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Akt activation protects pancreatic beta cells from AMPK-mediated death through stimulation of mTOR

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ABSTRACT

Sustained activation of AMP-activated protein kinase (AMPK) induces apoptosis in several cell types. In pancreatic beta cells this occurs under glucose limitation, or in the presence of the pharmacological AMPK activator 5-aminoimidazole-4-carboxamide-riboside (AICAR). It is unknown whether Akt activation can counteract AMPK-mediated apoptosis, nor whether mTOR activation downstream of Akt mediates any survival signal in these conditions. We report that expression of a constitutively active form of Akt increases mTOR activity and prevents apoptosis upon AMPK activation. Akt-mediated survival was inhibited by rapamycin. Expression of a constitutively active form of the mTOR target ribosomal protein S6 kinase (S6K) or of translation factor eIF4E reduced apoptosis by glucose limitation, and co-expression of S6K and eIF4E protected beta cells to the same extent as active Akt. The protective effects of active Akt and S6K were associated with increased cellular protein synthesis activity. It is concluded that Akt stimulation of mTOR and subsequent activation of the targets by which mTOR affects protein translation are required and sufficient mechanisms for Akt-mediated survival of beta cells undergoing sustained AMPK activation.

1. Introduction

AMP-activated protein kinase (AMPK) is a metabolic regulator which balances net energy production with the prevailing metabolic demands in eukaryotic cells. AMPK is activated by an increase of the intracellular AMP:ATP ratio in different stress conditions, e.g. glucose deprivation, hypoxia, ischaemia or muscle contraction. Once activated, the kinase inhibits energy consuming biosynthetic pathways such as fatty acid-, cholesterol-, and protein biosynthesis, while activating a number of ATP generating catabolic pathways including fatty acid oxidation [1]. AMPK plays a role in whole-body glucose homeostasis; its actions mimic the effects of insulin on glucose transport in muscle and glucose production by the liver [2]. This has suggested AMPK as a therapeutic target for treatment of hyperglycemia in forms of diabetes that are associated with insulin resistance [3]. The anti-hyperglycemic effect exerted by the longstanding type 2 diabetic drug metformin is indeed mainly ascribable to AMPK activation in the liver [4]. On the other hand, in pancreatic beta cells,

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Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide-riboside; AMPK, AMP-activated protein kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin; Ad, adenovirus; GFP, green fluorescent protein.

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AMPK activation inhibits insulin release [5]. In these cells, glucose limitation, the AMPK activators 5-aminoimidazole-4-carboxamide-riboside (AICAR) and metformin, and ectopic expression of constitutively active AMPK, have been shown to result in inhibition of glucose oxidation, depletion of $[ATP]_i$, decreased insulin gene expression, and diminished protein synthesis [2,6–9]. AMPK activation in these conditions appears responsible for these effects and for subsequent induction of apoptosis in the beta cell [6,8–11]. AMPK activation in the beta cell is likely a common event in diabetes and its treatment. AMPK activation was recently shown to be induced by hyperglycemic stress in beta cells [12], a condition which prevails in diabetes and results in beta cell dysfunction. Treatment of hyperglycemia in type 2 diabetes, currently based on metformin administration, involves a systemic induction of AMPK activity. When hyperglycemic and pharmacological AMPK activation are combined, the hyperglycemia-induced toxicity in beta cells may be potentiated [12]. It is unclear how beta cells avoid these detrimental effects of AMPK activation in vivo. Molecules that suppress pro-apoptotic steps of AMPK signaling could be useful for therapeutic intervention. In other cell types, active AMPK was shown to phosphorylate TSC2, which in turn inhibits mTOR [13] and affects two families of characterized mTOR substrates through which mTOR stimulates translation: the 70 kDa ribosomal protein S6 kinases (S6K1–2) which phosphorylate the S6 protein of small ribosomal subunits (S6), and the eIF4E-binding proteins (4EBP1–3) which bind to the mRNA cap-binding protein eIF4E [13,14]. Thus, mTOR inhibition through the AMPK/TSC2 pathway is consistent with AMPK's action to slow down protein synthesis. Branched chain amino acids and IGF-1 activate mTOR and stimulate protein translation in beta cells [15]. We showed recently that leucine and IGF-1 increase beta cell survival in conditions of glucose limitation [16]. This suggested that increasing the mTOR activity might inhibit beta cell death in AMPK-stimulating condition. There is evidence that Akt (protein kinase B) activity is crucial to function, survival, and adaptive growth of beta cells, and glucose and IGF-1-induced beta cell survival are mediated by Akt signaling [17]. This Akt action is generally ascribed to increased glucose metabolism and/or post-translational inhibition of pro-apoptotic proteins [17,18]. However, Akt is also known to stimulate mTOR via inhibition of TSC2, directly opposing the effect of AMPK activation on mTOR [13,18]. In this study, we investigated whether inducing mTOR activity or mimicking mTOR activity could suppress AMPK-mediated beta cell death. We found that Akt/mTOR signaling constitutes a survival pathway for stressed beta cells undergoing AMPK activation.

2. Materials and methods

2.1. Cell culture

Rat pancreatic beta cells were isolated by flow cytometry [19] and cultured overnight in suspension in 6-well plates (2×10^5 cells/well, Becton Dickinson, Lincoln Park, NJ) or as adherent cells in 96-well plates (5×10^3 cells/well) in Ham's F10 medium (Invitrogen, Life Technologies Inc., Paisley, Scotland) containing glucose (10 mM), 1% (w/v) charcoal-treated BSA (Sigma–Aldrich, St. Louis, MO), L-glutamine (2 mM), penicillin (0.075 mg/ml), and streptomycin (0.1 mg/ml), as previously described [20]. Subsequently, beta cells were incubated in identical medium containing 10 mM or 3 mM glucose, or 10 mM glucose and the AMPK activator 5-aminoimidazole-4-carboxamide-riboside (AICAR, 0.5–1 mM, Sigma–Aldrich), for the indicated time periods. In some cases, IGF-1 (100 ng/ml, a gift from Dr. R. Kooiman, VUB, Brussels) or rapamycin (25 nM, Cell Signaling Technology, Beverly, MA) was added before collecting the cells. Insulin producing INS-1 cells [21] of passages 30–35 were cultured in 24-well plates (3×10^5 cells/well) in RPMI 1640 medium (Sigma–Aldrich) containing 11.2 mM glucose and supplemented with 2 mM glutamine, 50 μM β-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, and 10% (v/v) fetal bovine serum (Invitrogen Life Science), penicillin (0.075 mg/ml), and streptomycin (0.1 mg/ml). AMPK was activated in INS-1 cells by transfer to medium without glucose, or by adding AICAR [5]. In studying HIF1a protein expression, $CoCl_2$ (200 mM, Sigma–Aldrich) was added to the culture medium, or cultures were transferred from 95% air/5% CO_2 to 95% N_2/5% CO_2 in an atmospheric chamber.

2.2. Adenovirus infection

Primary rat beta cells or INS-1 cells were cultured in 96-well plates (5×10^3 beta cells/well), 24-well plates (3×10^5 INS-1 cells/well) or 6-well plates (2×10^5 beta cells/well) for 24 h at 37 °C in 5% CO_2. Cells were then infected with adenovirus for 3–4 h at the requisite multiplicities of infection (MOI 0–30). Unbound adenovirus was removed by replacing the culture medium. Following infection, the cells were further incubated for 24 h, after which they were exposed in culture to the described conditions or agents. At the end of this period, cell viabilities were measured or the cells were collected for protein studies. Beta cells in suspension were directly collected, while adherent INS-1 cells were first detached using calcium- and magnesium-free Hank's balanced salt solution, containing (in g/l): 0.4 KCl, 0.06 KH_2PO_4, 8 NaCl, 0.35 $NaHCO_3$, 0.06 $Na_2HPO_4 \cdot 2H_2O$, 1 glucose, 0.2 EDTA, and 0.5 trypsin. Cells were washed in PBS, centrifuged (Allegra 6R, Beckman) at 1400 rpm for 3 min at room temperature, and the pellets stored at −20 °C.

