

Inhibition of AMP-Activated Protein Kinase Protects Pancreatic β -Cells From Cytokine-Mediated Apoptosis and CD8⁺ T-Cell–Induced Cytotoxicity

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OBJECTIVE—Apoptotic destruction of insulin-producing pancreatic β -cells is involved in the etiology of both type 1 and type 2 diabetes. AMP-activated protein kinase (AMPK) is a sensor of cellular energy charge whose sustained activation has recently been implicated in pancreatic β -cell apoptosis and in islet cell death posttransplantation. Here, we examine the importance of β -cell AMPK in cytokine-induced apoptosis and in the cytotoxic action of CD8⁺ T-cells.

RESEARCH DESIGN AND METHODS—Clonal MIN6 β -cells or CD1 mouse pancreatic islets were infected with recombinant adenoviruses encoding enhanced green fluorescent protein (eGFP/null), constitutively active AMPK (AMPK-CA), or dominant-negative AMPK (AMPK-DN) and exposed or not to tumor necrosis factor- α , interleukin-1 β , and interferon- γ . Apoptosis was detected by monitoring the cleavage of caspase-3 and DNA fragmentation. The cytotoxic effect of CD8⁺ purified T-cells was examined against pancreatic islets from NOD mice infected with either null or the AMPK-DN–expressing adenoviruses.

RESULTS—Exposure to cytokines, or expression of AMPK-CA, induced apoptosis in clonal MIN6 β -cells and CD1 mouse pancreatic islets. By contrast, overexpression of AMPK-DN protected against the proapoptotic effect of these agents, in part by preventing decreases in cellular ATP, and lowered the cytotoxic effect of CD8⁺ T-cells toward NOD mouse islets.

CONCLUSIONS—Inhibition of AMPK activity enhances islet survival in the face of assault by either cytokines or T-cells. AMPK may therefore represent an interesting therapeutic target to suppress immune-mediated β -cell destruction and may increase the efficacy of islet allografts in type 1 diabetes. *Diabetes* 57:415–423, 2008

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Received for publication 18 July 2007 and accepted in revised form 29 October 2007.

Published ahead of print at <http://diabetes.diabetesjournals.org> on 14 November 2007. DOI: 10.2337/db07-0993.

Additional information for this article can be found in an online appendix at <http://dx.doi.org/10.2337/db07-0993>.

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AICAR, 5-amino-4-imidazolecarboxamide riboside; AMPK, AMP-activated protein kinase; AMPK-CA, constitutively active AMPK; eGFP, enhanced green fluorescent protein; IFN- γ , interferon- γ ; IL, interleukin; KRB, Krebs Ringer bicarbonate medium; TNF- α , tumor necrosis factor- α ; TRITC, tetramethyl rhodamine isothiocyanate; TUNEL, transferase biotin dUTP nick-end labeling.

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Diabetes currently affects ~6% of the population in Westernized societies, an incidence expected to double by 2020 (1). Destruction of β -cells is now believed to be involved in the etiology of both type 1 and type 2 diabetes (2). Strategies that delay or reverse the loss of β -cell mass in either case are therefore likely to be of significant therapeutic value. Furthermore, such strategies may enhance the survival of islet allograft transplanted into type 1 diabetic patients. Indeed, current and successful human transplantation protocols involve a substantial (60–80%) loss of functional islet mass after transplantation, meaning several donors are still required for successful grafting (3). The development of strategies to enhance β -cell survival before and after transplantation should therefore provide clear therapeutic benefits.

In type 1 diabetes, autoreactive CD4⁺ and CD8⁺ T-cells recognize their target autoantigens (such as insulin, GAD65) as peptide fragments presented by major histocompatibility complex molecules, become activated, and travel to the pancreas, infiltrating (insulinitis) and finally destroying the insulin-producing β -cells (4). Both direct T-cell-mediated cytotoxicity and indirect cytokine-, nitric oxide (NO)- or free radical-, and Fas ligand (FasL)-dependent mechanisms are likely to be responsible for β -cell apoptosis. These all lead to the activation of caspases by cleavage of the inactive zymogen counterpart. Caspase-3 is an effector caspase leading to the characteristic apoptotic morphological changes such as membrane blebbing, cytoplasmic and nuclear condensation, DNA fragmentation, and formation of apoptotic bodies (5,6).

