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Biochemical Pharmacology 68 (2004) 409–416
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Metformin-induced stimulation of AMP-activated protein kinase in β-cells impairs their glucose responsiveness and can lead to apoptosis

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Received 6 January 2004; accepted 15 April 2004

Abstract

Metformin is an anti-diabetic drug that increases glucose utilization in insulin-sensitive tissues. The effect is in part attributable to a stimulation of AMP-activated protein kinase (AMPK). The present study demonstrates that metformin (0.5–2 mM) also dose-dependently activates AMPK in insulin-producing MIN6 cells and in primary rat β-cells, leading to increased phosphorylation of acetyl coA carboxylase (ACC). The maximal effect was reached within 12 h and sustained up to 48 h. After 24 h exposure to metformin (0.5–1 mM), rat β-cells exhibited a reduced secretory and synthetic responsiveness to 10 mM glucose, which was also the case following 24 h culture with the AMPK-activator 5-amino-imidazole-4-carboxamide riboside (AICAR; 1 mM). Longer metformin exposure (>24 h) resulted in a progressive increase in apoptotic β-cells as was also reported for AICAR; metformin-induced apoptosis was reduced by compound C, an AMPK-inhibitor. As with AICAR, metformin activated c-Jun-N-terminal kinase (JNK) and caspase-3 prior to the appearance of apoptosis. It is concluded that metformin-induced AMPK-activation in β-cells reduces their glucose responsiveness and may, following sustained exposure, result in apoptosis.

Keywords: Metformin; AMP-activated protein kinase; AICA-riboside; β-cell metabolism; Apoptosis; Diabetes

1. Introduction

The biguanide metformin was introduced as anti-diabetic drug several decades ago [1]. The agent has been found to lower blood glucose levels in type 2 diabetic patients by facilitating glucose utilization in skeletal muscle and reducing hepatic glucose production [2–5]. These effects appear mediated through inhibition of complex 1 in the mitochondrial respiratory chain [6,7] and/or stimulation of AMP-activated protein kinase (AMPK) [8]. AMPK is activated under conditions that deplete cellular ATP and elevate AMP, such as glucose deprivation, heat shock, hypoxia, and ischemia [9,10]. AMPK then phosphorylates and inactivates a number of metabolic enzymes involved in fatty acid and cholesterol synthesis, including acetyl coA carboxylase (ACC) and HMG coA reductase [11,12]. In liver, AMPK-activation by metformin results in reduced gluconeogenesis. Recent studies indicate that metformin may activate AMPK through AMP-independent pathways [13–15]. It is so far unknown whether the drug also activates AMPK in pancreatic β-cells and may thus influence the role of these cells in drug treated type 2 diabetic patients. According to an early in vitro study the drug can impair β-cell functions [16]. It has also been shown that a short-term activation of AMPK by AICAR inhibits glucose-induced insulin release in insulin-producing cell lines and pancreatic islets [9,17] and that a sustained activation by AICAR induces apoptosis in rat β-cells as well as in insulin-producing MIN6 cells [18,19]. In the present study, we investigated whether metformin stimulates AMPK in these cell preparations, and whether this affects their function and viability.

Abbreviations: AMPK, AMP-activated protein kinase; AICAR, 5-amino-imidazole-4-carboxamide-riboside; ACC, acetyl coA carboxylase; JNK, c-Jun-N-terminal kinase

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doi:10.1016/j.bcp.2004.04.003

2. Materials and methods

2.1. Cell culture

Pancreatic islets were isolated from male Wistar rats, dissociated, and β-cells FACS purified, as previously described [20]. Purified β-cells were cultured as single or re-aggregated cells in serum-free HAM's F10 medium (GIBCO) containing 10 mmol/l glucose, and supplemented with 0.075 mg/ml penicillin, 0.1 mg/ml streptomycin, 2 mmol/l L-glutamine, 0.5% (w/v) bovine serum albumin pretreated with charcoal (BSA, fraction V, Sigma), and 50 μmol/l 3-isobutyl-1-methylxanthine (IBMX, Janssen Chimica) [21]. MIN6 cells (passages 18–30) were grown in Dulbecco's modified Eagles medium (DMEM) containing 15% (v/v) heat-inactivated fetal calf serum, 25 mM glucose, and 50 μM β-mercaptoethanol. MIN6 cells (2×10^6) were cultured in six well-plates for 24 h and then exposed to the indicated conditions. Metformin (1,1-dimethylbiguanide, Sigma), 5-amino-imidazole-4-carboxamide-riboside (AICA-riboside), Sigma, were added to cultures as indicated. The general caspase inhibitor z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk, Bachem), and AMPK-inhibitor compound C (kind gift of Merck Research Laboratories) were added 1 h before AICA-riboside or metformin.