The following adenoviruses were used in this study: $AdAMPK^{CA}$ expressing GFP and, from an independent promoter, a Myc-tagged protein acting as a constitutively active kinase, comprising residues 1–312 of AMPK-subunit α1 containing mutation T172D [22,23]; AdS6K1 and AdS6K2 encoding Myc-tagged wild-type S6-kinases p70S6K1 and p70S6K2 [24,25]; $AdS6K1^{RR}$ encoding Myc-tagged rapamycin-resistant S6 kinase p70S6K1 ($S6K1^{RR}$) in which Thr 389 and Thr 421 had been mutated to Glu residues and Ser 411, Ser 418 and Ser 424 had been mutated to Asp residues [26–28]; $AdAkt^{myr}$ encoding haemagglutinin (HA)-tagged MyrAkt1 (Akt^{myr}) which is a constitutively active Akt1 molecule with a Src myristoylation sequence at its N terminus [29]; Adβ-gal encoding bacterial beta-galactosidase [29]; AdGFP encoding green fluorescent protein GFP [30]; AdeIF4E encoding HA-tagged wild-type eIF4E protein; $AdeIF4E^{209A}$ encoding an HA-tagged

mutant form of eIF4E bearing a mutation (S209A) which blocks eIF4E phosphorylation by upstream kinase Mnk-1 [31,32]. Eukaryotic expression plasmids (gift of Dr. P. Serup, Hagedorn, DK) for HA-tagged wild-type Akt and for the HA-tagged mutant Akt (K179M) in which the ATP-binding site was removed, had been described [33]. The kinase-dead Akt (K179M) mutant works as a dominant negative form of Akt (Akt^{DN}) in various cell types [34]; it was tested for blocking GF-induced GSK-3β phosphorylation in beta cells (see Section 3). Coding sequences of HA-tagged Akt^{WT} or HA-tagged Akt^{DN} were subcloned in the shuttle vector and constitutively expressed under control of the CMV promotor. pAdTrack-CMV also contained the eGFP cDNA downstream of a separate CMV promotor. Adenoviruses expressing Akt^{WT} in combination with GFP ($AdAkt^{WT}$), or Akt^{DN} in combination with GFP ($AdAkt^{DN}$) were generated following the standard procedure as described by He et al. [35]. All adenoviruses were propagated and purified as previously described [30].

2.3. Antibodies, immunological reagents, and immunofluorescent detection of proteins

Anti-Myc monoclonal antibody (clone 9E10, Gentaur, Brussels, Belgium) was used to detect Myc-tagged recombinant proteins $AMPK^{CA}$, $S6K1^{WT}$, $S6K2^{WT}$, and $S6K1^{RR}$, and anti-HA-tag polyclonal antibody (Clontech, Takara Bio company, USA) was used to detect the HA-tagged recombinant proteins Akt^{myr}, $eIF4E^{WT}$, and $eIF4E^{209A}$ in immunoblotting or immunocytochemistry. For immunofluorescent detection of proteins in cells, primary rat beta cells (10^4) were cultured on laminin-coated cover slips (Sigma–Aldrich) in 24-well plates and infected with adenovirus. After a 24-h culture period the cells were analyzed. For detection of HA-tagged proteins, cells were fixed in a solution of 4% (w/v) paraformaldehyde in PBS and permeabilized with PBS containing 0.2% (v/v) Triton X-100 for 10 min. For detection of Myc-tagged proteins, cells were fixed in methanol (−20 °C for 10 min) and then in acetone (−20 °C for 30 s). Secondary antibody detection was performed with a Alexa Fluor 555 goat anti-rabbit or Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes Inc., Eugene, OR). After staining with Hoechst 33258 the cover slips were mounted in Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA), and viewed on the fluorescence microscope.

All antibodies recognizing phospho-proteins were from Cell Signaling Technology (Beverly, MA). Specific antibodies recognizing the following proteins and epitopes were used in immunoblotting: phospho-(Thr 183/Tyr 185)-c-jun-N-terminal kinase (JNK), Phospho-(Thr 172)-AMPK, Phospho-Akt (Ser 473), total Akt (Cell Signaling Technology), Phospho-GSK3-α/β (Ser21/9), 4E-BP1 (Santa Cruz Biotechnology, Santa Cruz, CA), eIF4E (BD Bioscience Pharmingen, Erembodegem, Belgium), Phospho-p70S6 kinase (Thr 389), total p70S6 kinase (Cell signaling Technology), Phospho-S6 (Ser 235/236), HIF1α (BD Bioscience), Bcl-2 (N-19) and β-actin (Santa Cruz Biotechnology). Anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase were obtained from Amersham Bioscience (Bucks, UK), and anti-goat secondary antibody was from Santa Cruz Biotechnology.

2.4. Fluorescence microscopy

Adherent single beta cells or monolayer INS-1 cells were viewed in the inverted microscope (Axiovert 135M, Carl Zeiss), by fluorescence (or in phase contrast). The nuclei of propidium idodide-permeant (dead) cells were visible by a red fluorescence in the fluorescence microscope (Achroplan 20× lens, Chroma filter set 41002a, λ_{ex} 535/50x, λ_{em} 610/75 nm). Living cells displayed a blue nucleus (Hoechst3342, Chroma filter set 41002a, λ_{ex} 350/50x, λ_{em} 460/50 nm).

Cells infected with adenovirus that expressed GFP were detected by a green fluorescence (λ_{ex} 480/30x, λ_{em} 535/40 nm). Images from the inverted microscope were recorded by a AxioCam digital camera (Carl Zeiss) linked to AxioVision software (Carl Zeiss). Alexa Fluor probes, used in immunocytochemistry, were viewed on a Axioplan (Carl Zeiss) fluorescence microscope. Alexa Fluor 488 was detected by green fluorescence (λ_{ex} 489 nm/20x, λ_{em} 506 nm) and Alexa Fluor 555 by red fluorescence (λ_{ex} 545/20x, λ_{em} 610/75 nm), and images recorded with the Sensys digital camera (Photometrics) linked to the SmartCapture VP program (Digital Scientific Software).

2.5. Cell viability measurement

Primary beta cells were cultured in 96-well plates, and the number of viable, necrotic, and apoptotic cells was counted by fluorescence microscopy (see above) using propidium iodide (PI, Sigma) and Hoechst 3342 (HO342, Sigma). HO342 freely passes the plasma membrane and readily enters cells with intact membranes as well as cells with damaged membranes and stains DNA blue, whereas PI, a highly polar dye which is impermeable to cells with preserved membranes, stains DNA red. Medium (100 μl) was removed from the microtiter cups and replaced by the same volume containing 20 μg/ml HO342 and 10 μg/ml PI. After 20 min of incubation at 37 °C, the cells were examined in an inverted fluorescence microscope with ultraviolet excitation at 340–380 nm. Viable or necrotic cells were identified by intact nuclei with, respectively, blue (HO342) or red (PI) fluorescence. Apoptotic cells were detected by karyorrhexic or pyknotic nuclei which exhibited either a blue (HO342) or red (PI) fluorescence depending on the apoptotic stage [36]. The percentage of living cells was then calculated. For each individual experiment, at least 400 cells per condition were counted in triplicate.

Apoptosis was also analyzed by FACS, using the PI-lysis method. Briefly, cells were detached from the plate in 1× PBS containing 0.5% BSA, 1 mM EDTA, collected and centrifuged. Cells were then fixed in 1% (w/v) paraformaldehyde on ice for 15 min, washed in PBS and stored for 24 h in 70% (v/v) ethanol at −20 °C, before resuspension in PBS, addition of PI, and FACS analysis of DNA content.

