Interaction of glibenclamide and metformin at the level of translation in pancreatic β cells

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Abstract

Sulfonylurea and metformin are used in the treatment of diabetes. Their chronic effects on β cells are not well known. We have shown that sustained exposure of rat β cells to glibenclamide increased their protein synthesis activity, while metformin caused an inhibition. The effect of glibenclamide was attributed to an activation of translation factors. This study examines whether both drugs interact at the level of protein translation in β cells. Purified rat β cells were cultured with and without glibenclamide and metformin before measurement of protein and insulin synthesis, abundance of (phosphorylated) translation factors, and cell viability. A 24 h exposure to metformin stimulated AMP-activated protein kinase (AMPK), suppressed activation of translation factors—both the mammalian target of rapamycin

(mTOR; also known as mechanistic target of rapamycin, MTOR)—dependent ones (eukaryotic initiation factor 4E-binding protein 1 and ribosomal protein S6) and the mTOR-independent eukaryotic elongation factor 2-, and inhibited protein synthesis; a 72 h exposure resulted in 50% dead cells. These effects were counteracted by addition of glibenclamide, the action of which was blocked by the mTOR inhibitor rapamycin and the protein kinase A (PKA) inhibitor Rp-8-Br-cAMPs. In conclusion, metformin activates AMPK in β cells leading to suppression of protein translation through mTOR-dependent and -independent signaling. Glibenclamide antagonizes these metformin effects through activation of mTOR- and PKA-dependent signaling pathways. *Journal of Endocrinology* (2011) **208**, 161–169

Introduction

Sulfonylurea has been used in the treatment of diabetes for decades. Its hypoglycemic action is attributed to stimulation of insulin release that rapidly follows the drug's binding to the SUR receptor of β cells and the subsequent rise in cytoplasmic free calcium concentration (Sturgess *et al.* 1985, Henquin 1987, Proks *et al.* 2002). We have recently reported that glibenclamide, a second-generation sulfonylurea drug, also exerts stimulatory effects on β -cell protein and insulin synthesis when present over a prolonged period both *in vivo* and *in vitro* (Ling *et al.* 2006). This chronic effect was achieved through an activation of translation at the steps of initiation and elongation, as evidenced by phosphorylation of the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and of ribosomal protein S6 (rpS6), and dephosphorylation of eIF2 α and of eukaryotic elongation factor 2 (eEF2; Wang *et al.* 2008). It was shown to be calcium dependent and mediated through the mammalian target of rapamycin (mTOR; also known as mechanistic target of rapamycin, MTOR), protein kinase A (PKA), and MAPK/ERK kinases (MEKs; Wang *et al.* 2008). Thus, glibenclamide, which is used for its acute stimulatory effect on insulin release, exerts an

mTOR-dependent stimulation of insulin synthesis when present for prolonged periods (Wang *et al.* 2008). mTOR is a serine and threonine protein kinase that regulates protein translation through a rapamycin-sensitive pathway involving the rpS6 kinase (S6K1) and the 4E-BP1 (Gingras *et al.* 2001, Raught *et al.* 2001, Arsham & Neufeld 2006). It is activated by nutrients and growth factors (McDaniel *et al.* 2002, Kwon *et al.* 2004) and inhibited by cellular energy deficiency through a mechanism that is dependent on AMP-activated protein kinase (AMPK; Inoki *et al.* 2003, Gleason *et al.* 2007). AMPK activation inhibits protein translation in many cell types, including pancreatic β cells (Bolster *et al.* 2002, Kefas *et al.* 2004, Williamson *et al.* 2006, Dowling *et al.* 2007, Cai *et al.* 2008). Sustained inhibition of protein synthesis is known to impair β -cell function and survival (Hoorens *et al.* 1996, Garcia-Barrado *et al.* 2001, Cai *et al.* 2008). It is unknown whether glibenclamide-induced mTOR activation could preserve protein translation and consequently β -cell survival in conditions of AMPK activation.