2.2. Apoptosis measurement

The viable, necrotic, and apoptotic cells were determined by fluorescence microscopy using propidium iodide (PI, Sigma) and Hoechst 3342 (HO342, Sigma) [22]. Viable cells were identified by their intact nuclei with blue fluorescence (HO342), necrotic cells by their intact nuclei with yellow–red fluorescence (HO 342 + PI), and apoptotic cells by their fragmented nuclei with either blue (HO342, early apoptosis) or yellow–red fluorescence (HO 342 + PI, late apoptosis). Apoptosis and necrosis in β-cell cultures was quantified by counting >300 cells per condition in triplicate for each experiment. Apoptosis was also measured by FACS analysis of nuclear DNA content of both primary cells and cell lines, as previously described [19,23].

2.3. Caspase-3 assay and protein measurement

MIN6 cells were lysed in 10 mM HEPES-KOH buffer pH 7.5, containing 10 mM dithiothreitol (DTT), 0.1% CHAPS (Sigma), 1% Triton X-100, 2 mM EDTA, followed by a 30 min incubation on ice [19]. Cleared cell lysates were recovered after centrifugation for 3 min at 10,000 rpm and stored at −20 °C. Five or ten microliter of these extracts were incubated with the peptide DEVD-AFC for 1 h at 37 °C, and the cleaved product was detected fluorimetrically at 505 nm (excitation at 400 nm) [19]. Caspase-3 activity was expressed as fluorescence units per mg protein and per hour.

Protein concentration in lysates was measured using the Micro-BCA reagent (Pierce) with bovine serum albumin as a standard.

2.4. Western blot analysis

MIN6 or primary β-cells were harvested, washed with PBS, lysed in RIPA buffer containing protease inhibitors [24] and centrifuged at 12,000 rpm for 3 min to remove cell debris. Twenty five micrograms of protein was mixed with sample buffer to obtain 5% SDS, 0.05% w/v Bromophenol Blue, 10% glycerol, 5% β-mercaptoethanol in 80 mM Tris–HCl pH 6.8. Proteins were resolved by 5 or 10% (acrylamide:bisacrylamide, 37.5:1) SDS-PAGE, transferred to nitrocellulose membranes (Schleicher & Schuell) and specific proteins detected by immunoblotting as previously described [24]. Primary polyclonal antibodies against actin (Santa Cruz Biotechnology), phospho-(Thr 172)-AMPK, total-AMPK (α1 and α2 isoforms of catalytic subunit), phospho-(Thr183/Tyr185)-JNK1/2, total JNK (Cell Signaling Technology), phospho-(Ser79)-acetyl coA carboxylase (Upstate), and monoclonal anti-phospho-(Ser63)-c-Jun (Santa Cruz Biotechnology), were applied overnight at 4 °C, and secondary antibodies linked to horseradish peroxidase for 1 h at room temperature. Immunoreactive proteins were revealed using enhanced chemiluminescence detection (ECL, Amersham).

2.5. Protein biosynthesis and insulin release measurements

After overnight preculture in HAM's F10 medium, re-aggregated primary β-cells were cultured for 24 h with or without metformin or AICA-riboside. At the end of culture, medium was taken for measurement of insulin release, and cells were collected for analysis of their insulin content, glucose-induced insulin release, protein and insulin synthesis [25].

2.6. Glucose oxidation and utilization

Glucose utilization and oxidation were measured, as described previously [26], using duplicate samples of primary rat β-cell aggregates (4×10^4 cells) which had been cultured for 24 h with or without metformin or AICAR. The incubation was carried out for 2 h at 37 °C in 100 μl HAM's F10 medium containing 0.5% BSA buffered with 10 mM HEPES and 5 μCi D-[U-^{14}C] glucose, or 10 μCi D-[5-^{3}H] glucose (Amersham), and different concentrations of unlabeled D-glucose.