AMP-activated protein kinase (AMPK) is a multisubstrate, trimeric serine/threonine kinase composed of one 63-kDa catalytic α -subunit and two regulatory subunits, β and γ (7,8). AMPK activity is regulated allosterically by AMP (9) and through reversible phosphorylation at Thr172 of the α -subunit by upstream kinases such as LKB1 (10), calmodulin kinase kinase β (CaMKK β) (11) or transforming growth factor β -activated kinase (TAK1) (12). AMPK is thus a sensor of cellular energy charge that is activated by the fall in ATP-to-AMP ratios, an elevation in free Ca²⁺ concentration (13), or perhaps other mechanisms. In most cell types, activation of AMPK is associated with the phosphorylation of enzymes involved in ATP-consuming processes, such as fatty acid synthesis (acetyl-CoA carboxylase) and cholesterol biosynthesis (hydroxymethylglutaryl-CoA reductase), and the consequent activation of mitochondrial fatty acid oxidation (7,14). In this way, regulation of AMPK ensures that cellular ATP is spared during times of nutrient deprivation. However, in the pancreatic islet β -cell, the role of AMPK

may be more specialized and may represent a key part of the glucose-sensing machinery of these cells (7,14,15).

A good deal of data has emerged in the last 3–4 years showing that sustained AMPK activation can exert a proapoptotic effect in a variety of cell types (16,17). Thus, work in hepatocytes (18), gastric cancer (19), neuroblastoma (20), HT-29 colon cancer (21), and chronic lymphocytic leukemia (22) cells has implicated AMPK activation in cell death. Taken together, these data reinforce the view that whereas activation of AMPK is likely, in the short term, to reduce ATP consumption and thus to protect cells from transient metabolic stresses (23), sustained activation of the enzyme entrains a sequence of events ultimately leading to programmed cell death. Importantly, we have recently shown that adenovirus-mediated expression of a constitutively active form of AMPK (AMPK-CA) reduces the ability of syngeneic islets to reverse streptozotocin-induced diabetes, while a dominant-negative form of AMPK (AMPK-DN) tends to enhance graft efficiency (24).

Various cellular and molecular mechanisms are involved in β -cell apoptosis. CD8⁺ T-cells are increasingly recognized as key actors in the diabetes of the nonobese diabetic (NOD) mouse, which spontaneously develops diabetes remarkably similarly to human type 1 diabetes (25), and constitute a useful model to study this disorder. CD8⁺ T-cells are also likely to play a role in humans. Thus, in identical twins who received a transplant from their nondiabetic co-twin, recurrent disease occurred within 6 weeks, with CD8⁺ T-cells constituting a majority of the cells infiltrating the transplants (26). Biopsies performed in patients newly diagnosed with type 1 diabetes have also shown that CD8⁺ T-cells make up a considerable proportion of the infiltrate (27). Finally, a number of recent studies have indicated that T-cells recognizing proinsulin and IGRP (islet-specific glucose-6-phosphatase catalytic subunit-related protein) may be detected with high sensitivity at the onset of diabetes (28).

T-cells induce damage to islet β -cells by a number of mechanisms including lysis by perforin/granzymes and induction of apoptosis by Fas/FasL interactions (29). In addition, in the insulinitis lesion in type 1 diabetes, invading immune cells produce proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), and interleukin-1 β (IL-1 β), and therefore also constitute a key part of the type 1 diabetes mechanism (30).

Here, we demonstrate that AMPK is involved in regulating β -cell apoptosis induced by cytokines and in the cytotoxicity of CD8⁺ T-cells toward islets from NOD mice.

RESEARCH DESIGN AND METHODS

Collagenase, Histopaque (1,077), and Hoechst (type V) were from Sigma (St. Louis, MO). Mouse recombinant TNF- α , IFN- γ , and IL-1 β were from PreproTech EC (London, U.K.). BM Chemiluminescence Blotting Substrate, anti-rabbit and anti-mouse horseradish peroxidase, [³²P] γ ATP, and chromium sulfate (51) were from Amersham BioSciences (Buckinghamshire, U.K.). Firefly luciferase was from Promega (Madison, WI), and the In Situ Cell Death Detection Kit and tetramethylrhodamine red (TMR Red) was from Roche (Basel, Switzerland). The CD8a⁺ T-cell isolation kit was from MACS (Bergisch Gladbach, Germany). The culture media and cell dissociation buffer were from Invitrogen (Paisley, U.K.). Scintillation fluid Ultima Gold LLT was from Perkin Elmer (Waltham, MA), and the scintillation 96-well plate 1450 MicroBeta counter was from Wallac (Turku, Finland).

