Protein Kinase B/Akt Prevents Fatty Acid-induced Apoptosis in Pancreatic β-Cells (INS-1)*

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Free fatty acids (FFA) have been reported to reduce pancreatic β-cell mitogenesis and to increase apoptosis. Here we show that the FFA, oleic acid, increased apoptosis 16-fold in the pancreatic β-cell line, INS-1, over a 18-h period as assessed by Hoechst 33342/propidium iodide staining and caspase-3 and -9 activation, with negligible necrosis. A parallel analysis of the phosphorylation activation of protein kinase B (PKB) showed this was reduced in the presence of FFA that correlated with the incidence of apoptosis. At stimulatory 15 mM glucose and/or in the added presence of insulin-like growth factor 1, FFA-induced β-cell apoptosis was lessened compared with that at a basal 5 mM glucose. However, most strikingly, adenoviral mediated expression of a constitutively active PKB, but not a "kinase-dead" PKB variant, essentially prevented FFA-induced β-cell apoptosis under all glucose/insulin-like growth factor 1 conditions. Further analysis of pro-apoptotic downstream targets of PKB, implicated a role for PKB-mediated phosphorylation inhibition of glycogen synthase kinase-3α/β and the forkhead transcription factor, FoxO1, in protection of FFA-induced β-cell apoptosis. In addition, down-regulation of the pro-apoptotic tumour suppressor protein, p53, via PKB-mediated phosphorylation of MDM2 might also play a role in partially protecting β-cells from FFA-induced apoptosis. Adenoviral mediated expression of wild type p53 potentiated FFA-induced β-cell apoptosis, whereas expression of a dominant negative p53 partly inhibited β-cell apoptosis by ~50%. Hence, these data demonstrate that PKB activation plays an important role in promoting pancreatic β-cell survival in part via inhibition of the pro-apoptotic proteins glycogen synthase kinase-3α/β, FoxO1, and p53. This, in turn, provides novel insight into the mechanisms involved in FFA-induced β-cell apoptosis.

In insulin-resistant states such as obesity, there is a compensation of increased pancreatic β-cell mass and function so that insulin production is up-regulated and diabetes does not develop. However, with time and/or severity of the insulin resistance, pancreatic β-cell dysfunction and an inadequate β-cell mass develop, so that it can no longer compensate for the peripheral insulin resistance and type 2 diabetes results (1–3). Although the pathophysiological relevant factors that prevent expansion or lead to a reduction in β-cell mass have not been identified, studies have led to the proposal that chronic exposure to high levels of long chain free fatty acids (FFA)1 are a contributing factor (4). The FFA-induced reduction in β-cell mass has been attributed, in part, to a decrease in β-cell proliferation (5) and also decreased β-cell survival (1, 6, 7).

The correct balance between the level of apoptosis and cell proliferation is a crucial factor in maintaining an appropriate mass of fully functional β-cells within the pancreatic islet (8). A number of nutrients and growth factors can activate mitogenic signaling pathways to increase β-cell proliferation (3). For example, glucose can independently induce β-cell mitogenesis, and intriguingly growth factors such as insulin-like growth factor-1 (IGF-1) and growth hormone induce β-cell proliferation in a glucose-dependent manner (9, 10). Characterization of the pathways involved in this mitogenic stimulation have shown that the phosphatidylinositol 3'-kinase (PI3K) and mitogen-activated protein kinase kinase (ERK1/ERK2) signaling pathways, downstream of insulin receptor substrate-2 (IRS-2), play an important role in β-cell growth and survival (3, 11). However, little is known about the signaling mechanisms that are involved in preventing pancreatic β-cell death. Several studies in other cell types have implicated an important role for early upstream activation of protein kinase B (PKB, also known as Akt) in signaling pathways for maintaining cell survival (12). In this regard and relevant to pancreatic β-cells, it has recently been shown that β-cell-specific expression of a constitutively active form of PKB in transgenic mice markedly increases β-cell mass, mostly by preserving β-cell survival and increasing β-cell size (13). In contrast, there appears to be less of a role for PKB in promoting β-cell proliferation (14).

In β-cells, PKB activation can be mediated by IGF-1-induced tyrosine phosphorylation of IRS-2 leading to PI3K activation

