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Short Communication

AICA-riboside induces apoptosis of pancreatic beta cells through stimulation of AMP-activated protein kinase

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Abstract

Aims/hypothesis. Prolonged exposure of beta cells to low glucose concentrations triggers their apoptosis and is known to activate AMP-activated protein kinase (AMPK) in beta cell lines. We examined whether prolonged activation of AMPK can trigger apoptosis in rodent beta cells.
Methods. Primary beta cells were FACS-purified from rats, and from wild-type and AMPK(alpha2)-deficient mice. AMPK activation in beta cells was induced by the adenosine analog AICA-riboside and detected by immunoblotting using a phosphospecific antibody. Apoptosis of rodent beta cells was monitored by FACS analysis of beta cell DNA content, by direct counting of apoptotic cells using fluorescence microscopy, or by measurement of their caspase-3 activity.
Results. Dose-dependent and time-dependent apoptosis of the cells, concommittant with an activation of caspase-3, were suppressed by the caspase inhibitors zVAD-fmk and zDEVD-fmk. Apoptosis induction by AICA-riboside was also prevented by adding the MAPK-inhibitor SB203580 which blocked the AICA-riboside-induced phosphorylation of AMPK. Beta cells isolated from AMPK-(alpha2)-deficient mice were resistant against AICA-riboside induced apoptosis.
Conclusion/interpretation. Sustained activation of AMPK by AICA-riboside can trigger a caspase-dependent apoptosis of pancreatic beta cells. [Diabetologia (2003) 46:250–254]

Keywords AICA-riboside, AICAR, AMP-activated protein kinase, AMPK, knockout, apoptosis, signalling, beta cell, glucose, diabetes.

Prolonged exposure of pancreatic beta cells to low glucose concentrations results in apoptosis of the cells [1]. The underlying mechanism is not known but might be initiated by altered concentrations of the adenine nucleotides that express and signal the metabolic state of the cells. The rate of glucose oxidation influences cellular ATP, ADP and AMP concentrations and hence their regulatory role on beta cell functions. During glucose deprivation, the AMP over ATP ratio is expected to increase and thus to activate the AMP-activated protein kinase (AMPK). AMPK is a serine-threonine protein kinase that is composed of a catalytic subunit (α) and two regulatory subunits (β and γ) [2]. The control of its activity involves allosteric activation by AMP, as well as phosphorylation by AMPK-kinase, the upstream kinase [2]. In several cell types, this enzyme has been described as a sensor for the energy state of the cells and as a metabolic master switch [2, 3]. In beta cell lines, the AMPK activity is increased by culture at low glucose concentrations [4, 5]. 5-aminoimidazole-4-carboxamide (AICA)-riboside

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Abbreviations: AMPK, AMP-activated protein kinase; IBMX, 3-isobutyl-1-methylxanthine; zVAD-fmk, z-Val-Ala-Asp-fluoromethylketone; zDEVD-fmk, z-Asp-Glu-Val-Asp-fluoromethylketone; MAPK, MAP kinase, mitogen-activated protein kinase; Ac-DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin.

is known to activate AMPK after its phosphorylation into AICA-ribotide, also called ZMP, which is an analog of AMP [2, 3]. AICA-riboside suppressed glucose effects on gene expression and enzyme activities in MIN6 cells [5]. We investigated whether sustained activation of AMPK can mediate apoptosis in primary beta cells.

Materials and methods

Cell isolation and culture. Rat and mouse pancreatic islets were isolated, dissociated and purified to single alpha and beta cells as described [6]. The purified beta cells were either reaggregated or cultured as single cells using a HAM's serum-free medium [6] containing 10 mmol/l glucose and 50 μmol/l of the phophodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). AICA-riboside (Sigma) was added to the incubation medium as indicated. The general caspase-inhibitor z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk, Bachem) or the caspase-3-inhibitor z-Asp-Glu-Val-Asp-fluoromethylketone (zDEVD-fmk, R and D Systems) were added to cell cultures at a concentration of 50 μmol/l, and the MAPK inhibitor SB203580 (Sigma) at a concentration of 20 μmol/l, using equivalent volumes of DMSO for controls, 1 h before transferring the cells to apoptogenic culture conditions. During culture, the medium with or without pro-apoptotic or anti-apoptotic agents was replaced every 48 h.