2.7. Data analysis

Results from cell-counting are expressed as apoptosis indexes, calculated as [apoptotic cells in experimental

condition (%)−apoptotic cells in control (%)/living cells in control (%)] × 100.

Results are shown as means ± S.E.M., or representative traces and pictures, for the indicated number of independent experiments. Statistical significance of differences was calculated by ANOVA with Fisher PLSD-test.

3. Results

3.1. Metformin stimulates AMPK in MIN6 cells and in rat β-cells

Metformin (0.5–2 mM) dose-dependently increased the phosphorylation of AMPK in MIN6 cells (Fig. 1A). This effect was detectable after 3 h, reached its maximum within 12 h, and maintained this plateau upto 48 h (Fig. 1B). The agent also stimulated phosphorylation of acetyl coA carboxylase, a known substrate of AMPK; this effect was also present after 3 h and progressively increased upto 48 h (Fig. 1B). Exposure of rat β-cells to 1 mM metformin for 2 h induced phosphorylation of AMPK (Fig. 2A). This effect was comparable to that of

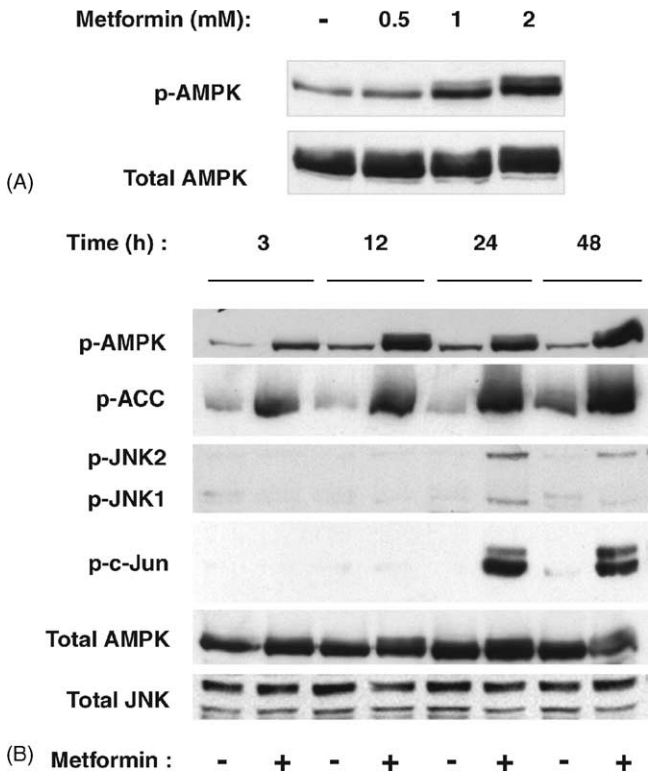

Fig. 1. Metformin activates AMP-activated protein kinase and c-Jun-N-terminal kinase in MIN6 cells. (A) MIN6 cells were exposed to the indicated concentrations of metformin for 24 h or (B) were exposed to 2 mM metformin for the indicated times. Protein was extracted and phosphorylated AMPK (p-AMPK), total-AMPK, phosphorylated acetyl coA carboxylase (p-ACC), phosphorylated JNK (p-JNK1/2), total JNK, and phosphorylated c-Jun (p-c-Jun) were detected by immunoblotting using specific antibodies. Results shown are representative of three independent experiments.