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1 The abbreviations used are: FFA, free fatty acid; IGF-1, insulin-like growth factor 1; PI3K, phosphatidylinositol 3'-kinase; ERK1/ERK2, extracellular signal-regulated protein kinases 1 and 2; IRS-2, insulin receptor substrate 2; PKB, protein kinase B (also known as Akt); PDK1, 3-phosphoinositide-dependent kinase; Fox, forkhead box; GSK3α/β, glycogen synthase kinase-3α and -β; BAD, Bcl-2/Bcl-XL-antagonist causing cell death; MDM2, murine double minute 2; FoxO1, forkhead rhabdomyosarcoma transcription factor (also known as FKHR); OA, oleic acid or cis-9-octadecenoic acid; HO, Hoechst 33342; PI, propidium iodide; Adv, adenovirus; m.o.i., multiplicity of infection; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; GFP, green fluorescent protein.
that generates phosphatidylserine 3,4,5-triphosphate (11). The increase in phosphatidylinositol 3,4,5-trisphosphate results in PKB translocation to the plasma membrane via its pleckstrin homology domain (15), where the constitutively active 3-phosphoinositide-dependent kinase-1 (PDK1) phosphorylates PKB at Thr-308 followed by another phosphorylation at Ser-473 thought to be by PKB autophosphorylation (16). PKB has a myriad of protein substrates that could influence apoptosis, including the family of forkhead box (Fox) transcription factors. Some of these genes, including glycogen synthase kinase-3α/β (GSK3α/β), BAD, caspase 9 (12), and murine double minute 2 (MDM2) (17). There are three known isoforms of forkhead box factors, FoxO1, FoxO3a, and FoxO4 (also known as FKHR, FKHR-L1, and AFX, respectively), which, upon phosphorylation by PKB, appear to be retained in the cytoplasm preventing their translocation into the nucleus, where they mediate transcription of pro-apoptotic factors. GSK3 is also an important pro-apoptotic signaling protein and the kinase activity of GSK3 is inhibited by PKB phosphorylation. PKB phosphorylation has also been shown to inhibit the activity of components of the apoptotic machinery including the Bcl2 family member, BAD, and the cysteine protease, caspase 9. A recent finding has identified the ubiquitin ligase protein, MDM2, as a direct target of PKB, and interestingly, when phosphorylated by PKB, MDM2 negatively regulates the tumor suppressor protein, p53, a known pro-apoptotic transcription factor. Although several pro-apoptotic substrates are directly inhibited by PKB-induced phosphorylation, it is currently unclear to what extent PKB activation is protective of β-cell apoptosis and, if so, which of the PKB substrates might be relevant in terms of promoting β-cell survival.

In this study, we have determined that FFA can induce apoptosis in the pancreatic β-cell line, INS-1, in a dose-dependent manner. Moreover, FFA also induced an inhibition of IGF-1-induced PKB phosphorylation activation complementary to previous observations of FFA-induced inhibition of PKB activity (5). Intriguingly, adenoviral mediated expression of constitutively active PKB completely protected β-cells from FFA-induced apoptosis. As such, we provide evidence that PKB plays an important role in protecting pancreatic β-cells against a physiologically relevant factor, FFA.

**EXPERIMENTAL PROCEDURES**

**Materials**—The oleic acid (OA) and OA methyl ester were purchased from Alltech (State College, PA). Hoechst 33342 (HO) and propidium iodide (PI) were purchased from Sigma, and the annexin V-fluorescein isothiocyanate (FITC) kit was form Molecular Probes (Eugene, OR). The p53wt and mutant vectors were purchased from BD Biosciences Clontech (Palo Alto, CA). Total-FKHR (for the detection of rat FKHR1) was from Upstate Biotechnology, Inc. (Lake Placid, NY), and phospho-Ser509 FKHR antibody (for the detection of rat phospho-FKHR1) was from Cell Signaling (Beverly, MA). The phospho-GSK3α/β (Ser21/9), total-PKB, phospho-Ser473 PKB, phospho-Thr308 PKB, p53, and the caspase-9 cleaved antibodies were from Cell Signaling (Beverly, MA). Caspase-3 antibody was a gift from Dr. Nancy Thornberry (Merck Research Laboratories, Rahway, NJ). The total and phospho-ERK1/2 antibodies were obtained from Promega Corp. (Madison, WI) and total GSK3α/β antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-rabbit and anti-sheep IgG horseradish peroxidase conjugates were from Jackson ImmunoResearch (West Grove, PA) and the anti-mouse IgG horseradish peroxidase conjugate was from Upstate Biotechnology, Inc. IGF-1 was purchased from Gen Porto Pty Ltd (East Leederville, Australia). DNA purification kits and Superfect transfection reagents were purchased from Qiagen (Valencia, CA). Restriction enzymes were from New England Biolabs. The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce. The chemiluminescence reagent was from NEN Life Sciences. Unless otherwise stated, all other reagents were of analytical grade from either Sigma or Fisher Scientific.

**Cell Culture**—The glucose-sensitive pancreatic β-cell line, INS-1 (18), was maintained in the complete medium RPMI 1640 (11.2 mM glucose) containing 10% (v/v) fetal calf serum, 50 μM β-mercaptoethanol, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin and incubated at 37 ºC, 5% CO2 as described (18).

**Construction of Adenoviruses**—The pUSEamp expression vectors containing Myc-His-tagged mouse Akt1 (wild type), myr-Akt1 (activated), and Akt1-K179M (kinase-dead) from Upstate Biotechnology, Inc. were used for construction of the PKB adenoviruses, which have been described previously (14). The constitutively active form has c-Src-derived residues fused to PKB, and the consequent myristoylation directly targets PKB to the plasma membrane leading to its constitutive activation (19). The kinase-dead form has a point mutation, K179M, removing the ATP-binding site, resulting in loss of kinase activity (20). The p53 wild type and p53 mutant cDNAs (from BD Biosciences Clontech) express the wild type or a dominant negative p53 (p53mt135) respectively. The dominant negative mutant differs form the wild type p53 by a G to A conversion at nucleotide 1017, which causes a conformational change that not only prevents interaction with p53 DNA binding sites, but may also bind to and inhibit wild type p53. The p53 cDNAs were digested out of the vector pCMV-p53 or pCMV-p53mt135 with the restriction enzymes HindIII and EcoRI, then inserted between the HindIII and EcoRI sites of pBUSEcript, providing the necessary restriction sites for insertion into pACMV pLPA between KpnI and XhoI. The p53 adenoviruses were generated and purified as previously described (14, 21, 22). As control a recombinant adenovirus expressing green fluorescent protein (GFP) was used and generated as previously described (21).