Wild-type CD-1 mice (20–25 g) and 8- to 12-week-old male NOD mice were used for islet isolation and killed by cervical dislocation immediately before the islet isolation procedure (see CELL CULTURE AND ISLET ISOLATION). Insulin-specific T-cell receptor (TCR) transgenic mice were generated from G9C8 cloned T-cells (31), which have previously been shown to have specific reactivity to amino acids 15–23 of the insulin β -chain (32). TCR- α and - β

founder lines were intercrossed to produce $\alpha\beta$ -TCR transgenic mice (G9.NOD). G9C $\alpha^{-/-}$.NOD mice expressing T-cells monoclonal for the transgenic TCR were generated by crossing the $\alpha\beta$ -TCR transgenic mice to NOD.C $\alpha^{-/-}$ mice (>20 generations backcross to NOD mice). All animal procedures were in accordance with the British Home Office Animals (Scientific Procedures) Act, 1986.

Antibodies. Rabbit anti-phospho-AMPK (Thr-172) and anti-cleaved caspase-3 (Asp-175) antibodies were purchased from Cell Signaling (Beverly, MA). Rabbit anti-ERK2 was from Santa Cruz (Santa Cruz, CA). Tetra methyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody against rabbit IgG was purchased from Jackson (West Grove, PA).

Adenoviruses. Adenoviruses encoding enhanced green fluorescent protein (eGFP) only, hereafter named pAd-GFP (Null), AMPK-CA, or AMPK-DN (AMPK α 1 [D^{157A}]) have been described by Carling and coworkers (33,34). AMPK-CA comprises the NH₂-terminal domain (amino acids 1–312) common to both AMPK α 1 and - α 2 and is rendered constitutively active by a T^{172D} point mutation. Islets and MIN6 cells were infected at a multiplicity of infection of 100 viral particles/cell overnight or 4 h and cultured for 48 h before experiments.

Cell culture and islet isolation. Clonal mouse pancreatic MIN6 β -cells were used between passage numbers 18 and 30 and grown in Dulbecco's modified Eagle's medium containing 25 mmol/l glucose and supplemented with 2 mmol/l L-glutamine, 15% heat-inactivated FCS, 50 μ mol/l 2-mercaptoethanol, 100 units/ml penicillin, and 100 g/ml streptomycin. Mouse mastocytoma P815 cells were grown in RPMI-1640 medium, 5% FCS, 2 mmol/l L-glutamine, 50 μ mol/l 2-mercaptoethanol, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Both were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Mice were killed by cervical dislocation. Collagenase (1 mg/ml in Krebs Ringer bicarbonate [KRB] medium comprising [in mmol/l]: 120 NaCl, 4.8 KCl, 2.5 CaCl₂, 1.2 MgCl₂, and 24 NaHCO₃ and 1 mg/ml bovine serum albumin, gassed with O₂/CO₂ [95%/5%]) to maintain a pH of 7.4) was then injected into the pancreatic duct (3 ml/mouse). The distended pancreas was then placed in a water bath at 37°C for 11 min, and the islets were hand picked. Mouse islets were maintained in RPMI-1640 medium supplemented with 2 mmol/l L-glutamine, 10% FCS, 11 mmol/l glucose, and antibiotics.

For immunocytochemistry, islets were dissociated with cell dissociation buffer before plating the liberated cells onto glass coverslips.

CD8⁺ T-cell purification and activation. G9C $\alpha^{-/-}$.NOD mice express monoclonal insulin-reactive CD8 T-cells. Cells were extracted from the spleen and activated overnight in the presence of insulin B15–23 peptide, in RPMI-1640 medium containing 5% FCS, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin, at 37°C in a humidified atmosphere containing 5% CO₂. The day after, activated CD8⁺ T-cells were purified from the splenocytes using a CD8⁺ T-cell isolation kit (MACS) according to the manufacturer's instructions. CD8⁺ T-cells obtained by this method were 90–95% pure.