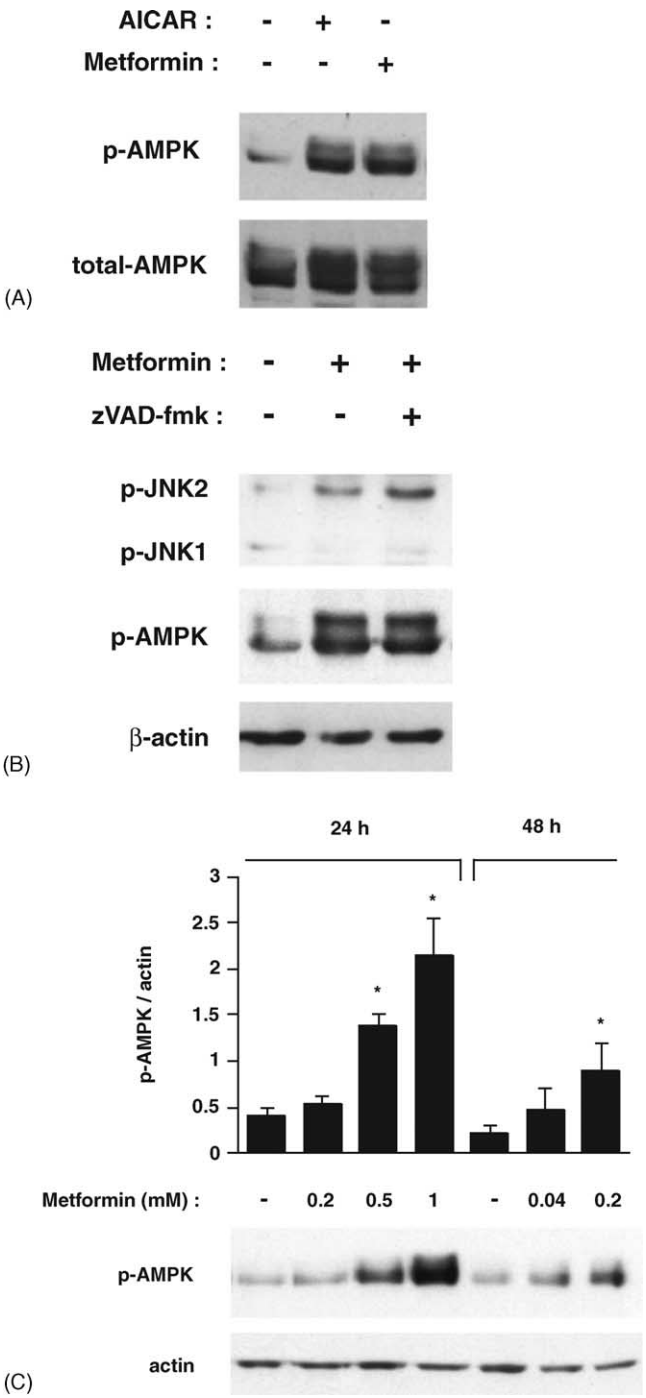

Fig. 2. Metformin-induced activation of AMP-activated protein kinase and c-Jun-N-terminal kinase in β-cells. (A) primary β-cells (2×10^5) were maintained for 2 h in medium with or without metformin (1 mM), or AICA-riboside (AICAR; 1 mM), as indicated. Cell lysates were analyzed for phosphorylated AMPK (p-AMPK) and total AMPK by immunoblotting as in Fig. 1. (B) β-cells were cultured for 24 h in the absence or presence of metformin (1 mM) and zVAD-fmk (50 μM), and the effects on phosphorylation of AMPK and c-Jun-N-terminal kinase examined by immunoblotting as described in (A). Actin was detected to evaluate sample loading. Results shown are representative of three independent experiments. (C) β-cells were cultured as in (A) for 24 or 48 h, at the indicated concentrations of metformin, afterwhich p-AMPK and actin were detected by immunoblotting. The results are expressed as the ratio of p-AMPK/actin and represent the mean ± S.E.M. of four independent experiments (*$P < 0.05$ by ANOVA).

Table 1
Effect of metformin and AICA-riboside on insulin content, synthesis, and release, and glucose oxidation and utilization in rat β-cells

Glucose	Insulin					Glucose	
	Content (ng/K cells/h)	Synthesis (dpm/cell/h)		Release (ng/K cells/h)		Oxidation (pmol/K cells/2h)	Utilization (pmol/K cells/2h)
		0 mM	10 mM	0 mM	10 mM		
Control	26.4 ± 2.2	1.0 ± 0.3	5.8 ± 0.7	0.5 ± 0.0	2.7 ± 0.4	11.1 ± 1.4	31.8 ± 6.7
Metformin 0.5 mM	22.4 ± 1.8	0.5 ± 0.1	3.6 ± 0.4*	0.4 ± 0.0	2.1 ± 0.3	6.7 ± 1.2*	36.0 ± 6.8
Metformin 1 mM	25.8 ± 1.3	0.2 ± 0.0**	1.4 ± 0.3**	0.5 ± 0.1	0.8 ± 0.1**	5.4 ± 0.7*	29.9 ± 4.7
AICAR 0.5 mM	22.0 ± 2.1	0.5 ± 0.0	4.0 ± 0.3	0.5 ± 0.1	2.0 ± 0.2	ND	ND
AICAR 1 mM	20.1 ± 7.8	0.3 ± 0.1*	1.9 ± 0.7**	0.3 ± 0.1	0.9 ± 0.3**	9.7 ± 2.0	27.7 ± 5.7