**Adenovirus (AdV) Infection**—Initially, the appropriate titer for each recombinant AdV was determined by the addition of various dilutions of each adenovirus to INS-1 cells subcultured in six-well plates (9.5 cm2) to 60% confluence (−2 × 104 cells) per well, giving a multiplicity of infection (m.o.i.) ranging from 50 to 2500 based on 0.5-3 × 104 plaque-forming units/ml as measured by A595. For the OA/BSA-treated experiments, INS-1 cells were subcultured on 10-cm plates to ~60% confluence and then infected with the indicated dose of adenovirus. For all infections, the viral stock was replaced with complete medium after 2 h and the cells were incubated at 37 °C in 5% CO2 for ~16 h. The AdV-infected cells were then used experimentally as indicated.

**FFA Treatment**—INS-1 cells were subcultured on 10-cm plates to ~60% confluence with or without recombinant AdV infection (see “Adenovirus (AdV) Infection” as indicated for each experiment. The cells were then incubated in the modified INS-1 cell RPMI 1640 medium at 5 or 15 mM glucose ≥ 10 ng/ml IGF-1 with 0.5% (w/v) BSA alone or 0.4 mM OA complexed to 0.5% (w/v) BSA for 16 to 18 h. Preparation of the 0.4 mM OA, 0.5% BSA complex solution was carried out as described previously (5). Briefly, a 100 mM OA stock solution was prepared in 0.1 M NaOH by heating at 70 °C. A 10% FFA-free BSA solution was prepared in H2O and maintained at 55 °C in an adjacent water bath. The appropriate amount of 100 mM OA stock solution was added to the BSA and incubated for another 30 min at 55 °C. The OA/BSA complex solution was then cooled to 25 °C, filter sterilized, and stored at 4 °C until use.

**Apoptosis Assay**—Apoptotic measurements were carried out using a flurometric method and counting cell numbers under a fluorescence microscope as described previously (23). This method involves the use of DNA-binding dyes HO and PI. The HO compound is known to cross the plasma membrane of all cells, whether they are damaged or not, causing a blue fluorescence of their nuclei. The polA only penetrates cells with damaged membranes and leads to nuclear fluorescence. The percentage of apoptotic cells were counted by viewing cells under an inverted fluorescence microscope. After treating cells for 18 h with various treatments (see “OA/BSA Treatment”), the cells were incubated with 20 μg/ml HO and 10 μg/ml PI at 37 °C, 5% CO2 for 15 min. The nuclei were aspirated, and the samples were stained once with PBS and then fixed by incubation with 4% formaldehyde for 15 min at room temperature. The formaldehyde was removed by aspiration, mounting fluid added and the number of apoptotic cells counted under an inverted fluorescence microscope. A minimum of 500 cells were counted for each plate under randomized conditions. In a few instances, the percentage of apoptosis and necrosis was also assessed with Hoechst 33342 and Annexin V-FITC staining kit (24) and complementary findings to that using the HO/PI method were found. In these studies, the incidence of necrosis only accounted for <1% of dead cells.

**Immunoblot Analyses**—Following the 18-h incubation at 5 or 15 mM glucose with or without IGF-1 ≥ 0.1 OA/BSA, the cells/media were centrifuged for 5 min at 3000 rpm for 5 min at 4 °C. The INS-1 cells were then lysed in ice-cold cell lysis buffer consisting of 50 mM HEPES (pH 7.5), 1% (v/v) Nonidet P-40, 2 mM activated sodium orthovandate, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin,
and 1 μg/ml aprotinin, then sonicated (25 watts; 10 s, on ice), and particulate material removed by centrifugation (10,000 × g; 10 min; 4 °C). The supernatants were collected and stored at −80 °C pending immunoblot analysis. For immunoblot analysis, cell lysates were first normalized for equivalent total protein levels, as determined using the BCA protein assay kit. Immunoblot analysis on cell lysates was then performed as previously described (9, 14, 21).

Other Procedures—Where appropriate, data are presented as a mean ± S.E. Statistically significant differences between groups were analyzed using Student’s t test, where p ≤ 0.05 was considered statistically significant.

RESULTS

FFA-induced Apoptosis in the Pancreatic β-Cell, INS-1—OA was used as a “model FFA” in these studies, especially because OA has been previously shown to inhibit glucose/IGF-1-induced β-cell mitogenesis independent of ceramide production (5), and induce apoptosis, while having only a minor effect on cell necrosis in β-cells (7, 25). In dose-response experiments, it was examined what concentration of OA would induce apoptosis in pancreatic β-cells, INS-1, over an 18-h period at a constant 5 mM glucose. The concentration of BSA present was fixed at 0.5% (w/v), and the OA concentration complexed was varied at 0.25, 0.3, 0.35, and 0.4 mM OA. The basal rate of apoptosis in the presence of 0.5% BSA and absence of OA was 2.9 ± 0.1% of the total INS-1 cell population undergoing apoptosis (n = 7; Fig. 1). At 0.25 mM OA and 0.5% BSA (Fig. 1), the rate of apoptosis was unaltered compared with the basal rate at 2.1 ± 0.2% (n = 3; Fig. 1). However, at 0.3 mM OA and 0.5% BSA, the level of apoptosis was increased to 3.8 ± 3.6% (n = 3), and at 0.35 mM OA and 0.5% BSA, it was significantly increased 4-fold above the basal rate at 11.1 ± 3.6% (p ≤ 0.05; n = 3) (Fig. 1). The highest rate of OA-induced apoptosis was observed at 0.4 mM OA and 0.5% BSA, which was 16-fold (p ≤ 0.05) above the basal rate at 46.4 ± 6.4% (n = 8) of INS-1 cells being apoptotic (Fig. 1). Interestingly, in INS-1 cells incubated with 0.4 mM methyl OA in the presence of 0.5% (w/v) BSA, the rate of apoptosis was significantly lower (p ≤ 0.01) compared with those exposed to 0.4 mM OA and 0.5% BSA in parallel experiments, and not significantly different from the basal rate at 3.7 ± 1.5% (n = 3) apoptotic INS-1 cells (Fig. 1). We found that there was negligible change in the occurrence of INS-1 cell necrosis under these OA/BSA incubation conditions, as judged by the HO/PI staining assay (Fig. 1), and also as assessed by annexin V-FITC analysis (data not shown), in agreement with previous studies (7). In a limited number of experiments, it was also found that palmitate could also induce INS-1 cell apoptosis similarly to that by oleate (data not shown), as previously observed (7).