Immunocytochemistry and apoptosis detection. MIN6 cells or dispersed islets were washed three times with PBS before fixation with 4% (vol/vol) paraformaldehyde in 0.1 mol/l NaH₂PO₄, pH 7.4. Cells were then permeabilized with 0.3% (vol/vol) Triton X-100 for 20 min and incubated for 1 h with primary rabbit polyclonal anti-cleaved caspase-3 antibody, at 1:200 dilution, at 4°C. Primary antibody was revealed using TRITC-conjugated secondary antibody against rabbit IgG (1:500 dilution). Nuclear staining was achieved by incubating the cells in wash buffer containing Hoechst for 10 min at room temperature. Apoptotic cells were imaged either on a Leica SP2 laser-scanning confocal or a Leica SP5 MP/FLIM inverted optics confocal microscope, using a \times 40 or a \times 63 oil immersion objective with excitation at 488 nm (Ar) and 543 nm (He-Ne) and emission detected at >515 (green, eGFP) and >560 nm (red, TRITC). A terminal deoxynucleotidyl transferase biotin dUTP nick-end labeling (TUNEL) assay was also performed using the In Situ Cell Death Detection Kit, TMR red, to visualize the DNA strand breaks of apoptotic cells by fluorescence microscopy, according to the manufacturer's recommendations. Briefly, the cells were treated as described above for fixation and permeabilization, and the TUNEL reaction mixture was added for 1 h. Imaging was performed with an excitation wavelength range of 520–560 nm, detecting in the range of 570–620 nm. Results were quantified by densitometry.

Western immunoblot analysis. MIN6 cells or mouse islets were incubated and lysed similarly as for measurements of caspase activity. Whole cellular extract (50 μ g) was denatured for 5 min at 100°C in 2% (wt/vol) SDS, 5% mercaptoethanol, and resolved by 10% or 7.5% SDS-PAGE before transferring to PVDF membranes and immunoblotting. Secondary antibodies were revealed using BM Chemiluminescence's blotting substrate. Intensities were measured by digital scanning of gels and quantified using Scion Image (www.scioncorp.com/).

ATP measurements. For total ATP assay, MIN6 cells were infected with adenoviruses and incubated with the indicated cytokines in culture medium and then at the given glucose concentrations in KRB medium for 30 min

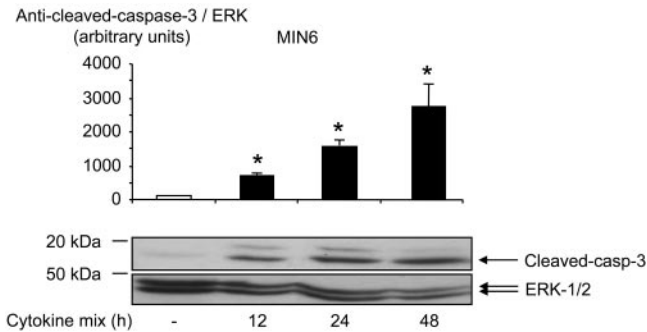


FIG. 1. Cytokines induce apoptosis in clonal MIN6 β -cells. MIN6 cells were cultured with a cytokine mix containing IL-1 β (50 units/ml), TNF- α (1,000 units/ml), and IFN- γ (1,000 units/ml), for the times indicated, in their culture medium (see RESEARCH DESIGN AND METHODS). Induction of apoptosis was measured by Western (immuno-) blotting for cleaved caspase-3 (Asp-175) (see RESEARCH DESIGN AND METHODS), and total protein content was estimated using anti-ERK1/2. Data are given as means \pm SEM of three independent experiments. * $P < 0.05$ for effect of cytokines compared with basal without cytokines.

before extraction into perchloric acid (10%, vol/vol). ATP was quantitated in extracts neutralized with Hepes-buffered KOH, using partially purified firefly luciferase and photon counting, as described before (35).

Cytotoxicity assay. A chromium release assay was performed similar to that previously described (31). Islets from 8- to 12-week-old NOD male mice were infected with either the Null adenovirus or the AMPK-DN for 48 h. They were then labeled with chromium sulfate (51) for 1.5 h, and washed (Fig. 8A). The purified activated CD8⁺ T-cells were added to the islets for 16 h, with or without added insulin B15-23 peptide (1 μ g/ml) at an effector-to-target ratio of 20:1 (assuming 1,000 cells/islet). Cytotoxicity using P815 cells, labeled with chromium sulfate (51) for 1 h and coated with insulin B15-23 peptide, at an effector-to-target ratio of 10:1 was used as a positive control. Results were expressed as the percentage of specific lysis determined as [(cytotoxic release - min)/(max - min)] \times 100, where the spontaneous lysis corresponds to the minimal release (min), and the lysis provoked by addition of hydrochloric acid corresponds to the maximal lysis (max).