Detection and quantification of apoptosis. Living, apoptotic, and necrotic cells were detected and quantified by fluorescence microscopy using propidium iodide (PI) and Hoechst 3342 (Sigma). The majority of apoptotic cells that accumulate during culture are microscopically recognized through their fragmented nuclei that fluoresce for both compounds [1]. Results from cell-counting are expressed as apoptosis or necrosis indexes, calculated as [(% necrotic or apoptotic cells in experimental condition−% necrotic or apoptotic cells in control)/(% living cells in control)]×100. Cells containing sub-G1 (apoptotic) nuclei were also identified and counted by FACS analysis [6]. Apoptotic index for conditions with zDEVD or SB203580 alone, were −4.4±0.6% and 4.9±0.4 %, respectively. Apoptosis was not converted into necrosis by the inhibitors zDEVD-fmk and SB203580, since necrotic index varied by less than 10% among all conditions tested. Apoptosis under control culture conditions was slightly increased in beta cells from AMPK(α2) −/− mice as compared to wild-type (16.7±2.5% vs 11.2±1.7%, $p>0.05$).

Assay of caspase-3 activity in cell extracts. Cells were lysed in a 10 mmol/l Hepes buffer (pH 7.4) containing 0.1% CHAPS (Sigma), 10 mmol/l dithiothreitol (DTT), 2 mmol/l EDTA, and protease inhibitors [6]. Protein concentration was measured by the Micro BCA assay (Pierce) with bovine serum albumin as a standard. Samples (25 to 50 μg protein) were incubated with 50 μmol/l N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) for 3 h at room temperature. The AMC-cleavage product was detected fluorimetrically at 460 nm (excitation at 360 nm). Caspase-3 specific activity was calculated as pmol of AMC formed per mg protein and per min, using the fluorescence of free AMC as a standard.

Western blotting. Western blotting was done [6], using rabbit polyclonal IgG antibodies (Cell Signalling Technology) directed against phopho-(Thr172)-AMPK or total AMPK (both α1 and α2 isoforms of the catalytic subunit), at 1:1 000 dilution.

Statistical analysis of data. Data are expressed as means ± SEM of at least three independent experiments. Statistical significance of differences with the mean is calculated by Student's two-way *t*-test for paired data and by ANOVA with Scheffe-F test for multiple comparisons.

Results

Induction of apoptosis in beta cells by AICA-riboside. Fluorescence microscopy indicated that 2 and 4 day cultures with AICA-riboside resulted in apoptosis of beta cells but not in their necrosis. Between 0.5 and 2 mmol/l, the AMPK activator induced a dose-dependent increase in the percent of apoptotic cells (Fig. 1A). FACS analysis confirmed the appearance of apoptotic cells as characterized by nuclei with subdiploid (sub-G1) amounts of DNA (Fig. 1B). These apoptotic nuclei were not detected when the cultures were conducted in the presence of the general caspase inhibitor zVAD-fmk (Fig. 1B). No additive effect of AICA-riboside was observed at low glucose concentration (3 mmol/l), a condition that is known to induce apoptosis by itself (Fig. 1C). Apoptosis induction by AICA-riboside was less pronounced in purified alpha cells: 16±1% (n=6) alpha cells underwent apoptosis during 4 days of exposure to 2 mmol/l AICA-riboside (compare Fig. 1A).

Apoptosis induced by AICA-riboside is dependent on caspase-3 activation. The appearance of apoptotic beta cells was associated with caspase-3 activation; an eightfold increase in activity was measured after 3 days of exposure to 2 mmol/l AICA-riboside (Fig. 1D). Apoptosis in beta cells exposed for up to 6 days to either 0.5 or 1 mmol/l AICA-riboside, was markedly reduced in the presence of 50 μmol/l of the caspase-3-inhibitor zDEVD-fmk (Fig. 2A), indicating that the AICA-riboside-induced activation of caspase-3 contributed to beta cell apoptosis.