Fatty-acid-Reduced PKB Phosphorylation Activation in Pancreatic β-Cells, INS-1—INS-1 cells ± OA as a model FFA (0.4 mM OA + 0.5% BSA, or 0.5% BSA), were incubated for 18 h in the presence of 5 or 15 mM glucose ± 10 ng/ml IGF-1 and then phosphorylation activation of PKB assessed by immunoblot analysis using PKB phosphospecific antibodies directed at phospho-Ser473 and phospho-Thr308. In the absence of FFA,
PKB phosphorylation was similarly stimulated in INS-1 cells by IGF-1, at either 5 or 15 mM glucose (Fig. 2), indicative of the glucose-independent aspect of IGF-1-induced PKB activation as previously reported (14). However, a slight increase in PKB phosphorylation activation was also observed in the absence of FFA in INS-1 cells chronically incubated for 18 h at a stimulatory 15 mM glucose compared with basal 5 mM glucose (Fig. 2). However, in INS-1 cells incubated with FFA, this modest 15 mM glucose-induced increase in PKB phosphorylation was not apparent (Fig. 2). Moreover, IGF-1-induced PKB phosphorylation at both 5 and 15 mM glucose was significantly reduced in the presence of FFA (Fig. 2). Immunoblot analysis of total PKB indicated equivalent levels of PKB were present in INS-1 cells under the various incubation conditions (Fig. 2). These observations of specific FFA-induced decrease in IGF-1-induced PKB phosphorylation correlate with our previous observations of FFA-induced decrease in PKB activity (5).

Adenoviral Mediated Expression of Constitutively Active PKB Prevented FFA-induced INS-1 Cell Apoptosis—Having ascertained that FFA can decrease the activation of PKB in association with FFA-induced β-cell apoptosis, we investigated the effect of expressing a constitutively active form of PKB on INS-1 cell apoptosis. INS-1 cells were infected with adenoviral vectors of Myc/His-tagged PKB, consisting of a wild type (AdV-PKB-WT), a constitutively active (AdV-PKB-CA), and a kinase-dead form (AdV-PKB-KD) as previously described (14). A GFP-expressing adenovirus (AdV-GFP) was used as a control (21). INS-1 cells were infected with equivalent levels (0.5–2.0 × 10⁶ plaque-forming units/ml) of AdV-PKB-WT, AdV-PKB-CA, and AdV-PKB-KD or the control AdV-GFP (14), and the rates of apoptosis were determined after 18 h treatment with 5 or 15 mM glucose ± 10 ng/ml IGF-1 with either control 0.5% BSA or 0.4 mM OA + 0.5% BSA for 18 h (see “Experimental Procedures”). The cell lysates were subjected to immunoblot analysis (as described under “Experimental Procedures”) using phospho-Ser⁴⁷³ PKB, phospho-Thr³⁰⁸ PKB, and total PKB antibodies. The results shown are representative of three independent experiments.

In AdV-PKB-KD-infected INS-1 cells, there was negligible difference in FFA-induced apoptosis compared with that in AdV-GFP-infected control INS cells, with comparable rates of apoptosis at the various incubation conditions of 5 or 15 mM glucose ± IGF-1 (Fig. 3). However, in AdV-PKB-CA-infected INS-1 cells, FFA-induced apoptosis was generally reduced compared with that in AdV-GFP-infected control cells. At 5 mM glucose, the incidence of apoptosis in AdV-PKB-CA-infected INS-1 cells was 12.5 ± 2.1% (n = 4), which was 8-fold higher than that in the absence of FFA under the same conditions (p ≤ 0.05; Fig. 3). The addition of IGF-1 at 15 mM glucose additionally reduced the degree of INS-1 apoptosis to 5.4 ± 1.8% (n = 4), yet this was 3-fold higher than that in the absence of FFA under the same conditions (p ≤ 0.05; Fig. 3).
treated AdV-GFP-infected control cells ($p \leq 0.05; n = 4$), and not significantly different from the baseline incidence of apoptosis in FFA-untreated AdV-GFP-infected control cells (Fig. 3). Likewise, in the added presence of IGF-1 at 15 mM glucose, FFA-induced apoptosis was not significantly different in AdV-PKB-CA-infected INS-1 cells compared with the basal rate of apoptosis in cells not exposed to FFA at 1.6 ± 0.7% ($n = 4$), which was a significant decrease compared with FFA-treated AdV-PKB-infected control cells ($p \leq 0.05$) (Fig. 3). As such, these data implicate an important role of PKB in maintaining β-cell survival.