2.6. Western blotting

Frozen or freshly collected cells were lysed and proteins extracted by a 1-min sonication in RIPA-buffer supplemented with phosphatase and proteinase inhibitor cocktails, as described [37]. Nuclear proteins were separated from

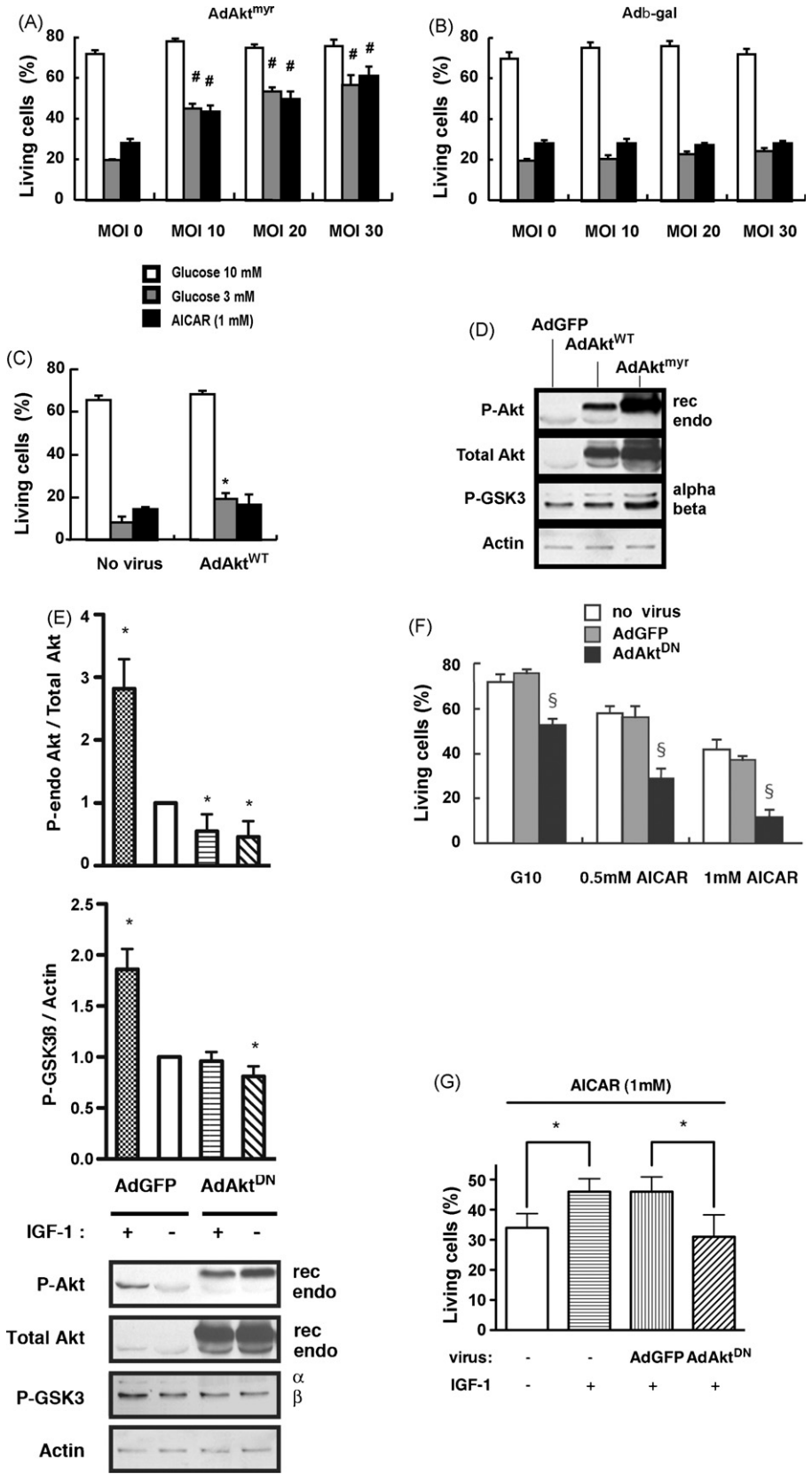

Fig. 1 – Effect of expression of wild-type and mutant forms of Akt on the survival of beta cells. Purified rat beta cells were cultured in 96-well plates and infected at the indicated MOI (from 0 to 30), or at MOI 30, with adenovirus AdAkt^{myr}, Adβ-gal, AdAkt^{WT}, AdAkt^{DN} and AdGFP expressing, respectively, constitutively active Akt, β-gal, wild-type Akt, dominant negative Akt, and GFP. Cells were then cultured for 3 days (A and B) or 5 days (C) in medium containing 10 mM glucose, 3 mM glucose or AICA-riboside (AICAR), as indicated. The viability (% living cells) was assessed by direct counting under the fluorescence

cytoplasmic proteins as described [37]. Protein concentration was determined by the micro-BCA assay (Pierce, Rockford, IL) using bovine serum albumin as the standard. Samples (25 μg protein) were mixed with SDS loading buffer and boiled, resolved by PAGE on 10 or 12% SDS-polyacrylamide gels, and transferred to a nitrocellulose membrane (Schleicher and Schuell Bioscience, Dassel, Germany). The membrane was incubated for 1 h at room temperature in 10 mM Tris–HCl (pH 7.4), 0.5 M NaCl, and 0.5% (v/v) Tween 20 (TBST) containing 5% (w/v) low-fat milk powder. Following overnight incubation at 4 °C with primary antibody diluted in TBST containing 5% milk powder, blots were washed extensively with TBST at room temperature. Blots were incubated for 1 h with the appropriate secondary antibody conjugated to horseradish peroxidase. After further washing with TBST, the blots were developed using enhanced chemiluminescence (Amersham Bioscience, Bucks, UK). Protein bands were subjected to densitometric analysis using open-source ImageJ software (v1.33u, NIH, USA).

2.7. Protein biosynthesis

The rate of total protein synthesis was measured as previously described [38]. After cells were infected with virus and precultured as indicated, they were collected and transferred to 5-ml Falcon tubes, washed in Ham's F10 without glucose, and incubated for 60 min in 200 μl Ham's F10 medium containing 0 mM or 10 mM glucose, 1% BSA, 50 μCi L-[3,5-^{3}H]tyrosine (specific activity 50 Ci/mmol (Amersham International, Buckinghamshire, UK). The total tyrosine concentration was 15 μM (5 μM [^{3}H] tyrosine and 10 μM unlabeled tyrosine), thus the specific activity of the tracer was 16.7 Ci/mmol. The labeling incubation was stopped by adding 0.8 ml cold Earle's Hepes containing 1 mM unlabeled tyrosine. After extensive washing, the cells were extracted in 1 ml of 2 M acetic acid/0.25% (w/v) BSA. Incorporation of [^{3}H]-tyrosine in total protein was determined by scintillation counting. The rate of protein synthesis was calculated from the radioactivity of incorporated tyrosine, and expressed in dpm per hour and per 1000 beta cells.

2.8. Statistical analysis of data

Quantitative data are presented as the mean ± S.E.M. of at least three independent experiments. Statistical analysis of data was done by Student's *t*-test, or by one-way ANOVA using Dunnett's test in multiple comparisons of means. Differences were considered statistically significant if the p value was <0.05.