In pancreatic β cells, AMPK can be activated by the anti-diabetic drug metformin (Kefas *et al.* 2004, Leclerc *et al.* 2004). Metformin is used in the treatment of diabetes as a facilitator of insulin sensitivity in peripheral tissues (Cusi and

Deffronzo, 1998, Rutter *et al.* 2003). It is so far unknown whether metformin treatment *in vivo* exerts also direct effects on β cells. *In vitro*, metformin inhibits glucose oxidation, suppresses insulin secretion and biosynthesis, and subsequently induces apoptosis (Schatz *et al.* 1972, Kefas *et al.* 2004, Leclerc *et al.* 2004). As in peripheral tissues, this effect appears mediated, at least in part, through inhibition of mitochondrial respiratory complex I and activation of AMPK (El-Mir *et al.* 2000, Owen *et al.* 2000, Zou *et al.* 2004, Hinke *et al.* 2007). It is conceivable that a metformin-induced activation of AMPK also results in an inhibition of mTOR-dependent translation in β cells. If that is the case, the combination of metformin and glibenclamide may exert opposing effects on mTOR activity, and thus on β-cell function and survival. In this *in vitro* study, we therefore examined whether the earlier observed metformin inhibition of β-cell function and survival was associated with inactivation of mTOR-dependent translation factors, and whether these effects could be prevented by glibenclamide. Likewise, since glucose activates mTOR in pancreatic β cells (Kwon *et al.* 2004, 2006) and chronically elevated glucose results in sustained translational activation in β cells (Ling *et al.* 1996), we examined whether high levels of glucose could counter metformin inhibition of translation.

Materials and Methods

Preparation and culture of purified rat β cells

Pancreatic islets were isolated from adult male Wistar rats as described previously (Pipeleers *et al.* 1985). The animals were housed according to Belgian regulation of animal welfare. Islets were dissociated into single cells in calcium-free medium containing trypsin and DNase (Pipeleers *et al.* 1985). Single β cells were purified by autofluorescence-activated sorting, using cellular light-scatter and flavin adenine dinucleotide-autofluorescence as discriminating parameters (Pipeleers *et al.* 1985).

Table 1 Effects of glibenclamide and glucose on metformin-induced suppression of protein synthesis. Rat β cells were cultured for 24 h at 10 mmol/l glucose, with or without 1 mmol/l metformin (Metf), and with or without 4 μmol/l glibenclamide (Glib) or additional glucose (20 mmol/l glucose instead of 10 mmol/l = Gluc). By the end of culture, protein and insulin synthesis were measured during subsequent 1 h incubations at 0 or 10 mmol/l glucose in the absence of these compounds. Data represent means ± s.e.m. of five to eight independent experiments

Addition to culture medium		Total protein synthesis (dpm/cell per h)		Insulin synthesis (dpm/cell per h)	
Stimuli	Metf	0 mmol/l glucose	10 mmol/l glucose	0 mmol/l glucose	10 mmol/l glucose
—	—	2.7 ± 0.3	8.5 ± 0.7	1.9 ± 0.4	6.8 ± 0.8
—	+	0.4 ± 0.1 [†]	2.3 ± 0.3 [‡]	0.2 ± 0.1 [†]	1.6 ± 0.1 [‡]
Glib	—	3.4 ± 0.4	8.4 ± 0.8	1.9 ± 0.3	6.0 ± 0.9
Glib	+	1.7 ± 0.3	5.1 ± 0.5 ^{‡,§}	0.9 ± 0.2	3.3 ± 0.4 [*]
—	—	1.6 ± 0.4	8.1 ± 0.8	1.3 ± 0.4	6.0 ± 0.9
—	+	0.3 ± 0.05 [*]	1.4 ± 0.3 [‡]	0.1 ± 0.02 [*]	1.4 ± 0.1 [†]
Gluc	—	3.1 ± 0.7 [§]	8.2 ± 1.4	2.4 ± 0.6	6.8 ± 1.3
Gluc	+	0.5 ± 0.1 [‡]	2.4 ± 0.3 [†]	0.3 ± 0.04 [†]	1.8 ± 0.2 [†]

Statistical significance of differences with and without Metf at the same glucose concentration: ^{*}*P* < 0.05, [†]*P* < 0.01, and [‡]*P* < 0.001; with and without stimuli at the same glucose concentration: [§]*P* < 0.05.