**Inhibition of FFA-induced β-Cell Apoptosis by Glucose, IGF-1, and PKB Inversely Correlated with Caspase-9 and -3 Activation**—The caspase family of cysteine proteases play a key role in the execution of apoptotic cell death (26). As such, we examined whether the degree of caspase activation correlated with FFA-induced β-cell apoptosis, and protection from that by PKB activation. INS-1 cells infected with the Ad-PKB constructs or control AdV-GFP were incubated with or without 0.4 mM OA and 0.5% BSA at 5 or 15 mM glucose ± IGF-1 for 18 h, as indicated (Fig. 4). The activation of the initiator caspase-9 and the effector caspase-3 were measured by immunoblot analysis with specific antibodies that only recognize the active, cleaved forms of these proteins in parallel with immunoblot analysis of PKB phosphorylation activation using the PKB phosphospecific antibodies. For INS-1 cells infected with the various Ad-PKB constructs, the “infected PKB” could be distinguished from the endogenous PKB on the immunoblot analyses as a result of the addition of a Myc-His tag that increased the apparent $M_r$ (Fig. 4A) as previously observed (14). In FFA-treated AdV-GFP-infected control cells as well as AdV-PKB-WT-, AdV-PKB-CA-, and AdV-PKB-KD-infected INS-1 cells, the 15 mM glucose- and IGF-1-induced Ser473 and Thr308 phosphorylation of the endogenous PKB was inhibited compared with AdV-GFP-infected control cells not incubated with FFA (Fig. 4A), as demonstrated elsewhere (Fig. 2). Nonetheless, despite the presence of FFA, in AdV-PKB-WT-infected INS-1 cells there was an IGF-1, and 15 mM glucose, stimulation of both Ser473 and Thr308 phosphorylation of the “infected PKB-WT” (Fig. 4A) that correlated with the modest decrease in FFA-induced apoptosis in AdV-PKB-WT-infected INS-1 cells over the 18-h incubation period (Fig. 3). As predicted, the PKB Ser473 and Thr308 phosphorylation activation state of the “infected PKB-CA” in AdV-PKB-CA-infected INS-1 cells was markedly increased to an equivalent extent regardless of the glucose/IGF-1 incubation conditions (Fig. 4A), consistent with the constitutive activation of this PKB variant and correlative with the prevention of FFA-induced apoptosis in AdV-PKB-CA-infected INS-1 cells (Fig. 3). As previously observed (14), glucose/IGF-1-induced PKB Thr308 phosphorylation of the “infected PKB-KD” in AdV-PKB-KD-infected INS-1 cells was equivalent to that of the “infected PKB-WT” in AdV-PKB-WT-infected INS-1 cells, but that of PKB Ser473 phosphorylation was very much reduced (Fig. 4A). This is consistent with the idea that Ser473 phosphorylation is an autophosphorylation by PKB and that infected PKB-KD lacks PKB activity (14, 16). Moreover, this observation correlated with a lack of protection from FFA-induced apoptosis in AdV-PKB-KD-infected INS-1 cells (Fig. 3). The total levels of endogenous PKB and infected PKB did not appreciably change in these experiments, underlining the specific effects observed on PKB phosphorylation (Fig. 4A).

The final stages of apoptosis are executed by the family of caspases, including caspase-9 and the effector caspase-3 (26). Consistent with the low incidence of apoptosis in AdV-GFP-infected control cells not incubated with FFA (Fig. 3), negligible activation of caspase-9 or -3 was detected (Fig. 4B). In contrast, in FFA-treated AdV-GFP-infected INS-1 cells, significant activated caspase-9 and -3 could be detected at 5 mM glucose that was correlatively decreased upon addition of IGF-1 and/or increasing to a 15 mM glucose concentration (Fig. 4B). A similar pattern of FFA-induced caspase-9 and -3 activation was observed in AdV-PKB-WT- and AdV-PKB-KD-infected INS-1 cells (Fig. 4B). However, in AdV-PKB-CA-infected INS-1 cells, negligible activated caspase-9 and -3 could be detected at 5 mM glucose that was correlatively decreased upon addition of IGF-1 and/or increasing to a 15 mM glucose concentration (Fig. 4B). Hence, the pattern of caspase-9 and -3 activities observed in these studies (Fig. 4B) correlated with the degree of FFA-induced apoptosis (Fig. 3) and inversely correlated with the extent of PKB phosphorylation activation (Fig. 4A). These measurements of caspase-9 and -3 activation corroborate well with the other determinations of apoptosis using the HO/PI staining technique (Figs. 1 and 3), and annexin V-FITC analysis. This further suggested that most of the FFA-induced β-cell death observed was via an apoptotic, rather than a necrotic, mechanism.
Inhibition of GSK3

