, according to SMBG levels might be related to this level of safety. The reduced dosages of SU at the initiation of BIAsp 30 in the