Purified β cells were suspended in Lux dishes (Miles, Naperville, IL, USA) containing 3 ml serum-free culture medium (Ling & Pipeleers 1994), reaggregated for 2 h in a rotatory shaking incubator (Braun, Melsugen, Germany), and then further cultured under static conditions in a CO₂ incubator (37 °C, 95% air/5% CO₂). Basal culture medium was Ham's F-10 containing 10 mmol/l glucose supplemented with 0.075 mg/ml penicillin, 0.1 mg/ml streptomycin, 2 mmol/l L-glutamine, 0.5% (weight/volume) BSA pre-treated with charcoal (BSA, fraction V, Sigma Chemical Co.), and 1.7 mmol/l calcium (final concentration 2 mmol/l). After overnight culture, rat β cells were further cultured for 24–48 h with or without metformin (0.04–1 mmol/l, 1,1-dimethylbiguanide, Sigma–Aldrich). In a second series of experiments, the effect of metformin was tested in the absence or presence of glibenclamide (0.04–4 μmol/l, MP Biomedicals, Eschwege, Germany) or 20 mmol/l glucose instead of 10 mmol/l. In a third series, its effect was examined with or without glibenclamide, mTOR inhibitor rapamycin (25 nmol/l) (Cell Signaling Technology, Danvers, MA, USA), and/or PKA inhibitor Rp-8-Br-cAMPs (100 μmol/l) (Biolog, Bremen, Germany). At the end of culture, cells were analyzed for their protein synthesis and expression.

Purified β cells were also cultured as single cells in the above-mentioned test media for analysis of cell viability.

Protein synthesis and expression

The rate of protein synthesis was measured in β-cell aggregates following culture under the above conditions. Samples of 3 × 10⁴ cells were incubated for an additional 1 h in Ham's F-10 medium at 0 or 10 mmol/l glucose without any of the tested compounds and with 1.85 MBq L-[3,5-³H]tyrosine (TRK200, Amersham) (Schuit *et al.* 1991). At the end of incubation, the cells were washed in Earle's-HEPES buffer containing 1 mmol/l unlabeled L-tyrosine and extracted in 2 M acetic acid containing 0.25% (weight/volume) BSA for

analysis of total protein and insulin biosynthesis. Total protein synthesis was measured as precipitable radioactivity after mixing the acid extracts with trichloroacetic acid (Pipeleers *et al.* 1973, Schuit *et al.* 1988). Insulin biosynthesis was measured as precipitable radioactivity after incubation of the cell extracts with excess anti-insulin serum (Schuit *et al.* 1988). Fractions of cell extracts (50 μ l) were dried and incubated at 37 °C for 1 h in insulin assay buffer (PBS with 0.5% BSA) containing guinea pig anti-insulin serum (kindly provided by Dr C Van Schravendijk). Thereafter, the samples were incubated for 15 min at room temperature with protein A-Sepharose CL 4B (5 mg/200 μ l 0.9% NaCl) (Pharmacia), centrifuged, and extensively washed. The pellets were resuspended in 2 M acetic acid, and their soluble fraction was mixed with scintillation cocktail (Perkin Elmer, Shelton, CT, USA) and counted in a β counter (Tri-Carb model 1600 TR, Packard, Canberra Company, Meriden, CT, USA). Nonspecifically bound radioactivity was determined by incubating the samples with normal guinea pig serum instead of anti-insulin serum. Insulin synthesis was calculated as the difference between both counts (Schuit *et al.* 1988).

Protein expression in cultured β cells was analyzed immediately after drug treatment by western blot as described previously (Ling *et al.* 1998). Samples of $2\text{--}3 \times 10^5$ cells were run on 12% SDS-polyacrylamide before protein transfer on a nitrocellulose membrane and incubation with antibodies against 4E-BP1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), total eIF2 α , phospho-eIF2 α (Ser51), total rpS6, phospho-rpS6 (Ser235/236), total eEF2, phospho-eEF2 (Thr56), total AKT, phospho-AKT (Ser473), total AMPK and phospho-AMPK (Thr172) (all from Cell Signaling Technology), and actin (Santa Cruz Biotechnology, Inc.). HRP-linked anti-rabbit, anti-mouse, or anti-goat Igs (1:1000, Santa Cruz Biotechnology, Inc.) were used as secondary antibodies, and peroxidase activities were detected by enhanced chemiluminescence (Amersham). Intensities of bands were quantified by Scion Image for Windows (Scion Corporation, Frederick, MD, USA) expressed in arbitrary units of optical density and normalized for actin intensity in the same blot.