Phosphorylation of GSK3

Inhibition of FFA-induced 

PKB/Akt Prevents Fatty Acid-induced Apoptosis in β-Cells

PKB/Akt Prevents Fatty Acid-induced Apoptosis in INS-1 Cells

Experimental Procedures

The phosphorylation state of FoxO1 was analyzed by immunoblotting with a phosphospecific antibody that recognizes FoxO1 when phosphorylated at Ser256. In AdV-GFP-infected cells incubated without FFA, GSK3β phosphorylation was similarly decreased, as it was in AdV-PKB-KD-infected cells, this glucose/IGF-1-induced FoxO1 phosphorylation was clearly decreased (Fig. 5B). Likewise, in FFA-treated AdV-PKB-KD-infected INS-1 cells, the glucose/IGF-1-induced FoxO1 phosphorylation was similarly decreased, as it was in FFA-treated AdV-PKB-WT-infected INS-1 cells, albeit to a lesser extent, particularly at 15 mM glucose + IGF-1 (Fig. 5A). In contrast, in FFA-treated AdV-PKB-CA-infected cells, the levels of FoxO1 phosphorylation were comparable with those for untreated AdV-GFP-infected control cells, especially under stimulated conditions at 15 mM glucose ± IGF-1 (Fig. 5A). Immunoblot analysis of the total FoxO1 indicated equivalent FoxO1 levels under all the conditions (Fig. 5A). It should be noted that several FoxO1 bands were observed on the immunoblot analysis, likely reflective of the phosphorylation state of FoxO1 at multiple Ser/Thr residues (27), a notion substantiated in resolving fewer FoxO1 bands after treating INS-1 cell lysates with alkaline phosphatase prior to immunoblot analysis (data not shown; Ref. 28).

Phosphorylation of GSK3αβ by PKB occurs at residues Ser9 and Ser21 on GSK3α and GSK3β, respectively (29). To assess the level of GSK3αβ phosphorylation, immunoblot analysis was performed using a phospho-GSK3αβ (Ser21/9) antibody, which only recognizes GSK3α when it is phosphorylated at residue Ser21 or GSK3β when phosphorylated at Ser9. In AdV-GFP-infected cells incubated without FFA, GSK3αβ phosphorylation inhibition was relatively low at basal 5 mM glucose, but increased by either addition of IGF-1 or increasing to a 15 mM glucose concentration for the 18-h incubation period (Fig. 4B). In contrast, in FFA-treated AdV-GFP-infected cells, glucose/IGF-1-stimulated GSK3αβ phosphorylation was decreased (Fig. 5B). A similar reduction in the phosphorylation of GSK3αβ in the presence of FFA occurred in the AdV-PKB-KD-infected cells (Fig. 5B). In AdV-PKB-WT-infected INS-1 cells, a glucose/IGF-1-induced GSK3αβ phosphorylation was observed comparable with that in AdV-GFP-infected control cells incubated in the absence of FFA (Fig. 5B). A marked increase of GSK3αβ phosphorylation was observed in AdV-PKB-CA-infected cells, which did not correlate with glucose/IGF-1 incubation conditions, but rather the constitutive activation of PKB in these β-cells (Fig. 5B). In this regard, the phosphorylation state of GSK3αβ observed was generally consistent with the PKB phosphorylated activation state at residue PKB Ser473 as previously found (Fig. 4A). Immunoblot analysis indicated that total levels of GSK3αβ did not appreciably vary (Fig. 5B).

Effects of Adenoviral Mediated Expression of p53 Wild Type and a p53 Variant on FFA-induced Apoptosis in INS-1 Cells—

One potential mechanism through which PKB may promote β-cell survival is by inhibition of the tumor suppressor protein, p53 (which has been shown to play a role in the induction of apoptosis; Ref. 30) via PKB-mediated phosphorylation of MDM2 (17). To examine the role of p53 in FFA-induced β-cell apoptosis, we generated adenoviruses of wild type p53 (AdV-p53-WT) and mutant p53 (AdV-p53-MT). The p53-MT carries a G to A mutation at nucleotide 1017 (Lys135 to Tyr), which not only leads to a DNA-binding deficient conformational change in mutant p53, but may also interact with and inhibit the wild type p53 (31, 32). The titer of the adenoviruses was examined by infecting INS-1 cells with 0–25 × 10⁴ m.o.i. and subsequent p53 immunoblot analysis of AdV-p53-WT- and AdV-p53-MT-infected cells (Fig. 6A).

The effect of p53-WT and p53-MT expression on FFA-induced apoptosis was examined. INS-1 cells were infected with...
15 × 10^2 m.o.i. of AdV-p53-WT and AdV-p53-MT, as well as AdV-GFP and AdV-PKB-CA as negative and positive controls, respectively, and treated with or without 0.4 mM OA plus 0.5% BSA at basal 5 mM glucose ± 10 ng/ml IGF-1 and 0.4 mM OA + 0.5% BSA for 18 h. As a control, AdV-GFP-infected cells were treated under the same glucose and IGF-1 conditions, but with 0.5% BSA only. Cell lysates were subjected to immunoblot analysis (as described under “Experimental Procedures”) using phospho-GSK3β and total GSK3β antibodies (A) and phospho-FoxO1 and total FoxO1 antibodies (B). The results shown are representative of three independent experiments.