AICA-riboside-induced apoptosis requires AMPK-activation. Exposure of suspension-cultured beta cells to 1 mmol/l AICA-riboside resulted in cellular phosphorylation of AMPK within 2 h, indicating rapid activation of the kinase (Fig. 2B). The AICA-riboside-induced AMPK-phosphorylation in beta cells was sustained for at least 15 h (Fig. 2B). Addition of 20 μmol/l of the MAPK-inhibitor SB203580 strongly reduced apoptosis by either 0.5 or 1 mmol/l AICA-riboside (Fig. 2A). Under these conditions, SB203580 also prevented the phosphorylation of AMPK (Fig. 2B). Identical results on AMPK-phosphorylation were obtained with cultures of single beta cells. These data strongly suggest that activation of AMPK is required for the AICA-riboside-induced apoptosis of beta cells.

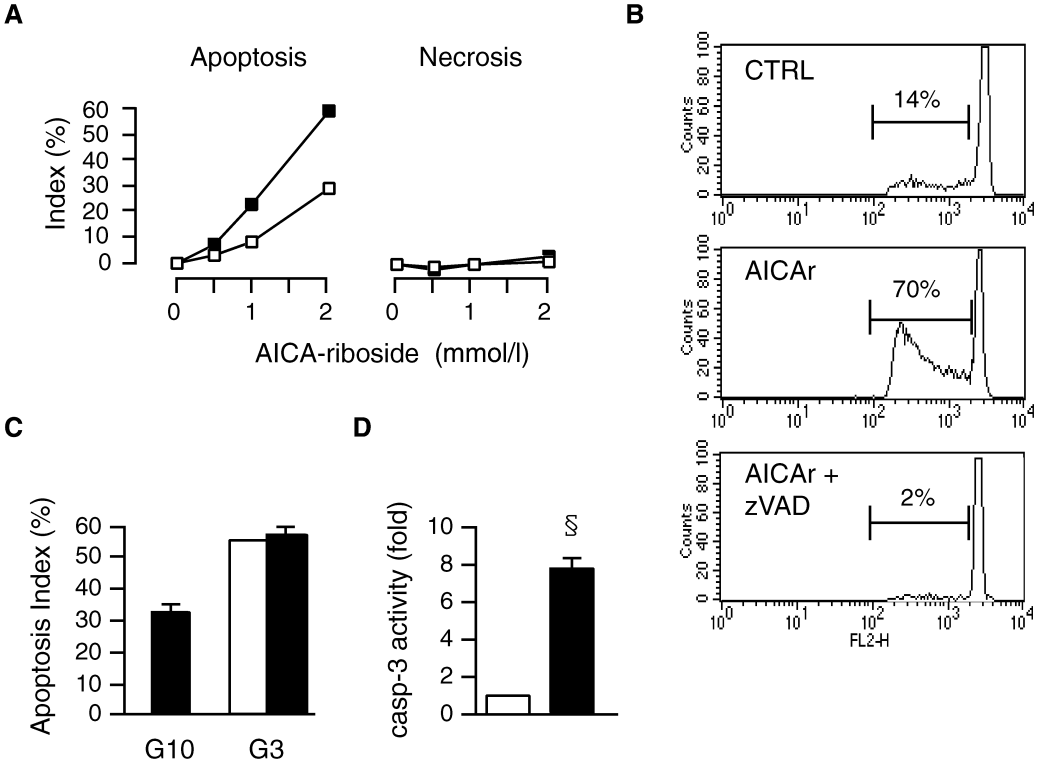

Fig. 1A–D. Apoptosis induction by AICA-riboside in beta cells. (**A**) Single rat beta cells were cultured in the presence of the indicated concentrations of AICA-riboside (AICAr). The apoptosis and necrosis indexes were scored after 2 or 4 days of culture (open and filled squares, respectively). The values shown are the means ± SE (n=8). Error bars are smaller than the size of data symbols. (**B**) Rat beta cells were suspension-cultured for 3 days in the absence (CTRL) or presence of 2 mmol/l AICA-riboside (AICAr), either with or without 50 μmol/l of the caspase inhibitor zVAD-fmk (zVAD). FACS-results are representative of three experiments. Apoptosis measured in the condition with zVAD-fmk alone was 1.1±0.3% (n=3). (**C**), Single rat beta cells were cultured for 6 days at both 10 mmol/l glucose (G10) and 3 mmol/l glucose (G3), either in the absence (white bar) or presence (black bars) of 1 mmol/l AICA-riboside, and apoptosis determined as in **A**. (**D**) Caspase-3 activity was measured in rat beta cells cultured in suspension for 3 days in the absence (white bar) or presence (black bar) of 2 mmol/l AICA-riboside. The data are expressed relative to casp-3 activity in untreated cells (means±SE, n=4, § p<0.01)