Viability assay

Viability assay consists of counting the percentage of dead cells and therefore requires the use of single cells. Purified single rat β cells (3×10^3 cells/well) were plated in polylysine-coated 96-well microtiter plates. After overnight culture in basal medium, cells were cultured for 72 h in test conditions. At the end of culture, cell viability was determined by fluorescence microscopy using propidium iodide (PI; Sigma) and Hoechst 3342 (HO342, Sigma; Hoorens *et al.* 1996). Viable cells were identified by their intact nuclei with blue fluorescence (HO342), and dead cells by nuclei with yellow-red fluorescence (HO342+PI). A cytotoxic test condition is detected by a lower cell survival than in control, and its toxicity index (TI) was calculated as $\text{TI} = ((\% \text{ dead cells in test} - \% \text{ dead cells in control}) / \% \text{ living cells in control}) \times 100\%$.

Statistical analysis

Results were expressed as mean $\pm$ S.E.M. for the indicated number of independent experiments. Statistical significance of differences was calculated by Student's *t*-test or ANOVA with the Newman-Keuls test.

Results

Metformin suppresses protein translation and synthesis in rat β cells

After 24 h culture in the presence of 1 mmol/l metformin, protein synthesis by β cells was suppressed by 80% (Table 1). However, protein synthesis was almost completely restored after an additional 24 h culture without metformin (84–93% of protein synthesis activity in control cells). An inhibitory

Figure 1 Effects of different concentrations of metformin on basal and glucose-induced protein synthesis. Rat β cells were cultured for 48 h at 10 mmol/l glucose, without or with 0.04, 0.2, and 0.5 mmol/l metformin. Total protein synthesis was measured during subsequent 1 h incubation at 0 mmol/l (a) or 10 mmol/l (b) glucose in the absence of metformin. Data are expressed as percentage of control (without metformin during 48 h culture) and represent means $\pm$ S.E.M. of four independent experiments. The rates of protein synthesis in control cells were 1.6 ± 0.2 dpm/cell per h in the absence of glucose and 7.0 ± 0.6 dpm/cell per h at 10 mmol/l glucose. Statistical significance of differences with control: * $P < 0.01$.

Figure 2 Effects of metformin on expression and phosphorylation of translation factors. Rat β cells were cultured for 24 h at 10 mmol/l glucose, without (C) or with 1 mmol/l metformin (M). The expression of translation factors was analyzed by western blot using specific antibodies (see Materials and Methods). Band intensities of 4E-BP1 γ , P-rpS6, P-eIF2 α , P-eEF2, P-AMPK, and P-AKT were normalized for the corresponding actin intensity. Data represent means $\pm$ S.E.M. of six independent experiments. Statistical significance of differences with control: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

effect of metformin was also observed at lower concentrations (0.2–0.5 mmol/l), but this required a 48 h exposure (Fig. 1).

A 24 h culture with 1 mmol/l metformin had decreased the phosphorylation state of translation initiation factors 4E-BP1 and rpS6 (Fig. 2a and b). The 4E-BP1 dephosphorylation was reflected in the shift of the highly phosphorylated γ -band to nonphosphorylated α -band and

an intermediate β -band (Fig. 2a). Metformin did not significantly affect the phosphorylation of eIF2 α (Fig. 2c), suggesting that the effect was initiation factor specific. Moreover, metformin inhibited translation elongation, as indicated by increased phosphorylation and thus inactivation of eEF2 (Fig. 2d).

Metformin also increased the phosphorylation of AMPK (Fig. 2e), indicating its activation, while the activity of AKT (protein kinase B) was unaltered (Fig. 2f).

Glibenclamide, but not glucose, prevents metformin suppression of protein translation

Addition of glibenclamide (4 μ mol/l) did not increase protein synthesis above the levels measured at 10 mmol/l glucose in the absence of metformin (Table 1) but counteracted the metformin suppression of protein synthesis (Table 1). This glibenclamide effect was also observed at lower concentrations (0.04 and 0.4 μ mol/l, Fig. 3). Glibenclamide prevented the metformin-induced dephosphorylation of 4E-BP1 (γ -form) and rpS6 (Fig. 4a and b), whereas it did not alter the phosphorylation of eEF2 (Fig. 4c), suggesting that glibenclamide counteracted the metformin suppression of protein synthesis by increasing translation initiation. Glibenclamide did not prevent metformin-induced AMPK activation (Fig. 4d) but markedly increased the phosphorylation of AKT (Fig. 4e).