Statistical analysis. Results are expressed as means \pm SEM of at least three independent experiments. Statistical significance was evaluated using the Student's *t* test for unpaired comparison with Bonferroni correction as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of cytokines on clonal MIN6 β -cell apoptosis. It has previously been reported that incubation of MIN6 cells (36) or islets (37,38) with different cytokine combinations can lead to the induction of apoptosis. Apoptosis can be prompted by several different mechanisms, but the one usually implicated in islet cell death in type 1 diabetes and allograft transplantation involves cytotoxic cytokines secreted by macrophages and possibly also β -cells. Here, we observed that incubation of MIN6 cells for 12, 24, or 48 h with a combination of the three cytokines, IL-1 β , TNF- α , and IFN- γ (Fig. 1), induced the cleavage of caspase-3, indicating the establishment of apoptosis in clonal MIN6 cells.

AMPK activation induces MIN6 cell apoptosis. Previous studies have shown that AMPK activators such as 5-amino-4-imidazolecarboxamide riboside (AICAR) (17) or chronic incubation of MIN6 cells or islets with low concentrations (0–3 mmol/l) of glucose (39) or metformin (40) can lead to β -cell apoptosis. Consistent with these earlier findings, when MIN6 cells were infected with Null/GFP (Null) or the AMPK-CA adenoviruses and cultured for 24–96 h, we observed a fourfold increase in the level of activated caspase-3 after 96 h of incubation with AMPK-CA compared with Null virus-expressing cells (Fig. 2). Thus, AMPK activation alone is sufficient to induce apoptosis in MIN6 cells.

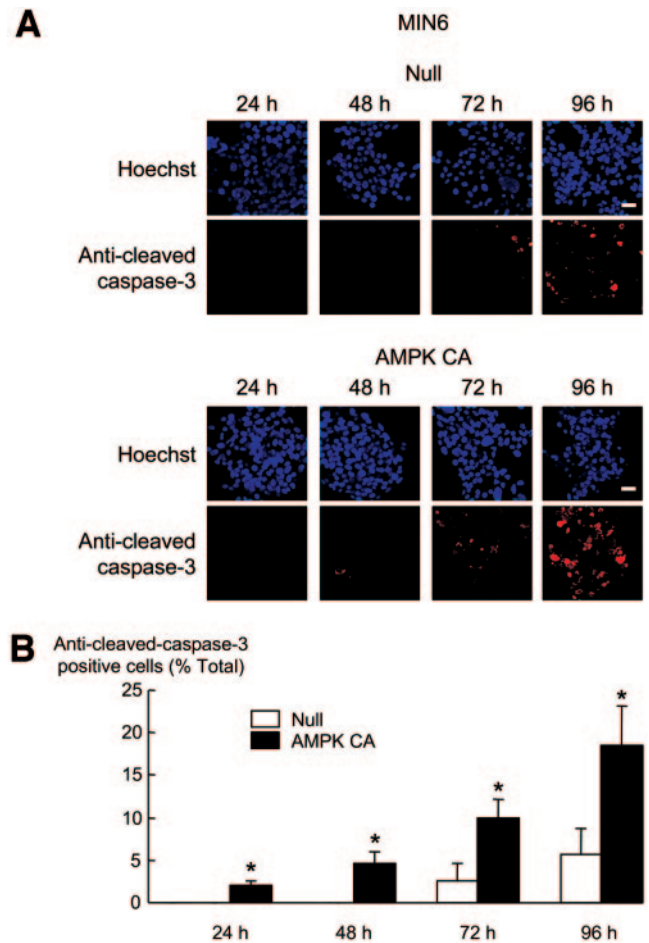


FIG. 2. Effect of AMPK-CA adenovirus on caspase-3 activity in MIN6 β -cells. **A:** Cells were infected with the indicated adenoviruses at 100 MOI (Null/GFP, AMPK-CA) for the times indicated. Induction of apoptosis was measured by immunocytochemistry with an antibody to cleaved caspase-3. Primary antibody was revealed using TRITC-conjugated secondary antibody against rabbit IgG, and a Hoechst coloration of the nucleus was performed. **B:** The data from A given as means \pm SEM of triplicate analyses from three independent experiments. Results were analyzed by densitometry, and statistical differences were assessed by unpaired Student's *t* test (* $P < 0.05$ for effect of AMPK-CA virus compared with Null virus). Scale bar, 20 μ m.

