AMP-activated protein kinase agonist dose dependently improves function and reduces apoptosis in glucotoxic β-cells without changing triglyceride levels

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Abstract

Prolonged hyperglycaemia leads to impaired glucose-stimulated insulin secretion (GSIS) and apoptosis in insulin-producing β-cells. The detrimental effects have been connected with glucose-induced lipid accumulation in the β-cell. AMP-activated protein kinase (AMPK) agonist, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), promotes utilization of nutrient stores for energy production. It was tested how impaired GSIS and elevated apoptosis observed in insulinoma (INS)-1E cells after prolonged culture at 27 mM glucose were affected by the inclusion of 0.3 or 1 mM AICAR during culture. Glucose-induced impairment of insulin release was reverted by the inclusion of 0.3 but not 1 mM AICAR, which did not affect insulin content. The glucose-induced rise in triglyceride (TG) content observed in the cells cultured at 27 mM glucose was not altered by the inclusion of either 0.3 or 1 mM AICAR. Inclusion of 1 but not 0.3 mM AICAR during culture induced phosphorylation of AMPK and its downstream target acyl-CoA carboxylase. Phosphorylation was paralleled by reduced number of apoptotic cells and lowered expression of pro-apoptotic C/EBP homologous protein (CHOP). In conclusion, AICAR dose dependently improves β-cell function and reduces apoptosis in β-cells exposed to prolonged hyperglycaemia without changing TG levels.

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Introduction

Pancreatic β-cells exposed to high glucose concentrations for an extended time period show impaired glucose-stimulated insulin secretion (GSIS) (Eizirik et al. 1992) and increased β-cell death (Leonardi et al. 2003), a phenomenon known as glucotoxicity. The manifestations of elevated glucose concentrations are also evident in the insulin-producing cell line INS-1 (Roche et al. 1998). Indeed, when the cells from the sub-clone INS-1E were cultured at 20 or 27 mM for 5 days, subsequent GSIS was significantly impaired (Nyblom et al. 2006). Exposure of β-cells to elevated levels of glucose up-regulates genes controlling lipogenesis (Wang et al. 2005a) and promotes glucose-induced lipid de novo synthesis (Berne 1975, Nyblom et al. 2008). Such lipid accumulation has been implicated in the deterioration of β-cell function (Unger et al. 1999). Under conditions of nutrient abundance, AMP-activated protein kinase (AMPK), a key regulator of cellular energy status, is inhibited (Winder & Hardie 1999, Kahn et al. 2005). By contrast, when ATP production is inhibited or ATP consumption accelerated with ensuing rise of the AMP:ATP ratio, AMPK is activated (Kahn et al. 2005). Activation of AMPK is associated with enhanced glucose utilization and fatty acid oxidation (Zhang & Kim 1995, Winder & Hardie 1999, Winder 2001, Zhou et al. 2001, Yamauchi et al. 2002). In addition, AMPK activation directs the cell away from lipogenesis (Zhang & Kim 1995, Zhou et al. 2001). Based on these results, it was hypothesized that AMPK activation would have positive effects on the impairment in function and mass of β-cells exposed to elevated levels of glucose. The hypothesis is supported by the results with metformin that activates AMPK indirectly (Zhou et al. 2001) through inhibition of the respiratory chain (El-Mir et al. 2000). This widely used anti-diabetic drug decreases basal and glucose-stimulated insulin plasma levels in patients, which have been attributed to increased peripheral insulin sensitivity (Rutter et al. 2003). To what extent metformin or the AMPK agonist, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR; Corton et al. 1995), have protective or detrimental effects on β-cell function and mass is unclear, however (Lupi et al. 2002a, Leclerc et al. 2004, Marchetti et al. 2004). To address the hypothesis, INS-1E cells were cultured for 5 days at elevated levels of glucose in the presence or absence of different concentrations of AICAR. The AMPK agonist improved GSIS and reduced apoptosis. The effects were dose dependent and not associated with alterations in the triglyceride (TG) content, however.
Materials and methods