The protein synthesis stimulation by glibenclamide was inhibited by the mTOR inhibitor rapamycin and by the specific PKA inhibitor Rp-8-Br-cAMPs, and more strongly when both inhibitors were added together (Fig. 5a–c). This suggests that glibenclamide stimulated protein synthesis through both mTOR- and PKA-dependent pathways.

We then examined whether the metformin suppression of protein translation could be prevented by increasing the

Figure 3 Effects of different concentrations of glibenclamide on metformin suppression of protein synthesis. Rat β cells were cultured for 24 h at 10 mmol/l glucose with 1 mmol/l metformin, in the absence or presence of different concentrations of glibenclamide (Glib, 0.04–4 μ mol/l). Protein synthesis was measured during subsequent 1 h incubations at 10 mmol/l glucose in the absence of these compounds. Data represent means $\pm$ S.E.M. of three independent experiments. Statistical significance of differences with and without Glib: * $P < 0.05$.

Figure 4 Effects of glibenclamide on metformin-induced suppression of translation factors. Rat β cells were cultured for 24 h at 10 mmol/l glucose, without or with 1 mmol/l metformin (Metf), and with or without 4 μ mol/l glibenclamide (Glib). The expression of translation factors was analyzed as described in Fig. 2. Data represent means $\pm$ S.E.M. of three or four independent experiments. Statistical significance of differences between conditions: * $P < 0.05$ and ** $P < 0.01$.

glucose concentration (20 mmol/l), a condition known to activate mTOR signaling and translation (Patel *et al.* 2001, McDaniel *et al.* 2002). Indeed, in the absence of metformin, 20 mmol/l glucose increased the basal protein and insulin synthesis of β cells (Table 1) and phosphorylation of 4E-BP1 and rpS6 (Fig. 6a and b). However, high glucose neither prevented the suppression of translational activity by metformin (Table 1 and Fig. 6a-c) nor the metformin-induced AMPK activation (Fig. 6d).

Glibenclamide decreases β -cell toxicity of metformin

Culture of rat β cells for 24 h with 1 mmol/l metformin or for 48 h with ≤ 0.5 mmol/l metformin, which were the conditions used for analysis of protein translation (see above), did not induce β -cell death (results not shown), whereas a 72 h culture period at the highest dose (1 mmol/l) did cause death of 50% of the cells (Fig. 7). This confirms the previous finding that metformin-induced apoptosis requires extended periods of time (Kefas *et al.* 2004). The metformin-induced β -cell toxicity at 72 h was markedly reduced by addition of

glibenclamide (Fig. 7). This protective effect of glibenclamide in β cells was less pronounced in the presence of the mTOR inhibitor rapamycin, or the PKA inhibitor Rp-8-Br-cAMPs, and disappeared completely in the presence of both inhibitors (Fig. 7). These data suggest that glibenclamide can protect β cells by stimulating mTOR and PKA.

Figure 5 Effects of mTOR and PKA inhibitors on glibenclamide protection against metformin-induced suppression. Rat β cells were cultured for 24 h at 10 mmol/l glucose, without or with 1 mmol/l metformin (Metf), supplemented with 4 μ mol/l glibenclamide (Glib) alone or in combination with the mTOR inhibitor rapamycin (25 nmol/l-Rapa), the PKA inhibitor Rp-8-Br-cAMPs (100 μ mol/l-PKai), or both. Total protein (a) and insulin synthesis (b) was measured during subsequent 1 h incubation at 10 mmol/l glucose. The expression of mTOR substrate p-rpS6 is shown in (c). Data represent means $\pm$ S.E.M. of three to six independent experiments or are representative for two independent experiments (c: last condition with both inhibitors). Statistical significance of differences versus Glib/Metf condition in the absence of inhibitors: * $P < 0.05$ and $^{\dagger}P < 0.01$.

Figure 6 Effects of glucose on metformin-induced suppression of translation factors. Rat β cells were cultured for 24 h at 10 or 20 mmol/l glucose (Gluc) and without or with 1 mmol/l metformin (Metf). The expression of translation factors was analyzed as described in Fig. 2. Data represent means $\pm$ S.E.M. of three or four independent experiments. Statistical significance of differences between conditions with and without metformin at the same glucose concentration: ** $P < 0.01$ and *** $P < 0.001$; 10 vs 20 mmol/l glucose in the absence or presence of metformin: † $P < 0.05$ and †† $P < 0.01$.