3. Results

3.1. Ectopic expression of active Akt confers beta cell protection while dominant negative Akt increases beta cell death under conditions of AMPK stimulation

As previously shown, more primary beta cells died when exposed for 3 days to a low glucose concentration (3 mM), or to AICAR (1 mM), as compared to culture in 10 mM glucose (Fig. 1A,B; Supplementary Fig. 1). The increased cell death is mediated by stimulation of AMPK under these conditions [8,10]. Infection of primary beta cells with AdAkt^{myr}, expressing a constitutively active form of Akt bearing a short myristoylation sequence [25] markedly increased cell survival in low glucose or AICAR (Fig. 1A, Supplementary Fig. 1), while control adenovirus Adβ-gal expressing the β-gal protein had no effect on beta cell survival (Fig. 1B, Supplementary Fig. 1). Infection of the beta cells with adenovirus AdAkt^{WT} expressing wild-type Akt, only slightly improved their survival in 3 mM glucose (Fig. 1C), although cells were transduced to similar extent by AdAkt^{WT} and AdAkt^{myr} (Supplementary Fig. 2). The expression of Akt^{myr} resulted in higher levels of phosphorylated Akt and GSK3β and thus cellular Akt activity (Fig. 1D). These results indicated that enhancement of Akt activity can counteract the beta cell death caused by AMPK stimulation. We next examined whether endogenous Akt activity contributed to beta cell survival in culture. Beta cells infected with adenovirus AdAkt^{DN}, expressed increased levels of a dominant negative (DN) form of Akt, and showed decreased basal phosphorylation of endogenous Akt and GSK3β, as compared to cells infected with control adenovirus AdGFP (Fig. 1E). Expression of DN-Akt also precluded activation of endogenous Akt by IGF-1 (Fig. 1E). Upon infection of beta cells with AdAkt^{DN}, the cell survival rates in the 10 mM glucose condition, or in AICAR (0.5 and 1 mM) were decreased, as compared to beta cells infected with AdGFP (Fig. 1F, Supplementary Fig. 1), while these viruses had similar beta cell infection rates (Supplementary Fig. 2). In addition, IGF-1 inhibited the cell death induction by AICAR, and this protective effect was abolished by expression of DN-Akt (Fig. 1G). The data indicate that lowering the level of endogenous Akt activity decreases beta cell survival in basal or AMPK activating culture conditions, while ectopic activation of Akt prevents AMPK-induced beta cell death.

3.2. Akt protects beta cells through activation of mTOR

Sustained AMPK activation in beta cells leads to a reduced beta cell function [2,7–9,39,40] and to beta cell death [8–12]. We therefore examined the effects of AMPK activation on

microscope as described in Section 2. Pictures are shown in Supplementary Fig. 1. Data represent the mean ± S.E.M. of 4–5 independent experiments done in triplicate ($^*p < 0.05$, $^\#p < 0.001$, infected *vs.* uninfected cells in identical culture conditions). (D and E) Expression of recombinant proteins was studied by immunoblotting using 2×10^5 beta cells at 24 h following infection with the indicated viruses at MOI10. Total Akt, phospho-Akt (Thr^{308}), and phospho-GSK-3α/β (Ser$^{21/9}$) were detected using specific antibodies. IGF-1 (100 ng/ml) was used to stimulate endogenous Akt. Immunoblotting results are expressed as indicated (mean ± S.E.M., $n = 4$, $^*p < 0.05$ *vs.* AdGFP in control condition). (F) Effect of AdAkt^{DN} on beta cell survival over a 3-day period (see also Supplementary Fig. 1), tested in medium containing 10 mM glucose (G10) or in G10 containing AICAR (mean ± S.E.M., $n = 4$, $^\S p < 0.01$ AdAkt^{DN} *vs.* AdGFP). (G) Protective effect of IGF-1 on death induction by AICAR over a 4-day period was abolished by expression of dominant negative Akt (mean ± S.E.M., $n = 4$, $^*p < 0.05$).