Cytokines induce an increase in MIN6 cells and mouse islet AMPK activity. Measured in either MIN6 cells (Fig. 3A) or islets (Fig. 3B), AMPK α phosphorylation at Thr-172 was markedly higher after incubation for 30 min in KRB containing low (0–3 mmol/l) than at elevated (17 mmol/l) glucose concentrations, consistent with previous findings (15). Likewise, incubation for 48 h with a combination of IL-1 β , TNF- α , and IFN- γ increased by 15- and 4-fold the level of phosphorylation on AMPK Thr-172 at 17 mmol/l glucose in MIN6 cells and mouse islets, respectively.

Cytokine-induced apoptosis requires AMPK activation in MIN6 cells. To determine whether cytokine-mediated apoptosis required AMPK activation, we used an adenovirus to express AMPK-DN (41). MIN6 cells were infected with the AMPK-DN adenovirus for 48 h and then treated with cytokines for a further 48 h. As expected, the increased phosphorylation of AMPK induced by 3 mmol/l glucose or by cytokines was reduced in cells and islets infected with AMPK-DN (data not shown). Inhibition of AMPK also reduced the pro-apoptotic effect of the cytokines. Thus, apoptosis was reduced about four- to fivefold

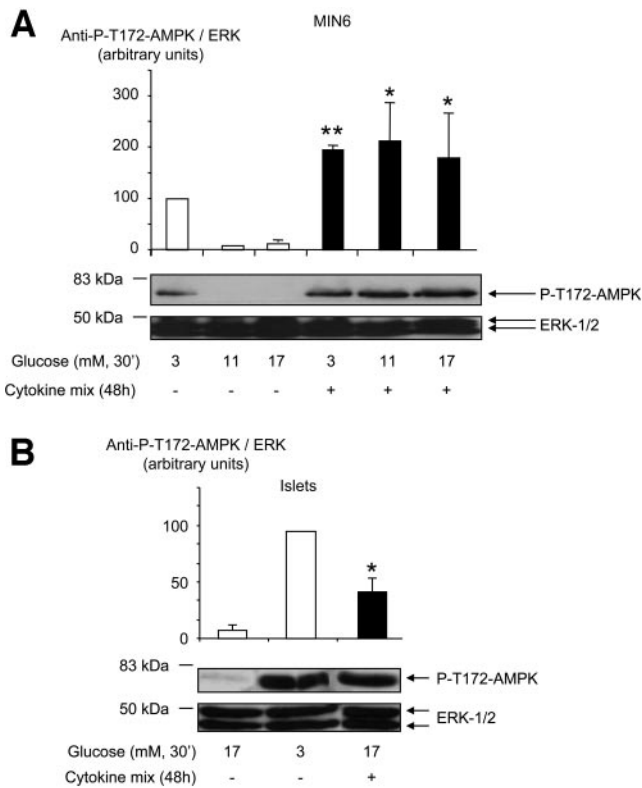


FIG. 3. Effect of cytokines on AMPK activity in clonal MIN6 β -cells and mouse pancreatic islets. MIN6 cells (A) or mouse pancreatic islets (B) were treated with cytokine mix for 48 h and incubated in KRB medium (see RESEARCH DESIGN AND METHODS) at the indicated concentrations of glucose for 30 min. Phospho-Thr-172 AMPK phosphorylation was assessed using the appropriate phospho-specific antibody. Total protein was estimated using anti-ERK1/2 antibody. Results shown are from three independent experiments, and statistical differences were assessed by unpaired Student's *t* test. **P* < 0.05 and ***P* < 0.01 for the effect of cytokines compared without cytokines.

in MIN6 cells infected with the AMPK-DN adenovirus and treated 48 h with the three cytokines, as shown by TUNEL assay (Fig. 4) or measuring anti-cleaved caspase 3 either in single cells by immunocytochemistry (where infection with adenovirus could be confirmed on a cell basis by the presence of eGFP; Fig. 5A and B) or in cell populations by Western (immuno-) blotting (Fig. 5C).

Effect of cytokines on primary mouse islets apoptosis and implication of AMPK and caspase-3. To examine the role of AMPK in primary β -cells, mouse islets were dispersed into single cells before infection with adenoviruses. This approach ensured high levels of infection such that >80% of cells were positive as judged by eGFP fluorescence. After treatment of the cells for a further 24 and 48 h with cytokines, apoptosis was assessed by TUNEL assay (Fig. 6A and B). The level of apoptosis was between 60 and 70% after the incubation with cytokines, and a significant decrease was apparent in cells expressing AMPK-DN versus those infected with Null adenovirus. Moreover, activation of AMPK by the AMPK-CA adenovirus during 72 h also led to an increase in apoptosis in primary mouse islets, reaching 70% (Fig. 6A and B). To determine whether caspase-3 was also involved in the latter process, we measured caspase-3 cleavage (Fig. 6C and D). Interestingly, the percentage of cells expressing cleaved caspase-3 reached 20% after 24 h in presence of cytokines. These results confirm the involvement of caspase-3 in cytokine-induced apoptosis of primary β -cells.