Discussion

The hypoglycemic effect of glibenclamide, a second-generation sulfonylurea drug, is attributed to its acute stimulation of insulin release (Sturgess *et al.* 1985, Henquin 1987, Proks *et al.* 2002). We recently demonstrated that treatment with glibenclamide also stimulates insulin synthesis, at least in rats (Ling *et al.* 2006, Wang *et al.* 2008). On the other hand, metformin – an anti-diabetic drug that increases insulin sensitivity in peripheral tissues (Cusi and DeFronzo, 1998) – was found to inhibit insulin synthesis in cultured rat β cells (Kefas *et al.* 2004). This study shows that both drugs exert opposing effects at the level of protein translation in β cells: glibenclamide counteracts the metformin-induced inhibition of protein synthesis.

The glibenclamide-induced elevation in insulin synthesis has been attributed to an activation of translation factors through calcium-dependent mTOR, PKA, and MEK signaling pathways (Wang *et al.* 2008). We now show that metformin suppresses activation of proteins involved in translation initiation and elongation, as evidenced by the lower amount of phosphorylated 4E-BP1 and rpS6 and the higher amount of phosphorylated eEF2. Total rpS6 was also lower, which is indicative of an associated decrease in ribosome biogenesis. Since the 4E-BP1 and rpS6 translation

initiators are well-known downstream targets of mTOR (Gingras *et al.* 2001, Raught *et al.* 2001, Arsham & Neufeld 2006), our data suggest that metformin suppression of translation results from an inhibition of mTOR. Interestingly, glibenclamide, but not glucose, counteracted the metformin suppression of protein synthesis, and of 4E-BP1 and rpS6 phosphorylation, and these glibenclamide effects were partially blocked by the mTOR inhibitor rapamycin. This suggests that glibenclamide prevents β -cell dysfunction, at least in part, through activation of mTOR. Metformin inhibition of growth of breast cancer cells was also found to suppress translation initiation through inhibition of mTOR (Dowling *et al.* 2007). In these cells, the action mechanism of metformin involved activation of AMPK (Dowling *et al.* 2007), as appeared also to be the case in our β -cell preparations (Fig. 2). This mechanism is further supported by our previous finding that sustained activation of AMPK by the pharmacological AMPK activator 5-aminoimidazole-4-carboxamide riboside also suppresses protein translation in β cells (Kefas *et al.* 2004, Cai *et al.* 2008).

The AMPK suppression of translation in β cells can be mediated via two pathways. In the first, active AMPK represses mTOR activity through phosphorylation of mTOR inhibitor TSC2 (Inoki *et al.* 2005), mTOR binding partner raptor (Gwinn *et al.* 2008), or mTOR itself on threonine

Figure 7 Effects of mTOR and PKA inhibitors on glibenclamide protection against metformin-induced cell death. Rat β cells were cultured for 72 h in medium containing 10 mmol/l glucose with or without 1 mmol/l metformin (Metf) and supplemented with 4 μ mol/l glibenclamide (Glib), alone or in combination with the mTOR inhibitor rapamycin (25 nmol/l-Rapa), the PKA inhibitor Rp-8-Br-cAMPs (100 μ mol/l-PKAi), or both. The toxicity index was calculated as described in Materials and Methods. Percentage of dead cells in control condition (without metformin, glibenclamide, rapamycin, and PKAi) was 32 ± 1 . Data represent means $\pm$ S.E.M. of six independent experiments. Statistical significance of differences with or without Glib, in the absence of inhibitors: * $P < 0.001$; with or without inhibitor, in the presence of Metf/Glib: † $P < 0.05$ and †† $P < 0.01$.

2446 (Cheng *et al.* 2004). In the second, active AMPK inhibits, in an mTOR-independent manner, the elongation factor eEF2 through activation of eEF2 kinase (Browne & Proud 2002, Browne *et al.* 2004). In β cells *in vitro*, both AMPK effector pathways seem operational. Indeed, the counteractive effect of glibenclamide on metformin inhibition of translation involved mTOR, supporting the first pathway, while increased phosphorylation of eEF2 by metformin suggested that the second pathway was involved as well. Our finding that glibenclamide partially restored protein synthesis in the presence of metformin can be explained by the observation that glibenclamide mainly interfered with the first AMPK pathway.