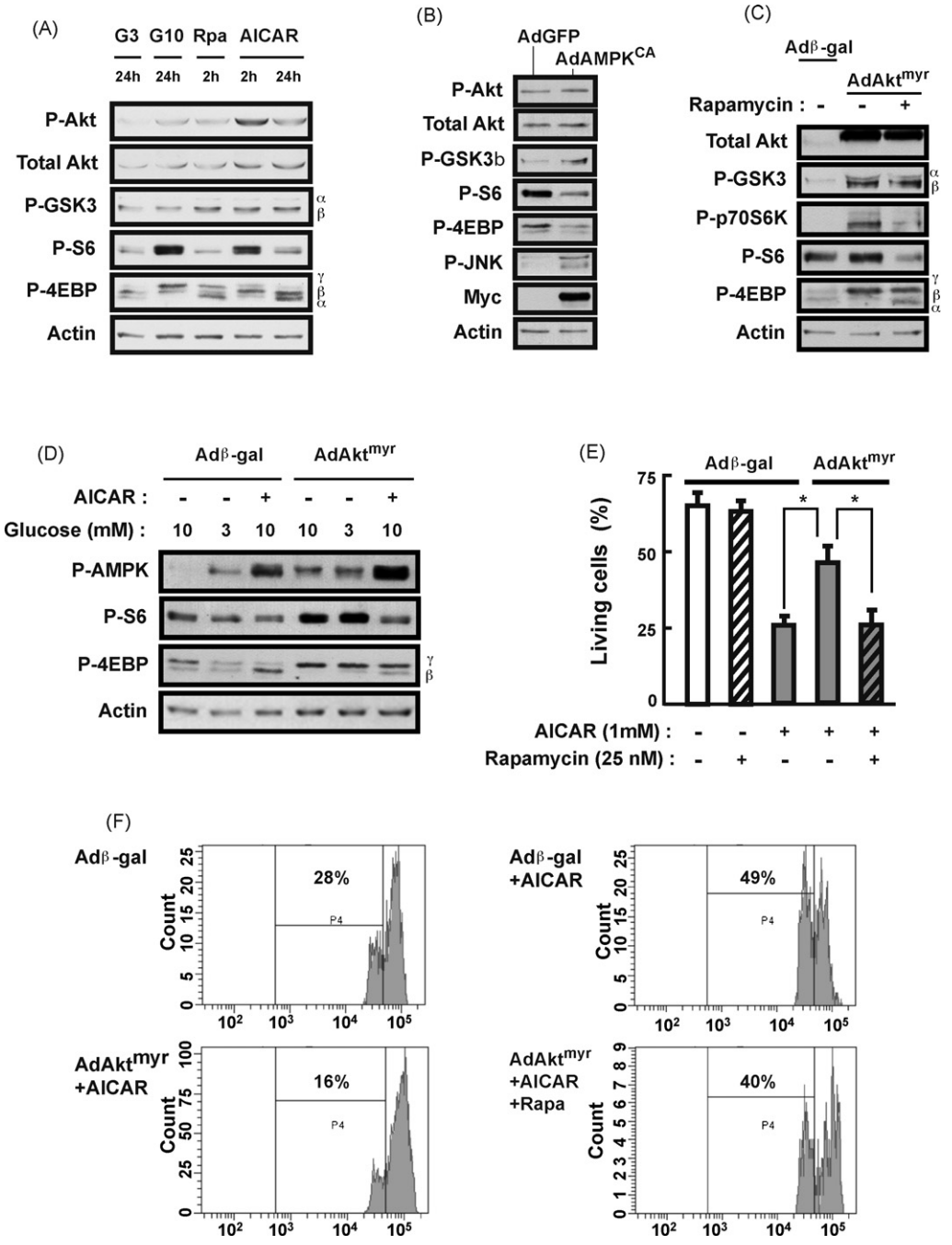

Fig. 2 – mTOR activity is modulated by AMPK and Akt and contributes to beta cell survival. Purified rat beta cells (2×10^5) were infected by adenovirus at MOI 30 as in Fig. 1, and 24 h later protein expression and phosphorylation was studied by immunoblotting using specific antibodies against total Akt, phospho-Akt (Thr^{308}), phospho-GSK3α/β ($Ser^{21/9}$), constitutively active AMPK (myc-tag), phospho-JNK (Thr^{183}/Tyr^{185}), phospho-p70S6K (Thr^{389}), phospho-S6 ribosomal protein ($Ser^{235/236}$), and 4E-binding protein (4E-binding protein appears as three different bands, with α, β, and γ, denoting increasingly phosphorylated states). β-actin was detected for evaluating protein loading. Proteins were compared in $AdAkt^{myr}$ *vs.* Adβ-gal infected cells, or in $AdAMPK^{CA}$ *vs.* AdGFP infected cells. The immunoblots shown are representative of four independent experiments. (A) Effect of culture in 3 mM glucose, and 10 mM glucose with or without 25 nM rapamycin (Rpa) or 1 mM AICA-riboside (AICAR), on phosphorylation of endogenous Akt and its target GSK3β, and on phosphorylation of mTOR targets ribosomal protein S6 and 4E-binding protein (4EBP). See Supplementary Fig. 3 for quantification of these results. (B) Effect of expressing constitutively active $AMPK^{CA}$ on phosphorylation of endogenous Akt, GSK3β, 4EBP, and ribosomal protein S6. Phosphorylation of JNK is a known effect of AMPK activation in beta cells [11]. 4EBP-γ indicates hyperphosphorylated 4EBP, while the β and α forms are decreasingly phosphorylated. (C) Effect of expressing constitutively active Akt^{myr} on phosphorylation of endogenous GSK3β, and mTOR targets p70S6 kinase, ribosomal protein S6, and 4EBP. Akt^{myr}-induced stimulation of S6-kinase, ribosomal protein S6, and 4EBP, was inhibited in the presence of 25 nM of the

endogenous Akt activity. Exposing primary beta cells to a condition that activates AMPK – culture in the presence of AICAR (1 mM) – did not decrease the phosphorylation of the kinases GSK3β and Akt. Their phosphorylation was even increased in the presence of AICAR (Fig. 2A, Supplementary Fig. 3). Likewise, Akt- and GSK3β phosphorylations were not decreased when cells were infected with adenovirus AdAMPK^{CA} expressing a constitutively active (CA-) form of AMPK (Fig. 2B). This suggested that AMPK-induced cell death did not result from lowering the endogenous Akt activity. However, both low glucose and AICAR decreased the phosphorylation of ribosomal S6 protein and eIF4E-binding protein (eIF4E-BP) (Fig. 2A, Supplementary Fig. 3), two downstream targets of the protein kinase mammalian target of rapamycin (mTOR). These effects were reproduced by 2 h-exposure of the cells to the mTOR-inhibitor rapamycin (Fig. 2A) or by virus-mediated expression of active AMPK^{CA} (Fig. 2B). The results showed that AMPK activation suppressed the mTOR activity in beta cells.

In contrast, adenovirus-mediated expression of active Akt^{myr} increased the phosphorylation of the mTOR targets S6 protein kinase (S6K) and eIF4E-BP, as well as the further downstream target protein S6 (Fig. 2C), indicating that increased Akt activity stimulated mTOR in beta cells. In addition, ectopic expression of active Akt^{myr} could restore the phosphorylation of mTOR targets eIF4E-BP and S6 protein in the low glucose culture condition and also restored eIF4E-BP phosphorylation in AICAR, despite the AMPK activation in these conditions (Fig. 2D). In presence of rapamycin, the stimulatory effects of active Akt on phosphorylation of S6K, S6, and eIF4E-BP, were blunted (Fig. 2C), showing that Akt affected these proteins via mTOR. Since ectopic Akt activation could increase beta cell mTOR activity in conditions of AMPK activation, we examined whether Akt protected the beta cells through this mTOR stimulation. The results in Fig. 2E and F show that the AdAkt^{myr}-conferred protection against AICAR-induced beta cell apoptosis, was blocked by mTOR inhibition (see also Supplementary Fig. 4). The data suggest that mTOR activation can mediate a beta cell protective mechanism.

3.3. *Ectopic expression of S6K and eIF4E can increase beta cell survival*

We next investigated whether mimicking mTOR activation by increasing the activity of (some of) its downstream effectors would also stimulate beta cell survival. As shown above, the activation of S6 protein kinase (S6K) and the phosphorylation of eIF4E-binding protein (eIF4E-BP), were mediated by mTOR. Therefore, beta cells were first infected with adenoviruses AdS6K1 or AdS6K2, which express two wild-type isoforms of p70S6 kinase [24,25], and the cell survival in 3 and 10 mM glucose was studied. This showed that neither AdS6K1- nor AdS6K2-infected cells survived better in low glucose than Adβ-gal-infected cells (Fig. 3A). We then infected beta cells with AdS6K1^{RR}, which expresses a rapamycin-resistant form of S6K1 that remains partly active even under mTOR inhibition [26–28]. Interestingly, AdS6K1^{RR} increased the survival of beta cells in 3 mM glucose (Fig. 3A). Infections of beta cells with either AdS6K1, AdS6K2, or AdS6K1^{RR} occurred with similar efficiency (Supplementary Fig. 5) and the viruses all increased the S6K activity under control culture conditions as seen by the phosphorylation levels of S6K and protein S6 (Fig. 3B and C). However, S6K1^{RR} retained higher activity than S6K1^{WT} under glucose limitation and mTOR inhibition in beta cells (Fig. 3C). This showed that ectopic expression of an active form of S6K1 can enhance beta cell survival. Since mTOR-mediated phosphorylation of eIF4E-BP results in the release of eIF4E [41], we next attempted to mimic mTOR activity by increasing the expression of the downstream effector eIF4E. Adenoviruses AdeIF4E^{WT} and AdeIF4E^{209A} express, respectively, the wild-type eIF4E protein, and a mutant form of eIF4E which cannot be phosphorylated by upstream mitogen activated protein kinases [31,32] (Fig. 3D). These adenoviruses infected beta cells with similar efficiency (Supplementary Fig. 6) and both increased the number of surviving beta cells in 3 mM glucose (Fig. 3A). The results indicated that increased expression of wild-type or a mutated form of eIF4E can increase beta cell survival, likely without the need for MAPK activity. When beta cells were co-infected with AdS6K^{RR} and AdeIF4E^{WT}, the cells were markedly protected over the 3-day culture period in low glucose (Fig. 3A). This level of protection could not be attained by any of the viruses alone (Fig. 3A), suggesting that S6K and eIF4E, two factors controlled by mTOR, acted synergistically to increase cell survival. However, in the condition of AICAR, where AMPK was stimulated most prominently (Fig. 2D), AdS6K1^{RR} could not increase beta cell survival (Supplementary Fig. 7).

3.4. *Stimulation of protein biosynthesis by active Akt or S6K correlates with beta cell survival*

We and others previously reported that beta cell apoptosis in conditions of AMPK activation is preceded by inhibition of protein synthesis [6,8,36]. It was therefore investigated whether survival stimulation by Akt and mTOR-dependent factors correlated with stimulation of protein synthesis. To study protein synthesis in the low glucose-induced cell death model, beta cells were infected with Adβ-gal, AdAkt^{myr} or AdS6K1^{RR}, pre-cultured in either 10 or 3 mM glucose for 24 h, and subsequently the basal protein synthesis was measured in the absence of glucose. The basal protein synthesis rate of

mTOR-inhibitor rapamycin. (D) Effect of Akt^{myr} expression on mTOR activity measured at 24 h of culture in 10 mM glucose, 3 mM glucose, or 10 mM glucose + 1 mM AICAR. Akt^{myr} expression partially restored phosphorylation of the mTOR targets ribosomal protein S6, and 4EBP, under conditions of AMPK activation. (E) Beta cells infected with the indicated adenoviruses at MOI 30, were cultured for 3 days in normal medium (−) or medium containing 1 mM AICAR and/or rapamycin. Cell viabilities (mean ± S.E.M., $n = 6$, $^{*}p < 0.01$) were assessed as in Fig. 1. (F) FACS detection of apoptotic cells for selected conditions (see E). Histograms for PI-fluorescence intensities are shown. Percent cells with a sub-G1 DNA content is indicated in window P4. The experiment shown was repeated once, with similar results.