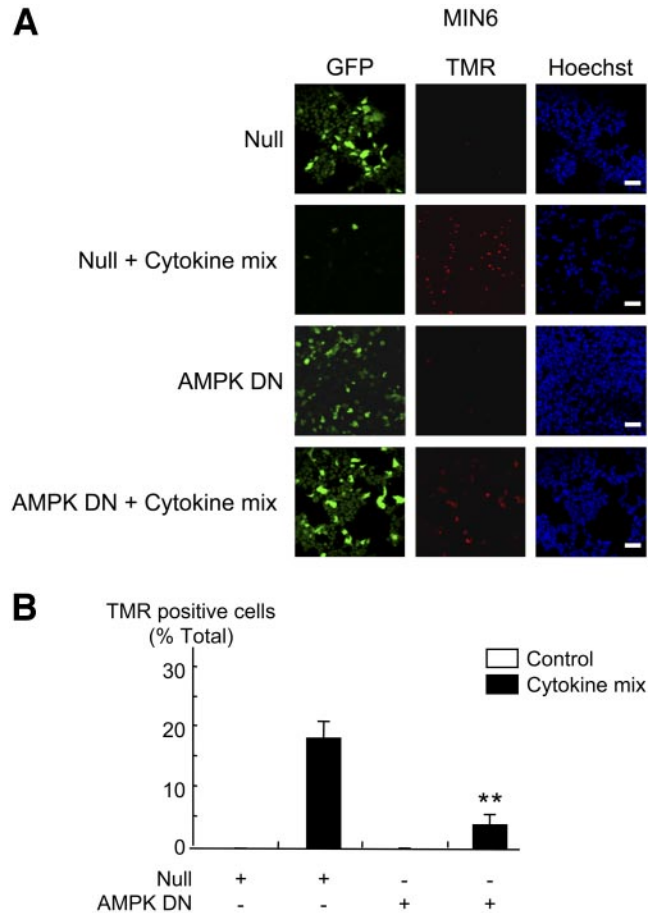


FIG. 4. Dominant-negative AMPK blocks cytokine-mediated apoptosis in MIN6 β -cells. A: MIN6 cells were infected with the indicated adenoviruses at 100 MOI (Null/GFP, AMPK-DN) for 48 h, before treatment with cytokines as in Fig. 1, for a further 48 h. Apoptosis was measured by TUNEL assay using the In Situ Cell Death Detection Kit, TMR Red, as described in RESEARCH DESIGN AND METHODS, followed by nucleus coloration with Hoechst dye. Results were analyzed by densitometry, and statistical differences were assessed by unpaired Student's *t* test. ***P* < 0.01 for the effects of AMPK-DN virus in the presence of cytokines compared with Null virus. B: The data from A given as means \pm SEM of triplicate analyses from three independent experiments involving 100 individual cells per condition. Scale bar, 20 μ m.

Nevertheless, this number is less than the percentage of apoptosis measured with the TUNEL assay (Fig. 6A and B), even after 24 h of incubation with cytokines, and thus implies the involvement of other (noncaspase 3-dependent) pathways.

Cytokines induce a decrease in total cellular ATP content. AMPK is an AMP-sensitive enzyme whose activity is expected, at least in large part, to be regulated by changes in intracellular ATP-to-AMP ratio. Given that the adenylate kinase reaction is likely to be at near equilibrium in β -cells, we measured the total cellular ATP content as a guide to ATP-to-AMP ratio (Fig. 7). As anticipated, cellular ATP content was increased as glucose concentrations were elevated, consistent with a lowering of AMP levels and inhibition of AMPK (15,41). Interestingly, when cells were incubated for 12 h to 48 h with the cytokine combination, the cellular ATP content was decreased at both three and 17 mmol/l glucose. These results thus suggest that the action of the cytokines on AMPK is likely to be due to a fall in ATP-to-AMP ratio and LKB1-mediated phosphorylation of AMPK α (13). By contrast, AMPK-DN