Glibenclamide not only antagonized metformin suppression of insulin and noninsulin protein synthesis but also protected β cells from the metformin cytotoxicity that is observed after longer culture periods. The counteractive effects of glibenclamide on metformin suppression of protein synthesis as well as cell survival observed in this study involved activation of both mTOR and PKA, as the glibenclamide effects were completely lost when both an mTOR inhibitor and a PKA inhibitor were present. These observations are consistent with previous work in which glibenclamide was found to activate translation through both mTOR-dependent

and PKA-dependent pathways (Wang *et al.* 2008). It is unlikely that glibenclamide abrogates the negative effects of metformin on β -cell function through inhibition of apoptosis, because the metformin inhibition of protein translation was reversible and preceded its effect on death. On the contrary, the protection of glibenclamide against β -cell apoptosis may occur through its stimulatory effect on protein synthesis, since sustained inhibition of protein synthesis may result in a deficit of anti-apoptotic proteins (Hoorens *et al.* 1996, Kefas *et al.* 2003).

In contrast to glibenclamide, high glucose could not counteract metformin inhibition of translation, although it activated mTOR and translation in the absence of metformin. These observations indicate that glibenclamide and glucose regulate different pathways upstream of mTOR. Glibenclamide-induced signals can activate mTOR when AMPK is active, suggesting that glibenclamide acts downstream of AMPK. In contrast, the glucose-induced mTOR activation is blocked by AMPK activation, suggesting that glucose acts upstream of AMPK.

Based on the present *in vitro* observations, we propose a model for the interaction of metformin and glibenclamide at the level of translation (Fig. 8). Metformin induces an activation of AMPK that can result in an inhibition of

Figure 8 Interaction of glibenclamide and metformin at the level of translation in β cells. Metformin activates AMPK, which will interfere with activation of mTOR and subsequently with the phosphorylation of rpS6 and 4E-BP1. Activated AMPK will directly phosphorylate eEF2 and thus inhibits its elongation activity. The metformin suppression of translation results in inhibition of protein synthesis. Glibenclamide partially counteracts these metformin effects through its activation of mTOR- and PKA-dependent pathways.

mTOR. This effect interferes with a nutrient or growth factor stimulation of mTOR signaling that activates protein translation through phosphorylation of rpS6 and 4E-BP1. In addition to interfering with mTOR signaling, activated AMPK also inhibits protein elongation through phosphorylation of eEF2. The resulting suppression of protein synthesis is responsible for a decreased hormone production and an increased cellular susceptibility to apoptosis. Combination of metformin and glibenclamide does not result in these detrimental effects, because glibenclamide stimulates mTOR- and PKA-dependent signaling pathways that activate translational proteins.

It is unknown to which extent our *in vitro* observations on rat β cells are relevant for *in vivo* conditions. Although the metformin concentrations used in this study (0.04–1 mmol/l) were generally higher than steady-state plasma levels of metformin in patients (0.01–0.04 mmol/l; Cusi and DeFronzo, 1998, Wiernsperger 1999), studies in rats have shown that tissue levels are several fold higher than in plasma (Wilcock & Bailey 1991). In an earlier report, we have shown that glibenclamide exerted the same effects in isolated rat β cells and in β cells in the rat pancreas (Ling *et al.* 2006). Adequate regulation of protein translation and synthesis in β cells is crucial for maintaining their survival and their insulin stores (Hoorens *et al.* 1996). Studies can thus be planned to investigate whether glibenclamide treatment of rats can help to maintain the functional β -cell mass through effects on β -cell protein synthesis and survival.

In conclusion, mTOR, PKA, and AMPK represent three protein kinases that are important targets of existing anti-diabetic pharmacologicals (Leibowitz *et al.* 2008, Rutter & Leclerc 2009) and that are expressed in β cells. Glibenclamide is now shown to act through stimulation of mTOR and PKA, while preventing the decline in protein synthesis and β -cell viability that are associated with AMPK activation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Q W, D P, and Z L designed the research; Q W and Y C performed the research; Q W and Z L analyzed the data; Q W, M V C, D P, and Z L wrote the manuscript.

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