Purified rat β-cells were cultured for 24 h with or without metformin and AICA-riboside (AICAR). Thereafter, cells were analyzed for their protein and insulin synthetic activity in the absence of metformine and AICAR. Data represent mean ± S.E.M. of three and five independent experiments. Statistical significance of differences with control was analyzed by ANOVA. Cell viabilities varied by <10% in the different pre-treatment conditions. ND: not determined.

* $P < 0.05$.

** $P < 0.01$.

1 mM of the AMPK-activator AICA-riboside (Fig. 2A), and was sustained for at least 24 h (Fig. 2B). Metformin increased the phosphorylation of AMPK time- and dose-dependently in β-cells (Fig. 2C); AMPK-stimulation was observed with metformin concentrations ⩾40 μM (Fig. 2C).

3.2. Metformin inhibits glucose-induced insulin release and biosynthesis

Culture of β-cells for 24 h in presence of metformin (0.5–1 mM) did not reduce their insulin content but lowered their basal and glucose-induced insulin synthesis and glucose-stimulated release. Similar effects were observed following culture with AICAR 1 mM (Table 1). Metformin also inhibited the rate of glucose oxidation in these cells but not glucose utilization; on the other hand, no inhibition of glucose oxidation was seen with 1 mM AICAR (Table 1).

3.3. Prolonged exposure to metformin induces apoptosis of rat β-cells

After longer exposures (3–14 days), metformin (⩾40 μM) induced a dose-and time-dependent increase in the number of apoptotic β-cells (Table 2). This effect was detected by a microscopic cytotoxicity assay (Table 2) and by FACS analysis (Fig. 3). The apoptosis was prevented by the general caspase inhibitor zVAD-fmk (Fig. 3). An apoptosis effect was also detected in MIN6 cells by FACS analysis, and by an assay of caspase-3 activity (Fig. 4).

3.4. Metformin-induced apoptosis depends on AMPK-activity and involves JNK

Compound C is a cell permeable inhibitor of AMPK-activity. The compound inhibited metformin-induced phosphorylation of acetyl coA carboxylase in rat β-cells (Fig. 5A). It also reduced metformin-induced apoptosis of these cells (Fig. 5B), which suggests that the latter depends at least in part on AMPK-activation. It was previously shown that AMPK-induced apoptosis of MIN6 cells and hepatoma cells involves JNK-activation [19,27]. Our experiments with metformin confirm this finding: the AMPK-activation by this agent is followed by phosphorylation of JNK and of its target c-Jun (Fig. 1B). This effect was detectable after 24 h exposure to metformin and coincided with the caspase-3 activation (Fig. 4). JNK-activation with metformin also occurred in rat β-cells even in the presence of 50 μM of the caspase inhibitor zVAD-fmk, placing it upstream of caspase-activation (Fig. 2B).

Table 2
Effect of metformin on β-cell apoptosis

Culture condition	Exposure time (days)	Apoptosis (%)	Live (%)	N
Control	3	10.5 ± 0.8	79.9 ± 1.2	7
	6	15.1 ± 0.3	74.4 ± 0.6	8
	14	18.8 ± 0.8	72.5 ± 1.0	7
Metformin 40 μM	14	25.1 ± 0.9	65.9 ± 0.9	7
Metformin 200 μM	14	30.9 ± 0.7	59.4 ± 0.9	7
Metformin 500 μM	3	18.1 ± 1.1	66.1 ± 4.8	4
	6	38.7 ± 0.3	42.0 ± 0.7	8
Metformin 1000 μM	3	39.7 ± 0.6	51.0 ± 3.9	8
	6	56.8 ± 0.6	15.4 ± 0.9	8

Single rat β-cells were cultured in medium with or without the indicated metformin concentrations for 3, 6, and 14 days. Apoptotic and live cells were scored by fluorescence microscopy after the addition of propidium iodide and Hoechst 3342, and data expressed as % of total cells, representing the mean ± S.E.M. of the indicated number of experiments. Statistical significance of differences between treated and control groups (no metformin) was analyzed by ANOVA; $P < 0.001$ for all comparisons of metformin vs. respective control.