Chemicals

Reagents of analytical grade and Milli-Q water were used. Culture plates were from Falcon (BD Biosciences Labware, Franklin Lakes, NJ, USA). RPMI 1640 culture medium, Dulbecco’s PBS, HEPES, fetal bovine serum (FBS), glutamine, sodium pyruvate, penicillin and streptomycin were purchased from Invtrogen. BSA was obtained from Roche Diagnostics. The antibody against BiP was from Abcam (Cambridge, UK). The antibodies against phosphorylated AMPK (p-AMPK), AMPK, phosphorylated acyl-CoA carboxylase (p-ACC), acyl-CoA carboxylase (ACC) and phosphorylated eIF2α (p-eIF2α) were purchased from Cell Signaling (Beverly, MA, USA). The anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

INS-1E cells were kindly supplied by Claes Wollheim and Pierre Maechler, Geneva, Switzerland, and cultured (passages 79–83) for 24 h in 6-, 12- or 24-well plates at 37 °C in a humidified atmosphere containing 5% CO2 in RPMI 1640 medium containing 11 mM glucose and supplemented with 10 mM HEPES, 10% heat-inactivated FBS, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin. After the initial culture period, the culture medium was replaced by an identical medium with the exception of the glucose concentration, which was 5.5, 11 or 27 mM with or without 0.3 or 1 mM AICAR. Culture was continued for 5 days, a time period required to obtain alterations in GSIS in INS-1E cells in response to culture in the presence of elevated glucose concentrations (Nyblom et al. 2006). Culture medium was changed every 48 h.

Insulin secretion and content

After 5-day culture in 24-well plates, when the INS-1E cells were confluent, insulin release and content were measured, as described previously (Nyblom et al. 2006). In short, after pre-incubation of the cells in glucose-free buffer supplemented with 0.1% (w/v) BSA, the glucose-free buffer was replaced by the same buffer supplemented with either 3 or 15 mM glucose and the cells were incubated for 30 min at 37 °C. Samples of insulin release were stored at −20 °C until analysis, which was performed by a competitive ELISA (Bergsten & Hellman 1993). After insulin release measurements, the cells were washed twice with PBS and lysed with a buffer containing 10 mM Tris, 150 mM NaCl, 0-1% (w/v) SDS, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 5 mM EDTA and adjusted to pH 7.2. PIC was added to the lysis buffer prior to the sample preparation. Total protein content was determined by the DC Protein Assay (Bio-Rad).

Apoptosis assay

Apoptosis was measured with the Cell Death Detection ELISA PLUS kit (Roche), which determines the amount of apoptotic mono- and oligonucleosomes in a sample. After the culture period, INS-1E cells from a 24-well plate were washed with PBS and lysed with 200 μl of the supplied lysis buffer. After a 30-min incubation at room temperature, the lysate was spun at 177 g for 10 min. The assay was performed using 20 μl supernatant in the ELISA, according to the manufacturer’s instructions. Apoptosis, determined by optical density, was correlated with total protein determined by the DC Protein Assay.

TG content

INS-1E cells from two wells in a 6-well plate were scraped and suspended in 100 μl buffer containing 20 mM Tris, 150 mM NaCl, 2 mM EDTA and 1% (v/v) Triton X-100 (pH 7-5). TGs were extracted in 3 ml chloroform: methanol (2:1, v/v). Samples were resuspended in 50 μl chloroform from which 20 μl, in duplicate, were transferred to microtubes and air dried. Thesit (5 μl, 10% w/v) was added to the dry pellet. After the Thesit had dried, 10 μl H2O was added (Briaud et al. 2001). TGs were measured using a commercial kit (Infinity TGs Liquid Stable Reagent; Thermo Electron, Melbourne, Australia) and the TG content was correlated with total protein determined by the DC Protein Assay. The triolein standard curve, used to determine the TG content, was treated in parallel with the samples.