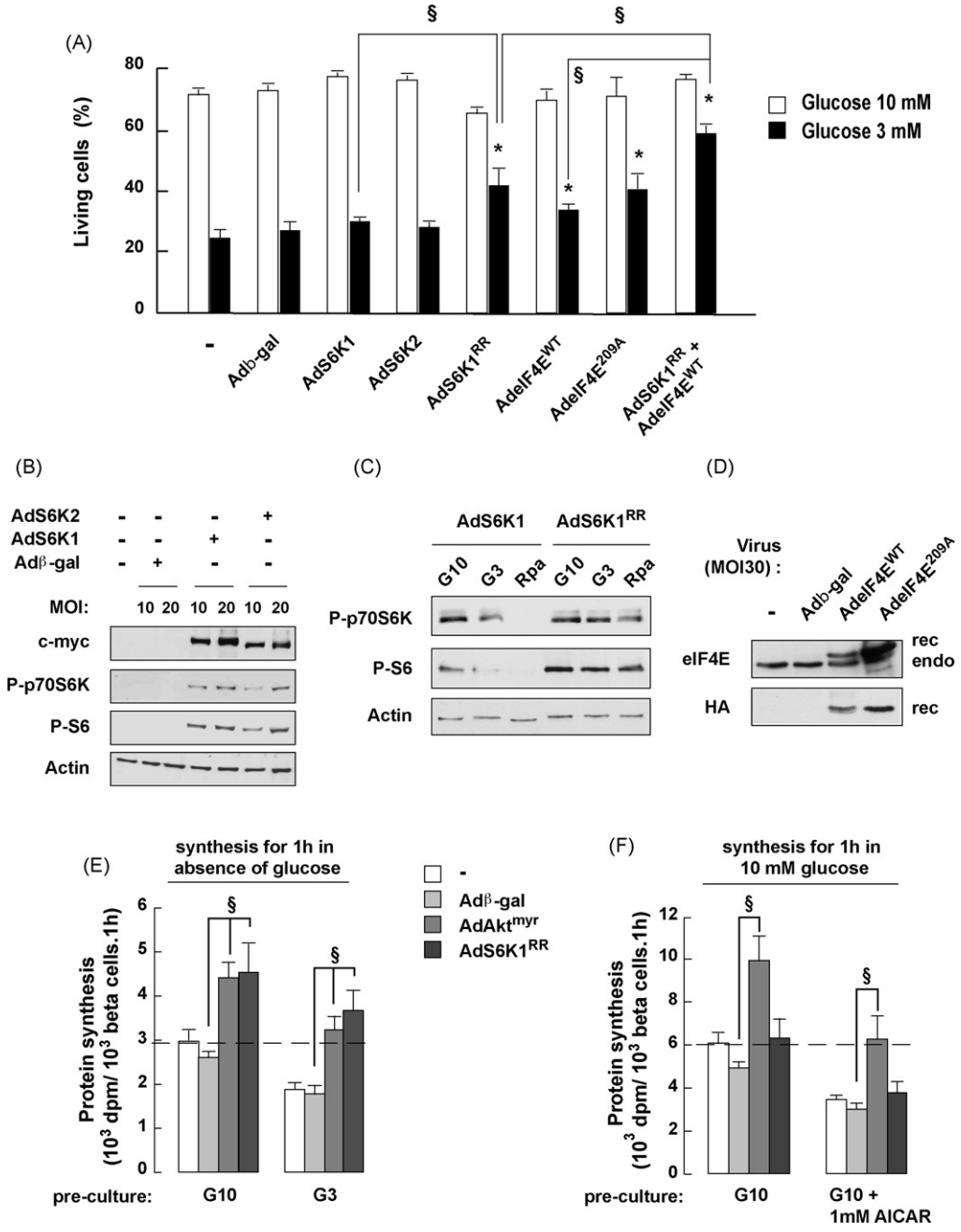

Fig. 3 – Ectopic expression of active S6 kinase and/or eIF-4E increases beta cell survival in low glucose. (A) Purified rat beta cells infected with the indicated viruses at MOI 30 were cultured for 3 days, in 3 or 10 mM glucose, and cell viabilities were determined as in previous figures (mean ± S.E.M., $n = 5$, $^{*}p < 0.01$ *vs.* Adβgal; $^{\S}p < 0.05$). (B) Effect of adenovirus-mediated expression of wild-type S6K1 and S6K2, and (C) of rapamycin-resistant S6K1^{RR}, on the phosphorylation of p70S6K and ribosomal protein S6 measured as in Fig. 2. S6K1^{RR} was more resistant than S6K1^{WT} against inhibition by low glucose (3 mM, G3) or by rapamycin (Rpa). Recombinant S6 kinases were also detected using an antibody against the myc-tag (not shown for S6K1^{RR}). (D) Expression of endogenous eIF4E and wild-type or mutant forms of eIF4E in beta cells that had been infected with Adβ-gal, AdeIF4E^{WT} or AdeIF4E^{209A}. Specific antibodies against total eIF4E protein or the HA-tag (in recombinant eIF4Es) were used. Immunoblots shown are representative of three experiments. (E and F) Effect of adenovirus-mediated expression of kinases Akt^{myr} or S6K1^{RR} on total protein synthesis in beta cells. (E) Beta cells (5×10^4) were cultured for 24 h in 3 mM (G3) or 10 mM glucose (G10) and subsequently the protein synthesis rate was measured in the absence of glucose. (F) Cells were cultured for 24 h in 10 mM glucose (G10) with or without 1 mM AICAR, as indicated, and the protein synthesis rate was subsequently measured in 10 mM glucose (as described in Section 2). Protein synthesis rates (mean ± S.E.M., $n = 3$, $^{\S}p < 0.05$) are expressed as counts per minute (dpm) per 1000 cells, and per hour.

uninfected or Adβ-gal-infected beta cells was decreased by culture in 3 mM glucose as compared to culture in 10 mM (Fig. 3E) but the basal synthesis rate of AdAkt^{myr} or AdS6K1^{RR} infected cells that had been cultured in 3 mM glucose was similar to that of uninfected cells cultured in 10 mM glucose (Fig. 3E). This demonstrated that expression of active S6K1 mimicked the effects of active Akt, sustaining the protein translation in cells that were under long-term glucose limitation.

To study translation in the AICAR-induced cell death model, beta cells were infected with Adβ-gal, AdAkt^{myr} or AdS6K1^{RR}, cultured for 24 h in 10 mM glucose either in the absence or presence of AICAR, and subsequently protein synthesis was measured in 10 mM glucose. This indicated that the protein synthesis rate of uninfected, and Adβ-gal or AdS6K1^{RR}-infected beta cells was decreased by pre-culture in AICAR as compared to culture in control condition (Fig. 3F), whereas the protein synthesis rate of AdAkt^{myr}-infected cells that had been exposed to AICAR was similar to that of uninfected cells cultured in control conditions (Fig. 3F). Thus, in AICAR, expression of active Akt, but not of active S6K1, could rescue the protein synthesis activity of beta cells. Taken together, the results showed that the capacity of active Akt or S6K1 to stimulate beta cell survival in either low glucose or AICAR correlated with the capacity of the kinases to functionally stimulate protein synthesis in the respective conditions.