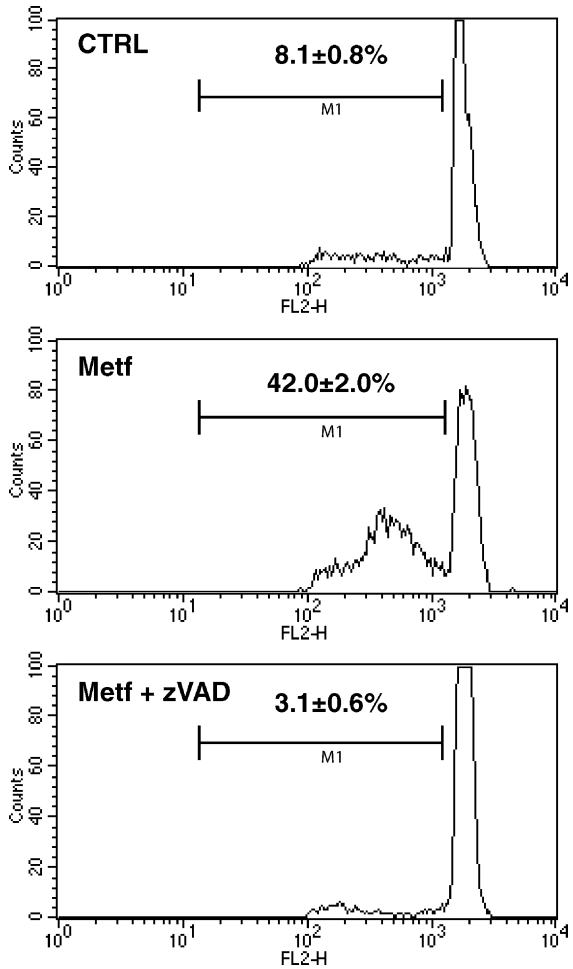

Fig. 3. Metformin-induced apoptosis in β-cells is inhibited by caspase inhibitor zVAD-fmk. Primary β-cells were cultured in the absence (CTRL) or presence of 1 mM metformin (Metf) either alone or with 50 μM of the general caspase inhibitor zVAD-fmk (zVAD) for 3 days. The percentage of cells displaying sub-G1 quantities of DNA was determined by FACS; representative FACS histograms are shown with horizontal bars indicating the gate setting used to detect sub-G1 (apoptotic) cells. Data represent the mean ± S.E.M. of three experiments.

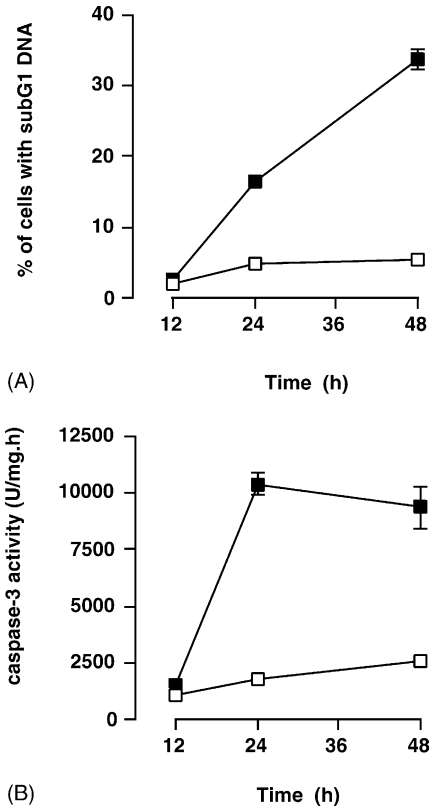

Fig. 4. Effects of metformin on apoptosis and caspase-3 activity in MIN6 cells. (A) MIN6 cells were cultured for the indicated times in medium with (closed symbols) or without (open symbols) metformin (2 mM), and apoptosis (% cells with sub-G1 DNA) was quantified by FACS as in Fig. 3. (B) Caspase-3 activities were measured (see Section 2) in MIN6 cell preparations exposed as in (A); the results were expressed in fluorescence units per mg protein and per hour (U/mg h). Data represent the mean ± S.E.M. of six experiments.