Western blot

INS-1E cells were lysed in a buffer containing 10 mM Tris (pH 7.2), 150 mM NaCl, 0-1% (w/v) SDS, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 5 mM EDTA, 1 mM sodium orthovanadate and PIC. Total protein content was determined by the DC Protein Assay.
Protein Assay. The samples were separated by SDS-PAGE (10%), electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes and probed with antibodies against p-AMPK (1:1500 dilution), AMPK (1:3000 dilution), p-ACC (1:1000 dilution), p-eIF2α (1:1000 dilution), BiP (1:10,000 dilution) or CHOP (1:2000 dilution). Signal detection was performed using enhanced chemiluminescence (ECL) (Advanced or Plus) detection kit (Amersham Biosciences) and the Fluor-S MAX Multi-Imager (Bio-Rad). Signals were quantified using the Quantity One software (Bio-Rad). Subsequently, the PVDF membranes were stained with Coomassie, imaged, scanned and quantified with Quantity One software. The expression level of each protein was normalized to the corresponding Coomassie-stained lane.

**Statistical analysis**

Differences in insulin secretion, TG content, apoptosis and differences in protein expression levels were evaluated using ANOVA with Tukey’s post hoc test. \( P < 0.05 \) was considered significant. Values were expressed as means ± S.E.M.

**Results**

**GSIS and AMPK activity of INS-1E cells exposed to elevated glucose concentrations in the presence or absence of AICAR**

Insulin secretion in response to 3 or 15 mM glucose was measured from INS-1E cells cultured for 5 days at 5.5, 11 or 27 mM glucose (Fig. 1). As reported previously (Nyblom et al. 2006), insulin release in response to 15 mM glucose was drastically reduced for the cells cultured at 27 mM glucose compared with the cells cultured at 11 mM glucose. When AMPK agonist AICAR (0.3 mM) was included during culture of INS-1E cells at 27 mM glucose, insulin secretion in response to 15 mM glucose was similar to the levels observed in control cells cultured in the presence of 11 mM glucose (Fig. 1). Insulin release in the presence of 3 mM glucose was raised, however. When the AICAR concentration was increased to 1 mM, basal insulin release increased further and stimulatory release was curtailed. Similar results were obtained for the cells cultured in the presence of 11 mM glucose with enhanced insulin secretion at 15 mM glucose in the presence of 0.3 mM AICAR, which was reversed when 1 mM of the agonist was added to the culture medium. Basal insulin secretion at 3 mM glucose was dose dependently increased by AICAR also for the cells cultured at 11 mM glucose (Fig. 1).

Insulin content of INS-1E cells cultured at 5.5, 11 or 27 mM glucose was determined after the 5-day culture period and normalized to total protein (Fig. 2). Significantly lower amounts of insulin were detected in the cells cultured at 11 compared with 5.5 mM glucose. This effect was further accentuated for the cells cultured at 27 mM glucose, as reported previously (Nyblom et al. 2006). Inclusion of AICAR in the culture medium did not change the insulin content of the cells, however.

High glucose concentrations have been shown to inactivate AMPK in the β-cell (Salt et al. 1998, da Silva Xavier et al. 2003). When the degree of phosphorylation of AMPK was determined by measuring the ratio between p-AMPK and total AMPK, it was decreased in INS-1E cells cultured in the presence of 11 mM glucose compared with 5.5 mM glucose (Fig. 3). No further reduction was observed in the cells cultured in the presence of 27 mM glucose. When p-AMPK and AMPK
were measured in the presence of 0.3 or 1 mM AICAR, the lower concentration did not affect the ratio between phosphorylated and total AMPK (Fig. 3). At 1 mM of the agonist, the ratio was increased, however. In the presence of the higher AICAR concentration, p-AMPK and AMPK levels were similar to those measured in the cells cultured in the presence of 5.5 mM glucose. The activity of AMPK was determined by measuring the ratio between phosphorylated and total amounts of its downstream product ACC. As observed previously (Roche et al. 1998), total amounts of ACC were induced with increased glucose concentration (Fig. 3). When the p-ACC:ACC ratio was analysed, it tended to be lower already at 11 mM glucose, although 27 mM of the sugar was required to obtain a significant difference in the ratio compared with the cells cultured at 5.5 mM glucose (Fig. 3). When 0.3 mM AICAR was added to the culture medium, there was no change in the ratio between p-ACC and ACC, similar to the finding with p-AMPK and AMPK. Also in agreement with the results obtained with 1 mM AICAR on the p-AMPK:AMPK ratio, the higher concentration of the agonist enhanced the p-ACC:ACC ratio (Fig. 3).