3.5. *AMPK suppresses HIF1α and Bcl-2, and active Akt or S6K oppose this action*

Hypoxia inducible factor 1α (HIF1α) has an important impact on beta cell function through stimulation of glycolytic metabolism [42] and contributes to hypoglycemic survival of other cell types [43]. To study how mTOR-dependent translation factors could increase beta cell survival in conditions that activate AMPK, we investigated in INS-1 cells whether the expression of HIF1α as well as the anti-apoptotic protein Bcl-2 were inhibited in these conditions and whether they were reactivated by Akt and/or S6K.

Like in other cells, the expression of HIF1α protein in INS-1 cells appeared to be controlled by regulation of its breakdown: (i) HIF1α protein level was increased by $CoCl_2$ (Supplementary Fig. 8A), an inhibitor of the prolyl hydoxylase enzyme which is responsible for HIF1α degradation, and (ii) the HIF1α protein level was transiently increased by hypoxia also known to stabilize HIF1α (Supplementary Fig. 8B). It was also increased by serum (Supplementary Fig. 8A) suggesting a modulation of its synthesis as well [44]. Interestingly, both low glucose and AICAR decreased the expression of HIF1α protein in INS-1 cells, an effect that was reproduced by the mTOR-inhibitor rapamycin (Fig. 4A). This suggested that HIF1α synthesis was inhibited by AMPK activation and mTOR inhibition. HIF1α expression was not decreased by low glucose or AICAR in cells infected with AdAkt^{myr} (Fig. 4A). Infection of INS-1 cells with

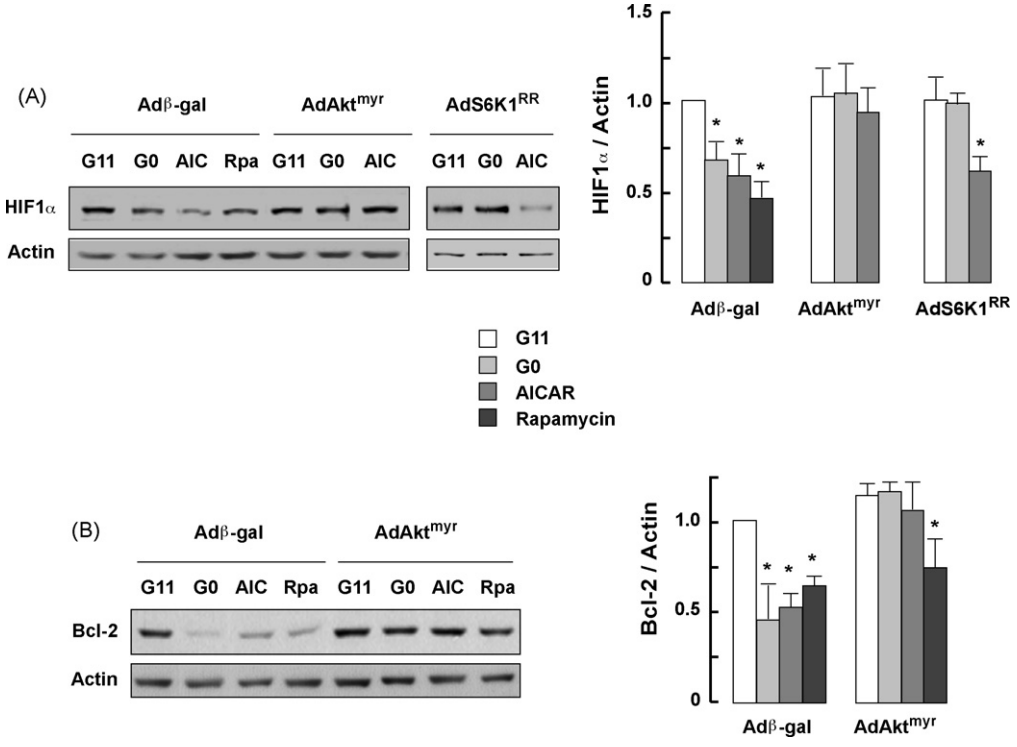

Fig. 4 – Expression of active kinases Akt^{myr} or S6K1^{RR} restores the HIF1α and Bcl-2 protein expression in INS-1 cells. (A and B) Insulin producing INS-1 cells were cultured as described in Section 2, and were infected with adenoviruses encoding either β-gal, Akt^{myr}, or S6K1^{RR} and cultured in 11 mM glucose (G11), no glucose (G0), 2 mM AICAR in G11 (AIC), or 25 nM rapamycin in G11 (Rpa) for 24 h. HIF1α (120 kDa), Bcl-2, and actin were detected by immunoblotting on total cell extract (25 μg protein), and the corresponding band densities recorded (see Section 2). Results are expressed as the ratio of the HIF1α or Bcl-2 signal over the actin signal, and data (mean ± S.E.M., $n = 3$, $^*p < 0.05$ *vs.* G11) normalized to G11 + Adβ-gal (white bars).

AdS6K1^{RR} also prevented the downregulation of HIF1α expression in low glucose, though not in AICAR (Fig. 4A). The results suggested that AMPK activation inhibited HIF1α synthesis and that this effect was abrogated by Akt or S6K activation in INS-1 cells.

When the anti-apoptotic protein Bcl-2 was examined in INS-1 cells, we found that low glucose, AICAR and rapamycin all decreased Bcl-2 expression (Fig. 4B). Infection of these cells with AdAkt^{myr} restored the expression of Bcl-2 in low glucose and AICAR (Fig. 4B), while AdAkt^{myr} did not restore Bcl-2 expression in the rapamycin condition (Fig. 4B). These results indicated that Akt prevented downregulation of the anti-apoptotic protein Bcl-2 in conditions of AMPK activation, and suggested that this action of Akt was mTOR-dependent.

4. Discussion

We previously showed that sustained AMPK activation triggers apoptosis in beta cells and insulin producing cell lines. Sustained AMPK activation in these cells can be provoked by culture in low glucose or in the presence of the AMPK-stimulator AICAR [5]. These models have been used to identify mechanisms that contribute to AMPK-mediated beta cell death [6,9,11,12]. AMPK-mediated cell death has also been observed in other cell types. The present study investigated whether AMPK-mediated cell death can be prevented by activation of Akt. Expression of a constitutively active form of Akt, Akt^{myr}, was sufficient to prevent cell death in both low glucose and AICAR. A wild-type form of Akt, which, as seen from its effect on GSK3β phosphorylation, was less active than Akt^{myr} when expressed in beta cells, was also less effective in protecting these cells. Our data thus indicate that high levels of Akt activity can potently suppress AMPK-mediated apoptosis.

Since cultured beta cells are subject to autocrine stimulation of Akt, we also examined whether this basal stimulation of Akt activity conferred some protection from cell death. Expression of a kinase-dead Akt mutant which acted as a dominant negative Akt protein in beta cells, indeed caused more beta cells to die in control conditions and increased further the cell death in AMPK-stimulating conditions. Exposing beta cells to AICAR in vitro did not decrease their basal Akt activity, indicating that suppression of endogenous Akt activity was not the cause of the beta cell death induction by AMPK activation. The results suggest that the beta cell endogenous Akt activity determines their sensitivity to AMPK-mediated death.