Apoptosis in beta cells from wild-type and AMPK(α2)-deficient mice. AMPKα2 –/– mice were undistinguishable from their control littermates with no statistically significant difference in body composition, adiposity or food intake, while being negative for AMPK (α2) mRNA expression in the pancreas. Glucose and L-arginine-stimulated insulin secretion was normal in islets isolated from AMPKα2 –/– mice (Vaulont, manuscript in preparation).

Beta cells isolated from wild-type and AMPK(α2)-deficient mice were cultured as single cells for 6 days in normal medium, or in medium containing either 0.5 or 1 mmol/l AICA-riboside, after which apoptosis induction was assessed by fluorescence microscopy. This study showed that beta cells from AMPK(α2)-deficient animals were more resistant to AICA-riboside induced apoptosis as compared to wild-type beta cells, in particular at the lower AICA-riboside concentration (Fig. 2C).

Discussion

Previous studies have shown that rat beta cells undergo apoptosis during prolonged exposure to low glucose concentrations [1]. Since this condition was shown to activate AMPK in beta cell lines [4, 5], we examined whether this enzyme triggers a mechanism that leads to apoptosis of primary beta cells. Activation of AMPK was induced by exposing the cells to AICA-riboside, a well-known specific activator of AMPK.

Culture of primary beta cells with AICA-riboside resulted in a time-dependent and dose-dependent apoptosis which was mediated by caspases. Our results indicate that exposure of primary beta cells to AICA-riboside results in rapid and sustained activation of AMPK. The MAPK inhibitor SB203580 prevented AICA-riboside-induced apoptosis, apparently through blocking AMPK phoshorylation. It is still not known through which mechanism SB203580 abolishes the AICA-riboside induced AMPK phosphorylation. Nevertheless, our observations suggest that AMPK activation is required for AICA-riboside induced apoptosis, which is further supported by studies on beta cells isolated from AMPK(α2)-deficient mice.