TG levels in INS-1E cells exposed to elevated glucose concentrations in the presence or absence of AICAR

Extended culture of INS-1E cells at elevated glucose levels leads to glucose-derived de novo lipid synthesis and accumulation (Nyblom et al. 2008). After 5 days of culture at elevated glucose levels, a fivefold increase in fatty acyls was observed (Nyblom et al. 2008). In the present study, it was tested whether the observed beneficial effect of 0.3 mM AICAR on GSIS was connected with decreased levels of TGs. To this aim, TG levels were determined in INS-1E cells cultured for 5 days at 5.5, 11 or 27 mM glucose with or without AICAR (Fig. 4). The TG content increased twofold for the cells cultured at 27 mM glucose when compared with the cells cultured at 11 mM glucose and more than threefold when compared with the cells cultured at 5.5 mM glucose, which is comparable with our previous NMR-based measurements (Nyblom et al. 2008). When 0.3 or 1 mM AICAR was included during culture of INS-1E cell, the TG content was not affected in the cells cultured in the presence of 11 or 27 mM glucose, however.

Figure 3 Levels of phosphorylated and total AMPK and ACC in INS-1E cells cultured for 5 days at 5.5, 11 or 27 mM glucose in the presence or absence of AICAR. Ratios between phosphorylated and total AMPK and ACC are shown as means ± S.E.M. (n=4). *P<0.05 compared with 5.5 mM glucose. #P<0.05 compared with the absence of AICAR. Cells cultured at 5.5 mM glucose denote control.

Figure 4 Triglyceride content in INS-1E cells cultured for 5 days at 5.5, 11 or 27 mM glucose in the presence or absence of AICAR. Results show means ± S.E.M. (n=4). *P<0.05 compared with 5.5 mM glucose.
Apoptosis in INS-1E cells exposed to elevated glucose concentrations in the presence or absence of AICAR

Deterioration of GSIS in β-cells exposed to elevated levels of glucose is connected with the loss of β-cell mass (Eizirik et al. 1992, Butler et al. 2003, Leonardi et al. 2003). Based on the improved secretory response observed in the presence of 0.3 mM AICAR (Fig. 1), we hypothesized that AICAR reversed such loss of β-cells. Apoptosis was measured in INS-1E cells cultured for 5 days at 5·5, 11 or 27 mM glucose in the presence or absence of AICAR. Apoptosis was not affected in the cells cultured at 11 mM glucose but increased sevenfold in the cells cultured in the presence of 27 mM glucose compared with the cells cultured in the presence of 5·5 mM glucose (Fig. 5). When 1 mM AICAR was included in the culture medium, the number of apoptotic cells observed after culture at 27 mM glucose was significantly reduced. No decrease in apoptosis was observed when 0·3 mM AICAR was included.

ER stress markers in INS-1E cells exposed to elevated glucose concentrations in the presence or absence of AICAR

Apoptosis has been connected with endoplasmic reticulum (ER) stress in INS-1E cells exposed to elevated glucose concentrations for extended time periods (Wang et al. 2005a). When the levels of ER stress-related protein CHOP (Harding & Ron 2002) were measured, there was a glucose-regulated increase in the pro-apoptotic protein (Fig. 6). After 1 mM AICAR was included during culture of INS-1E cells, CHOP levels were diminished both in the cells cultured at 11 and 27 mM glucose. No reduction in the CHOP levels was observed when 0·3 mM AICAR was included during culture. When the levels of p-eIF2α and BiP were measured in INS-1E cells cultured at elevated glucose concentrations in the presence or absence of 0·3 or 1 mM AICAR, neither glucose nor AICAR altered the levels of the proteins.