Fig. 5. FFA-induced inhibition of glucose/IGF-1 stimulated GSK3 and FoxO1 phosphorylation in INS-1 cells is rescued by expression of constitutively active PKB. INS-1 cells were cultured on 10-cm plates and infected with AdV-GFP, AdV-PKB-WT, AdV-PKB-CA, or AdV-PKB-KD at an m.o.i. of 10 × 10^2 (as described under “Experimental Procedures”). The infected cells were treated with 5 or 15 mM glucose ± 10 ng/ml IGF-1 and 0.4 mM OA + 0.5% BSA for 18 h. As a control, AdV-GFP-infected cells were treated under the same glucose and IGF-1 conditions, but with 0.5% BSA only. Cell lysates were subjected to immunoblot analysis (as described under “Experimental Procedures”) using phospho-GSK3β and total GSK3β antibodies (A) and phospho-FoxO1 and total FoxO1 antibodies (B). The results shown are representative of three independent experiments.

Fig. 6. Effects of adenoviral mediated expression of p53 wild type and a p53 variant on FFA-induced apoptosis in INS-1 cells. INS-1 cells were infected with AdV-p53-WT and AdV-p53-MT using an m.o.i. from 2 to 25 × 10^2 (as described under “Experimental Procedures”), and an uninfected control was included. After the 16-h incubation in complete medium, cell lysates were subjected to immunoblot (IB) analysis as described under “Experimental Procedures” using the total p53 antibody (A). INS-1 cells were cultured on 10-cm plates and infected with AdV-GFP, AdV-PKB-CA, AdV-p53-WT, or AdV-p53-MT at an m.o.i. of 10 × 10^2 (as described under “Experimental Procedures”). The infected cells were treated with 5 mM glucose and 0.5% BSA or 0.4 mM OA + 0.5% BSA for 18 h. The percentage of apoptotic cells was measured as described under “Experimental Procedures.” The results shown are representative of four to six independent experiments.
further increased 35-fold to 50.0 ± 3.9% of the INS-1 cell population compared with the AdV-GFP-infected control cells in the absence of FFA (p = 0.01; n = 4) (Fig. 6B). In contrast, FFA-induced apoptosis was reduced in AdV-p53-MT-infected INS-1 cells to an incidence of 15.6 ± 3.5% (n = 4; Fig. 6B), significantly decreased in comparison to FFA-treated AdV-p53-WT-infected cells (p = 0.005), and AdV-GFP-infected INS-1 cells (p = 0.02). However, FFA-induced apoptosis in AdV-p53-MT-infected INS-1 cells was still a significant 11-fold higher (p = 0.05) compared with AdV-GFP-infected control cells incubated in the absence of FFA (Fig. 6B). Nonetheless, these results indicated that, at least in part, FFA-induced β-cell apoptosis might involve regulation of p53, perhaps mediated downstream of PKB activation (17).

DISCUSSION

In this study we have shown that prolonged exposure to the FFA, oleate, induced β-cell apoptosis in INS-1 cells, as did palmitate (data not shown), similar to the FFA-induced apoptosis previously observed in islet β-cells (7). It has been proposed that FFA-induced β-cell apoptosis might occur, in part, via intracellular production of ceramide from palmitate (6). However, because, unlike palmitate, oleate is not a significant source for de novo synthesis of ceramide (33, 34), the oleate-induced β-cell apoptosis observed in this study was most likely ceramide-independent. Intriguingly, we also found that methyl oleate did not induce β-cell apoptosis. This verifies that the oleate-induced β-cell death we observed was mediated via a programmed apoptotic mechanism, rather than a necrotic one caused by a nonspecific detergent effect of the FFA. In addition, because methylated FFA cannot undergo esterification to fatty acyl-CoA (35), these data indicate that oleate-induced apoptosis was mediated by a prerequisite for oleoyl-CoA formation, as previously indicated in studies where inhibition of fatty-acyl CoA synthetase reduced FFA-induced apoptosis (6).

Increasing the glucose concentration from a basal 5 mM glucose, and/or addition of IGF-1, tended to decrease the degree of FFA-induced β-cell apoptosis (Fig. 3), suggesting a requirement for activation of signaling pathways to promote β-cell survival. In this regard, we found that glucose/IGF-1-mediated phosphorylation activation of the anti-apoptotic signaling protein, PKB, was reduced in the presence of FFA, correlating with previous findings of FFA-induced inhibition of PKB activity in β-cells (5). This is consistent with a number of reports in other cell types, which have shown that FFA and ceramide inhibit insulin-induced PKB activation (36, 37). Currently, the mechanism of FFA-induced inhibition of PKB activation in β-cells is unclear; however, several possibilities have been proposed in other cell types. For example, inhibition of PKB by FFA and/or ceramide does not appear to be caused by the down-regulation of signaling components upstream of PKB (5), such as PI3K or PDK1, but instead is thought to prevent PKB translocation to the plasma membrane (38, 39). Alternatively, it has been proposed that PKB dephosphorylation may be increased by FFA-induced activation of certain protein phosphatases, such as protein phosphatase 2A, in turn reducing PKB activity (40, 41). There are also reports that suggest there can be a FFA-induced activation of certain PKC isoforms that increase Ser/Thr phosphorylation of IRS-1/2, dampening downstream IRS signal transduction, including decreased PI3K/PKB activation (42–44). For the moment, it remains to be shown whether any of these potential mechanisms might be pertinent to FFA-induced inhibition of PKB in β-cells.