4. Discussion

We previously demonstrated that exposure of pancreatic β-cells and mouse insulinoma MIN6 cells to 5-amino-imidazole-4-carboxamide riboside triggered sequential activation of AMP-activated protein kinase, c-Jun-N-terminal kinase (JNK) and caspase-3, followed by apoptosis of the cells [18,19]. Sustained AMPK-activation was also shown to mediate AICA-riboside-induced apoptosis in liver cells [27] lymphocytic leukemia cells [28] and neuroblastoma cells [29]. AICA-riboside is widely used to activate AMPK pharmacologically; it is converted in cells to ZMP, which mimics the effect of AMP on the AMPK cascade. However, AICA-riboside has been suggested to induce apoptosis independently of ZMP synthesis and AMPK-activation in some cell types [28,30]. The finding that metformin activates AMPK [8], by a mechanism that appears to be independent of changes in adenine nucleotides [13–15], provides an alternative approach to further examine whether the kinase can affect the survival of cells. The present work supports this view by demonstrating that the metformin-induced AMPK-activation in β-cells can lead to their apoptosis. Our results also indicate that JNK may be a mediator of metformin-induced β-cell death, downstream of AMPK-activation; JNK-activation occurred at the time of the caspase-activation but was not dependent on it. This pathway is similar to that previously observed for AICA-riboside-induced β-cell death. It is consistent with the identification of JNK as a downstream target of AMPK [31], and as a mediator of AICA-riboside-induced apoptosis in liver cells [27,31].

Metformin induces an AMPK-activation in primary β-cells within 2 h, while apoptosis was only noticed after an exposure of 24 h. Such delay was also observed in β-cells exposed to AICA-riboside [18], indicating that the cell death pathway is only triggered after sustained activation of AMPK. However, shorter exposure did impair β-cell functions as shown by the reduced glucose-induced insulin release and synthesis in preparations that were cultured for

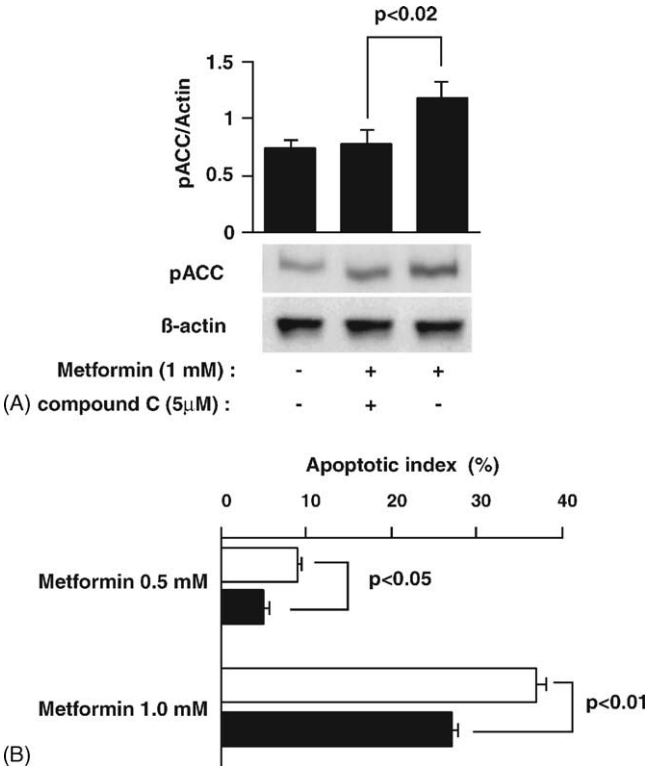

Fig. 5. Effects of AMPK-inhibitor compound C on metformin-induced AMPK-activation and apoptosis in β-cells. Primary β-cells (2×10^5) were maintained for 10 h in control medium or medium containing 1 mM metformin alone or with 5 μM of the AMPK-inhibitor compound C. (A) Cell lysates were subjected to electrophoresis and phosphorylation of acetyl coA (p-ACC) was determined by immunoblotting as a measure for AMPK activity. ACC-phopshorylation was measured in densitometric scans of the blots and expressed as a p-ACC/actin signal ratio. Data represent mean ± S.E.M. of six experiments. (B) Single β-cells were cultured for 3 days in the presence of the indicated concentrations of metformin, either with (black bars) or without (white bars) 5 μM of compound C, and apoptosis induction was determined by cell-counting using fluorescence microscopy (see Section 2). Results are expressed as apoptotic indexes and are the means ± S.E.M. of six experiments.