Discussion

In the present study, the ability of AICAR to protect INS-1E cells from the negative effects of extended hyperglycaemia was investigated. Inclusion of AICAR in INS-1E cells cultured at elevated glucose concentrations decreased apoptosis and improved GSIS. The AICAR concentrations at which these beneficial effects occurred differed, however. Whereas functional improvement was obtained at a lower (0·3 mM) AICAR concentration, decreased apoptosis required a higher (1 mM) concentration of the AMPK agonist. The concentration-dependent effects may contribute to explain why the inclusion of AICAR during culture has been associated with improved (Akkan & Malaisse 1994, Malaisse et al. 1994, Yamashita et al. 2004, Wang et al. 2005a), inhibited (Salt et al. 1998, da Silva Xavier et al. 2003) or not affected (Zhang & Kim 1995) GSIS. In addition, duration of agonist exposure varies between the studies and may also contribute to explain the divergent effects of the agonist. The dose-dependent secretory improvement was not related to the effects of the AMPK agonist on insulin content, which was not affected by either of the AICAR concentrations used.

Activated AMPK was determined by measuring phosphorylated levels of AMPK and ACC as in other studies (da Silva Xavier et al. 2003, Yamashita et al. 2004). Using this approach, increased phosphorylation of the kinase and its downstream target was observed in...
the presence of 1 mM but not 0.3 mM AICAR. It could be concluded that improved GSIS observed in the presence of the lower AICAR concentration was not caused by an increase in the ratio of p-AMPK and AMPK or p-ACC and ACC. The present study demonstrated that AICAR had pleiotropic effects on insulin secretion. First, AICAR enhanced GSIS but this insulinotropic effect of the AMPK agonist was abolished in a concentration-dependent manner. K\textsubscript{ATP} channel conductivity has been proposed to be the target of the stimulating effect of AICAR for insulin release (Wang et al. 2005b), whereas diminished glucose metabolism with the reduction in ATP generation and Ca\textsuperscript{2+} influx may be a mechanism of the inhibiting effect of the agonist (da Silva Xavier et al. 2003). Secondly, AICAR elevated basal insulin release in a concentration-dependent manner irrespective of the culture glucose concentration. The rise in basal insulin release induced by AICAR has been observed previously (Akkan & Malaisse 1994, Salt et al. 1998, da Silva Xavier et al. 2003, Wang et al. 2005b) and has been attributed to enhanced glucose metabolism and has also been observed when β-cells are exposed to elevated levels of fatty acids (Zhou & Grill 1994, Milburn et al. 1995). By contrast, INS-1E cells exposed to chronic hyperglycaemia have shown impaired GSIS without increase in basal insulin secretion, although elevated levels of total fatty acyls were recorded (Nyblom et al. 2008). Enhanced glucose metabolism is probably not responsible for elevated basal release in the presence of AICAR since glucose metabolism has been reported to be unaffected or even decreased by the agonist (da Silva Xavier et al. 2003). Instead, activation of AMPK is associated with enhanced glucose utilization and fatty acid oxidation (Zhang & Kim 1995, Winder & Hardie 1999, Winder 2001, Zhou et al. 2001, Yamauchi et al. 2002) and decreased lipogenesis (Zhang & Kim 1995, Zhou et al. 2001).