The present study explored the mechanism by which Akt can prevent AMPK-mediated cell death in beta cells. In various other systems, the pro-survival effect of active Akt is in part mediated by phosphorylation/inactivation of Bad, GSK-3α/β and FoxO1, and inactivation of caspase 9 or p53 [18]. We could not find alterations at the level of Bad or caspase 9, neither by AMPK activators, nor by expression of Akt^{myr} (not shown). We further examined a possible involvement of mTOR activation in beta cell survival, because we observed previously that IGF-1, glutamine, and leucine, which are known to stimulate mTOR in beta cells [15], increased beta cell survival in low glucose concentrations [16,45]. We report herein that IGF-1 can also stimulate beta cell survival in AICAR, strongly suggesting that upstream activators of Akt signaling can interfere with AMPK-induced apoptosis. Our study shows that AMPK activation decreased the phosphorylation of the mTOR targets S6 kinase, ribosomal protein S6, and 4E binding protein. Our results thus confirm the findings of a recent study [46] and show that mTOR is inhibited by forced activation of AMPK, in beta cells. Expression of Akt^{myr} had the opposite effect on S6 kinase, ribosomal protein S6, and 4E binding protein. Thus, whereas AMPK inhibited mTOR, Akt stimulated mTOR. Interestingly, expression of Akt^{myr} fully restored the mTOR activity in low glucose and partially in AICAR. The opposed effects of AMPK activity and Akt activity on the mTOR activity, correlated with their effects on the protein synthesis rate in beta cells: (i) AMPK activation was found to markedly decrease protein translation, (ii) protein synthesis was elevated by Akt^{myr} expression and (iii) when exposed for 24 h to conditions that activated AMPK, Akt^{myr} expressing cells still showed a protein synthesis rate that was similar to control cells. Thus, expression of Akt^{myr} in beta cells stimulates and restores the protein synthesis rate under AMPK-activating conditions. Our data suggest that this action is a consequence of mTOR stimulation by active Akt. Furthermore, Akt stimulated beta cell survival in AMPK activating conditions; this effect was mTOR dependent, as it was lost in the presence of rapamycin. We therefore conclude that mTOR activation can mediate an anti-apoptotic function in beta cells. To investigate whether the mTOR activation could also be sufficient to inhibit cell death, mTOR activation was mimicked by increasing the expression of the effectors S6 kinase and eIF4E, through which mTOR stimulates protein translation. We found that a mutated form of S6K1, named S6K1^{RR}, was more resistant to inhibition of its kinase activity by rapamycin or by glucose limitation in beta cells, than wild-type S6K1^{WT}, which is in agreement with the increased resistance of S6K1^{RR} towards mTOR inhibition demonstrated in other cells [25]. Our finding that S6K1^{RR}, but not S6K1^{WT}, stimulated beta cell survival in low glucose concentrations, indicated that increasing the S6K1 activity was necessary and sufficient to mediate a survival effect in low glucose. Likewise, increased expression of eIF4E, another mTOR-regulated effector, also stimulated beta cell survival in this condition. Interestingly, when both S6K1^{RR} and eIF4E were expressed in beta cells, these cells were markedly protected from low glucose-induced cell death. In fact, the viability was similar to that observed in cells expressing Akt^{myr}. Our data indicate that S6K1 and eIF4E acted in a synergistic fashion, suggesting that mTOR activation increases beta cell viability through both these effectors. Activation of mTOR and its downstream translation-effectors, may thus be a necessary and sufficient mechanism of Akt^{myr}-mediated survival in low glucose. Our observations are in agreement with findings reported in other cells that Akt and eIF4E expression can in part determine resistance to apoptosis [47], and studies that have indicated a similar role for S6 kinase. Novel findings in our study are (1) the survival effect of Akt appears highly dependent on mTOR, (2) individual 'Akt-like' survival effects of S6K and eIF4E, within one and the same cell death model, (3) full recapitulation of Akt's inhibitory action on cell death by combined action of mTOR effectors S6K and eIF4E and (4) mTOR activation is lifesaving in conditions of

AMPK activation in beta cells, whereas in cancer cells mTOR inhibition by AMPK mediates survival in glucose limitation [48].

When AMPK was activated by AICAR, cells could be rescued by expression of Akt^{myr}, but neither expression of $S6K1^{RR}$, nor of eIF4E, could stimulate beta cell survival. We showed that AICAR, when compared to low glucose, induced a stronger AMPK activation (Fig. 2D), which in turn resulted in a stronger mTOR inhibition as seen from the phosphorylation status of S6 and 4EBP (Fig. 2D). It is thus possible that the expression of $S6K1^{RR}$ (or eIF4E) was not sufficient to reboot the survival program in the AICAR condition. The $S6K1^{RR}$ mutant $E_{389}D_3E$ used in this study is indeed still in part sensitive to mTOR inhibition [49] and could therefore be less active in AICAR than in low glucose. This is consistent with our finding that $S6K1^{RR}$ could stimulate protein synthesis in low glucose, but not in the presence of AICAR (Fig. 3E and F).

We further explored how mTOR activity in beta cells can contribute to maintaining their function and viability. Previous studies have shown that mTOR activity is important for HIF1α expression and activity [50,51]. An Akt/mTOR regulation of HIF1α expression has also been reported; mice transgenic for constitutively active Akt displayed increased expression of HIF and its targets, while TSC2-deficiency or treatment of these mice with a rapamycin analog normalized the levels of HIF and its target genes [52]. Consistent with these studies, we observed that expression of Akt^{myr}, which increased mTOR activity, or expression of rapamycin-resistant $S6K1^{RR}$, which mimicked mTOR activation, both led to increased expression of HIF1α in insulin secreting INS-1 cells. As recently reported by others [53], we found that HIF1α was repressed by AMPK activation (Fig. 4A). Interestingly, HIF1α expression was dependent on mTOR activity, and repression of HIF1α by AMPK activation was counteracted by active Akt in INS-1 cells (Fig. 4A).

HIF1-α forms heterodimers with HIF1β, and HIF1α/β-mediated transcription appears at the center of metabolic control of beta cells, as disruption of HIF1β in these cells clearly leads to altered expression of genes involved in regulation of glucose metabolism and insulin secretion [42]. We have shown that mTOR activation can be effective in stimulating beta cell survival in stress conditions associated with AMPK activation. Whether this can, at least in part, be attributed to effects on HIF1α expression in beta cells needs further investigation. Our findings suggest that increasing the mTOR activity in beta cells may contribute to increased transcription and translation of those genes that are crucial for an active nutrient metabolism, which in turn may help the cells to cope with metabolic stress. Similar conclusions regarding a possible role for metabolic effects in mTOR-mediated survival have been reached by studying thymocytes in conditions of growth factor withdrawal [54].

We have shown that IGF-1 inhibits apoptosis in AMPK-activating conditions via Akt (Fig. 1G), and that Akt-mediated survival can at least in part be explained by stimulation of the mTOR pathway. Since insulin is also known to activate Akt [17], together this may explain how deleterious effects of prolonged AMPK activation in beta cells could be suppressed in vivo. Our results show that Akt activation can stimulate beta cell survival via mTOR signaling, but do not exclude that other mechanisms play a role in counteracting AMPK. For instance, Gleason et al. recently demonstrated a direct effect of glucose on mTOR [46]. In the present study we have not examined the contributions made by secreted insulin to both signaling and survival under AMPK activating conditions. We and others previously reported that prolonged AMPK activation inhibits insulin synthesis and secretion [2,5–9,39], while short-term AMPK activation was recently shown not to inhibit but actually to potentiate glucose-induced insulin secretion [46]. Our present observation that AICAR exposure transiently increased the phosphorylation of Akt (Fig. 2A, Supplementary Fig. 3) might be related to these recent findings. However, no clear consensus exists on the regulation of insulin secretion by Akt [17].

We previously showed that apoptosis induced by AMPK activation can be inhibited by over-expression of Bcl-2 [9]. The present study demonstrated a downregulation of the endogenous expression of Bcl-2 protein by AMPK activation or mTOR inhibition (Fig. 4B). Over-expression of active Akt increased the Bcl-2 expression under AMPK activation in INS-1 cells, and this effect was dependent on the mTOR activity. Together, these data identify stimulation of Bcl-2 expression as another mechanism by which mTOR activity can inhibit beta cell death.

In conclusion, our results suggest that increased Akt activity enhances beta cell survival through stimulation of mTOR and subsequent co-activation of p70S6K and eIF4E. Stimulation of this Akt/mTOR pathway can prevent the death of cells that would otherwise die upon AMPK activation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.02.019.

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