Notwithstanding, insufficient PKB activation can induce β-cell apoptosis, especially in the light of our findings in this study, which show activation of PKB plays a key role in promoting pancreatic β-cell survival. Adenoviral mediated gene transfer of a constitutively active PKB variant into β-cells almost completely prevented FFA-induced apoptosis especially in the presence of IGF-1. These findings are complementary to that of transgenic expression of a constitutively active PKB-α (also known as Akt-1) specifically in mouse pancreatic β-cells (myr-Akt1 mice), where there was increased β-cell mass attributable to increased β-cell survival and increased size of β-cells, but not up-regulation of β-cell proliferation where the actual number of β-cells per islet was decreased (13). We have previously shown that increased expression of constitutively active or wild type PKB in β-cells has little effect on glucose/IGF-1-induced β-cell proliferation (14), and because in this study we have used the INS-1 cell line, PKB-mediated changes in β-cell neogenesis are irrelevant. As such, our findings emphasize the importance of PKB activation in promoting β-cell survival.

Remarkably, the β-cell specific myr-Akt1 mice are resistant to low dose streptozotocin-induced diabetes (13). Here we show that a marked increase in PKB activity in β-cells is protective against a physiologically relevant mediator of β-cell death, FFA. In the pathogenesis of obesity-linked type 2 diabetes, chronic exposure to FFA has been proposed to be a key factor in promoting reduced β-cell mass, so that peripheral insulin resistance can no longer be compensated for and, together with β-cell dysfunction, the disease is acquired (3, 4). Although we believe our findings to be informative, for the moment it is premature to consider that PKB protection of the β-cell from FFA-induced apoptosis has therapeutic possibilities in protecting β-cell mass delaying the onset of obesity-linked type 2 diabetes (13, 45). We recognize the limitation of this study in using the INS-1 cell line, and it will be important, first of all, to examine whether these observations are reflected in the in vivo setting in animal models of obesity-linked type 2 diabetes. Moreover, because PKB has a plethora of protein substrates, it will also be important to establish which are the appropriate substrates downstream of PKB involved in promoting β-cell survival.

In this regard, we examined several downstream PKB targets to determine whether their regulation, as influenced by PKB, could be associated with the degree of FFA-induced β-cell apoptosis. Phosphorylation of two known pro-apoptotic PKB targets, GSK3α/β and FoxO1, leads to their inactivation (12). Here we found that increased phosphorylation state of GSK3α/β and FoxO1 correlated well with increased PKB phosphorylation activation (especially in cells expressing the constitutively active PKB), and this in turn inversely correlated with the extent of FFA-induced apoptosis. Hence, PKB-mediated phosphorylation inactivation of GSK3α/β and FoxO1 likely promotes β-cell survival. Inhibition of GSK3α/β has indeed previously been implicated to play a role in the protection of cells from apoptosis (12); however, little is known in regard to GSK3 activity in β-cells, and it will be important to elucidate the pro-apoptotic protein phosphorylation substrates of GSK3 in future studies. FoxO1 is one of a family of forkhead transcription factors, suggested to be involved in the expression of a number of pro-apoptotic genes, as well as the transcriptional activation of other genes (27). PKB phosphorylation of FoxO1 prevents its translocation to the nucleus, and it is retained in the cytoplasm (via interaction with 14.3.3 proteins), thus decreasing FoxO1 transcriptional activity in driving expression of pro-apoptotic factors such as p27, a cyclin inhibitor, and Fas ligand (27). However, as with GSK3 substrates, the potential
pro-apoptotic genes regulated by FoxO1 in the β-cells need to be better determined. In addition to GSK3 and FoxO1, MDM2, an ubiquitin-protein isopeptide ligase, has also been identified as a substrate for PKB (17). MDM2 is involved in the regulation of the tumor suppressor protein, p53, which is a transcription factor known to induce apoptosis (30). Following phosphorylation by PKB, MDM2 translocates to the nucleus, whereupon it interacts with p53, suppressing the activity and promoting p53 degradation, which in turn further reflects the anti-apoptotic action of PKB activation (17). We did not have the tools at hand to directly examine MDM2 phosphorylation by PKB; however, we determined whether p53 might be involved in FFA-induced β-cell apoptosis. It was found that adenosin-mediated increase in wild-type p53 expression increased the incidence of β-cell apoptosis in the presence or absence of FFA, suggesting it plays a role, at least in part, in the general mechanism of β-cell apoptosis. In contrast, adenosin-mediated expression of a negative variant of p53, which is deficient in DNA binding (31, 32), gave a partial protection of FFA-induced apoptosis. These data implicate a contributing role for p53 in the mechanism of FFA-induced β-cell apoptosis, perhaps because of reduced p53 down-regulation as a consequence of FFA-induced inhibition of PKB activation. However, because protection from FFA-induced β-cell apoptosis in Adv-p53-MT-infected cells was only partial, and to a lesser extent than that of expressing constitutively active PKB, clearly phosphorylation inhibition of other factors downstream of PKB, including GSK3 and FoxO1, will play contributing roles in promoting β-cell survival.

In conclusion, chronic exposure of FFA can promote β-cell apoptosis, at least in part by dampening PKB activation. This may have implications for the development of an inadequate β-cell mass that no longer compensates for peripheral insulin resistance in the pathogenesis of obesity-linked type 2 diabetes (3, 4). However, it will be important to first substantiate that our findings in this study can be replicated in an in vivo setting. Nonetheless, it is interesting to note that FFA has been implicated to interfere with insulin signal transduction in muscle, including downstream inhibition of PI3K and PKB, which contribute to an insulin-resistant state (46). As such, there might well be some intriguing parallels between FFA-induced inhibition of IRS-mediated signal transduction pathways that causes insulin resistance, as well as promoting apoptosis of pancreatic β-cells.

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