24 h with either AICAR or metformin. These data confirm the studies by Da Silva Xavier et al., where AICA-riboside as well as expression of constitutively active AMPK affected glucose-stimulated insulin secretion and gene transcription in β-cells [17,32]. Activation of AMPK was also shown to suppress glucose metabolism and to lower cellular ATP levels in MIN6 cells [17]. We also found metformin to inhibit glucose oxidation but not glucose utilization. Since we did not detect an inhibition of glucose oxidation following 24 h culture with AICA-riboside, we believe that the metformin-induced inhibition of glucose oxidation occurs independently of the AMPK-activation, conceivably through its inhibitory action on complex 1 of the mitochondrial respiratory chain [6,7] and could thus affect glucose oxidation at this level. The question is then which common pathway accounts for the reduced functions of β-cells following 24 h culture with metformin or AICAR. A likely mechanism is the inhibition of protein synthesis through AMPK-induced phosphorylation of the translation elongation factor 2 [33]. A similar action may explain the observed reduction in the β-cell protein/insulin synthetic activity and the subsequent decrease in insulin release.

Our results appear to be somewhat at variance with a number of reported effects of metformin. First, metformin has been shown to lower blood glucose levels and to ameliorate insulin sensitivity, however, this beneficial effect is believed to involve predominantly periferal actions of the drug. Second, metformin has been suggested to restore the normal secretory pattern in rat pancreatic islets whose glucose responsiveness was impaired by chronic exposure to elevated glucose or free fatty acid levels in vivo or in vitro [34,35], and the compound appears to protect β-cells under such conditions [36]. While the mechanism of these beneficial actions of metformin on β-cells is unknown, our results suggest that metformin can partially inhibit biosynthesis- and release-pathways in β-cells, which may prevent their functional exhaustion in conditions of chronic exposure to high concentrations of free fatty acids and glucose. Thirty years ago, Schatz et al. did already report that metformin suppresses insulin synthesis and release by isolated rat islets, and suggested that the drug might, in addition to its effects on peripheral metabolism, also exert direct actions on the β-cells [16]. It is of course unknown whether the in vitro concentrations and exposure times are reached in vivo. Steady state plasma levels of metformin in humans are reported to be as high as 40 μM [8,37]. Metformin is known to accumulate in tissues. Studies in rodents have shown that administration of metformin at doses less than those required to lower hyperglycemia in diabetic animals resulted in tissue concentrations of ±200 μM [38,39]. Concentrations of 0.5–2 mM have been used in many in vitro studies on the peripheral effects of metformin, and were also examined in the present work on β-cells. We show that the effects of metformin were dose- and time-dependent in both MIN6 cells and primary β-cells, and that prolonged culture of β-cells with near-therapeutic concentrations (40–200 μM) of the drug [8] activated their AMPK and caused apoptosis. Our finding that low concentrations of metformin can induce AMPK-mediated alterations in β-cells, should be further investigated for its possible in vivo relevance, although there is at present little or no indication of impaired β-cell function in patients treated with metformin. During revision of the present manuscript, Leclerc et al. [40] reported metformin-induced activation of AMPK and decreased β-cell function in cultured human islets.

Acknowledgments

We thank G. Stangé, R. De Proft, and S. Yosef for their assistance. K.K. and G.M. are recipients of a Ph.D. research fellowship, and H.H. of a postdoctoral research fellowship, from the Belgian Fonds voor Wetenschappelijk

Onderzoek. This work was supported by the Interuniversity Poles of Attraction Program (P5/17)-Belgian Science Policy, and grants from the Belgian Foundation for Scientific Research (G.0376.97 and 1.5133.00) and the Brussels Free University, VUB (OZR-898).

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