Lowered apoptosis in the presence of AICAR has been demonstrated in β-cells exposed to elevated levels of glucose and fatty acids (El-Assaad et al. 2003) and attributed to redirection of fatty acids from esterification to oxidation (Corton et al. 1995, Merrill et al. 1997, Velasco et al. 1997, Muoio et al. 1999, El-Assaad et al. 2003). In β-cells exposed to elevated glucose concentrations, lipid de novo synthesis occurs (Berne 1975, Dunlop & Larkins 1985, Roche et al. 1998, Nyblom et al. 2008). Given the results that AICAR did not affect the TG content of the β-cells in the present and a previous similarly designed study (Yamashita et al. 2004), the explanation of redirection from esterification to oxidation seems less plausible under conditions of elevated glucose levels alone. In addition, metformin has been reported to affect insulin secretion in β-cells positively without lowering the TG content of the cell (Lupi et al. 2002b). Deposition of excess fatty acids as the TGs has been both positively and negatively correlated with fatty acid-induced β-cell death (Shimabukuro et al. 1998, Higa et al. 1999, Cnop et al. 2001, Lupi et al. 2002a). It appears that the constituent fatty acids incorporated into the TGs are the determinants to what extent the lipid accumulation is detrimental or not, where both chain length and degree of saturation could play roles (Cnop et al. 2001, Busch et al. 2005). In this context, it was observed that β-cell lipid accumulation in the presence of externally applied saturated fatty acid palmitate was clearly harmful, affecting the morphology of the ER (Moffitt et al. 2005). Under such conditions, redirection from esterification to oxidation of fatty acids becomes critical and may be operative (El-Assaad et al. 2003). An explanation between the difference in lipid accumulation in the presence of glucose and fatty acids was offered when it was demonstrated that lipogenesis in response to hyperglycaemia resulted in the generation of both saturated and unsaturated fatty acid species in proportions similar to those found in control cells (Nyblom et al. 2008), which is less harmful for β-cell function than when exposed to saturated fatty acids (Moffitt et al. 2005). It remains to be determined to what extent these proportions are altered when sterol regulatory element binding protein (SREBP)-1c is overexpressed, making the cell more prone to lipogenesis. When overexpressing the lipogenic transcription factor (Wang et al. 2003, Diraison et al. 2004), increased TG levels were observed in islets and INS-1E cells, which were normalized by AICAR (Diraison et al. 2004, Yamashita et al. 2004). The observed decrease in apoptosis without changes in the TG content in the present study renders further support for the view that lipid accumulation per se is not detrimental for the β-cell (Cnop et al. 2001). In two recent studies, it was reported that AICAR induced apoptosis (Cai et al. 2007, Kim et al. 2007). The fact that p-AMPK levels were not reduced but rather increased after prolonged exposure to elevated glucose levels in the insulinoma cell line used may contribute to explain the divergent effect of the agonist in these cells.

Prolonged elevated glucose concentrations have also been associated with ER stress (Wang et al. 2005a). ER stress is induced under conditions of enhanced protein synthesis. If the protein load surpasses the capacity of the ER to handle cargo proteins, accumulation of unfolded or misfolded proteins in the ER occurs, which elicits the unfolded protein response (UPR). The UPR is a cellular programme by which the cell attempts to alleviate ER stress (Rutkowski & Kaufman 2004). If not alleviated, signalling pathways are initiated leading to apoptosis, where CHOP is a component protein (Harding & Ron 2002). From the observation that AICAR reduced the levels of the pro-apoptotic protein CHOP, it can be proposed that ER stress alleviation is a contributing mechanism by which AICAR reduces apoptosis under glucotoxic conditions. However, no
change in the phosphorylation of eIF2α was observed. Although the lack of effects on p-eIF2α may be due to time kinetics in the phosphorylation of the protein (Laybutt et al. 2007), a more plausible explanation is that the enhanced CHOP expression is the result of mechanisms not related to ER stress.

In conclusion, although AICAR-induced activation of AMPK reduced apoptosis and improved insulin release in β-cells exposed to high glucose concentrations, these positive effects occurred at different concentrations of the agonist. Indeed, when AICAR at a given concentration positively affected one β-cell parameter, other β-cell parameters deteriorated. These effects of AICAR on β-cell function and mass make the administration of the agonist questionable as a strategy to treat individuals with type 2 diabetes mellitus.

**Declaration of Interest**

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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