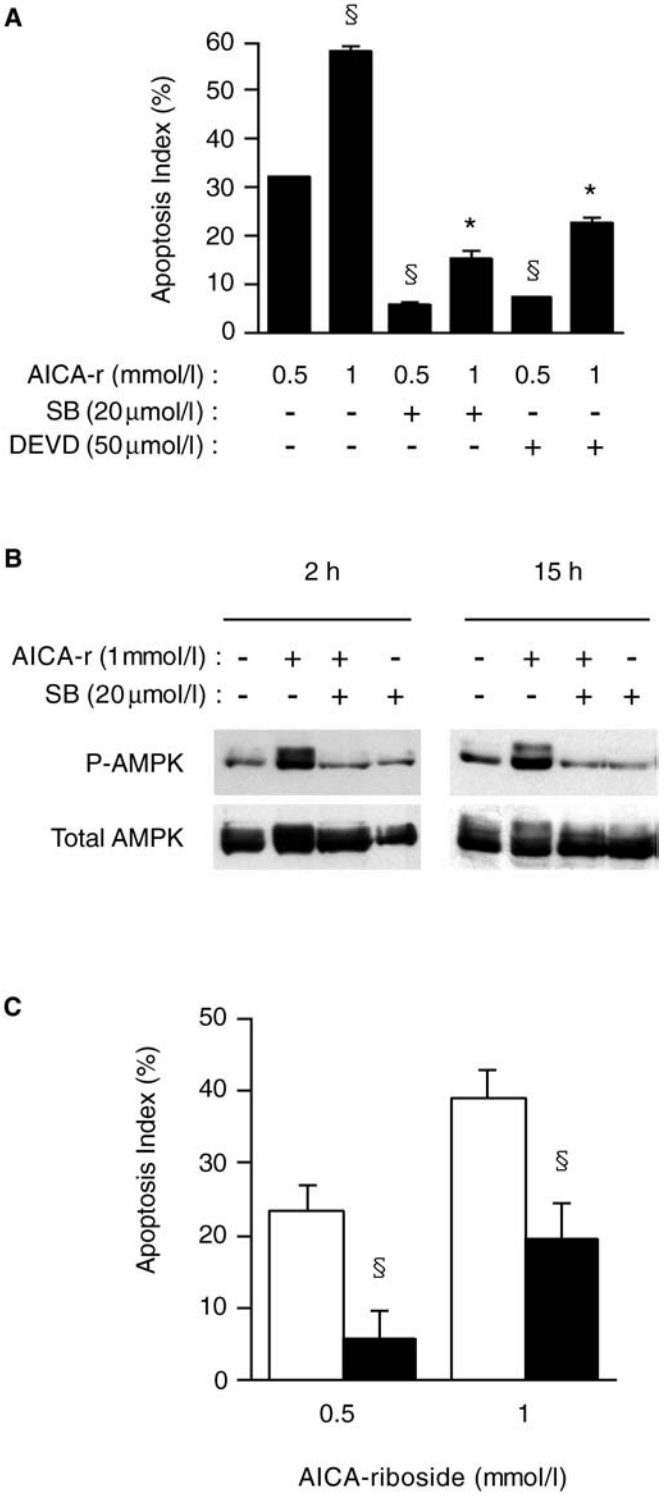

Fig. 2A–C. Apoptosis induction by AICA-riboside in beta cells is mediated by activation of AMPK and caspase-3. (**A**), The effects of adding the caspase-3 inhibitor zDEVD-fmk or the MAPK-inhibitor SB203580 on apoptosis induction by AICA-riboside. Single rat beta cells were cultured for 6 days ; apoptosis was determined as in Fig. 1A and expressed as apoptotic index. The values shown are the means ± SE (n=5, p<0.001 by ANOVA, § vs 0.5 mmol/l AICA-riboside, and * vs 1 mmol/l AICA-riboside). (**B**) Rat beta cells (2.5×10^5) were cultured in suspension, and exposed to normal medium (−) or medium containing 1 mmol/l AICA-riboside (AICA-r), either in the absence or presence of SB203580. Western blotting was done by using specific antibodies for detection of phosphorylated AMPK and total AMPK. Results shown are representative of six experiments. (**C**) Apoptosis induced by AICA-riboside in beta cells from wild-type and AMPK(α2)-deficient mice (white and black bars, respectively; § p<0.05 vs. wild-type. Data represent the means ± SE, n=3). Mouse beta cells were cultured for 6 days in the presence of the indicated concentrations of AICA-riboside, and apoptosis was measured as in **A**

Our findings imply that sustained activation of AMPK in beta cells can result in their apoptosis, or make them more susceptible to apoptotic conditions. The presently described AMPK-mediated apoptosis in beta cells contrasts with earlier reports on AICA-riboside inhibition of apoptosis in other cell types [7, 8]. This apparent discrepancy might be related to differences in cell lineage, in environmental conditions, and in the duration of exposure. In our model, the AMPK activation was sustained for at least 15 h. It is also conceivable that the pro-apoptotic or anti-apoptotic effects of AMPK vary with the AMPK isoform. Indeed the cellular content of the two isoforms of the catalytic subunits depends on the cell type, and the expression of the regulatory (β, γ) subunits might also differ. Activated AMPK has been found to phosphorylate and inactivate a number of key enzymes involved in biosynthetic pathways, thereby conserving ATP [2]. This could explain the rather long delay preceding the onset of apoptosis. On the other hand, preservation of ATP also represents a crucial parameter in favoring apoptosis over necrosis in damaged cells [9]. Likewise, an ATP-preserving effect of AMPK could explain selective appearance of apoptosis in our study, and in a previous study on glucose deprivation in beta cells [1]. The mechanism(s) via which caspase-3 is activated by AMPK have yet to be identified. Activation of AMPK in primary beta cells leads to activation of c-Jun-N-terminal kinase, suggesting this MAPK could be a potential mediator of the caspase activation.

The use of pharmacological AMPK activators could have side effects at the level of beta-cell survival. This should be assessed particularly in conditions of Type 2 diabetes where use of agents such as AICA-riboside has been proposed as a way to reduce insulin resistance in view of their stimulatory effects on glucose transport in skeletal muscle [3, 10].

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