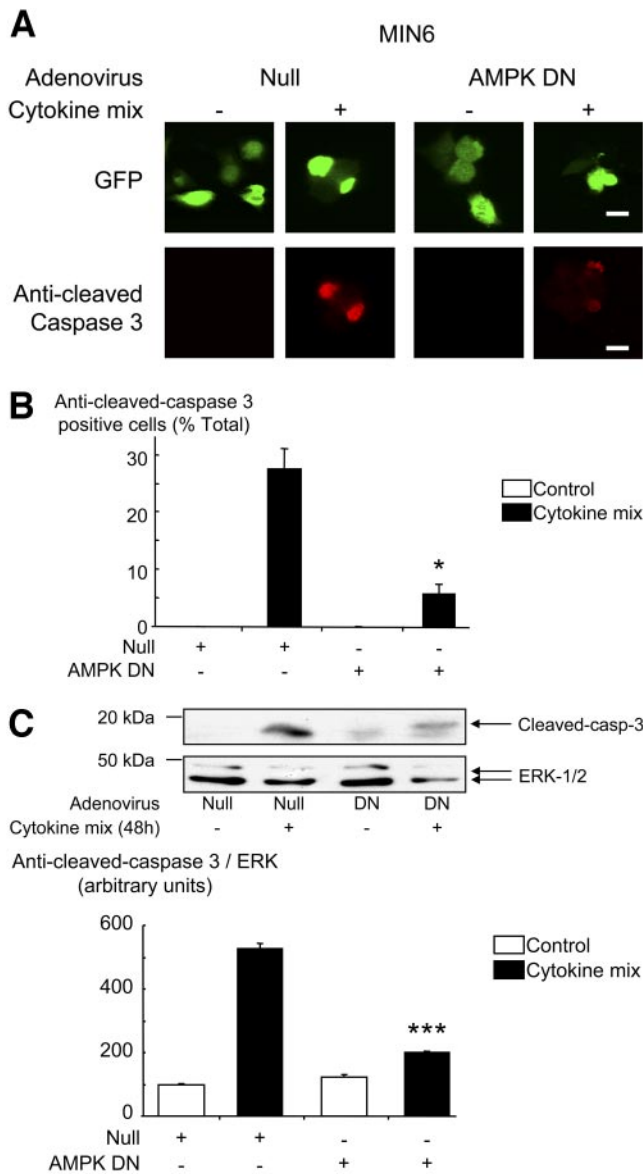


FIG. 5. Dominant-negative AMPK blocks cytokine-activation of caspase-3 in MIN6 β -cells. MIN6 cells were treated as in Fig. 4, and active caspase-3 was measured by immunocytochemistry (A and B) or Western blot (C) with anti-cleaved caspase-3 antibody. Results were analyzed as in Fig. 4. * $P < 0.05$ and *** $P < 0.001$ for the effect of AMPK-DN virus in the presence of cytokines compared with Null virus. Data are given as means \pm SEM of triplicate analyses from three independent experiments involving 50 individual cells per condition for the immunocytochemistry (B) and as means \pm SEM of three independent experiments for the Western blot (C). Scale bar, 10 μ m.

had no significant impact on the action of the cytokine combination to lower cellular ATP content (Fig. 7).

Inhibition of AMPK decreases the cytotoxic effect of CD8⁺ T-cells on NOD pancreatic islets. CD8⁺ T-cell cytotoxicity toward islets probably occurs via a combination of effects including cytokine-mediated killing, Fas/FasL apoptosis, and lysis by perforin/granzymes. To determine whether AMPK might be involved in the cytotoxicity of CD8⁺ T-cells against islets during type 1 diabetes, we used a cytotoxic assay (Fig. 8A) involving insulin B15-23-reactive CD8⁺ T-cells as effectors and islets isolated from NOD mice and infected either with the Null or the AMPK-DN adenoviruses as targets. The insulin-specific T-cells used in this study are low avidity and

require a relatively high peptide concentration to stimulate them (42). Although NOD β -cells do express endogenous peptide, the concentration is low. However, β -cells (both wild-type and NOD) also express major histocompatibility complex class I molecules so the added insulin peptide can be presented at their surface. In other words, β -cells have a dual role in this assay, serving both as the targets of insulin-specific cytolytic T-cells and as antigen-presenting cells for these T-cells. Inhibition of AMPK led to a 50% decrease of CD8⁺ T-cell cytotoxicity toward the islets (Fig. 8C). Lysis of B15-23-coated P815 target cells was used as a positive control for CD8⁺ T-cell cytotoxicity (Fig. 8B).

DISCUSSION

The main aim of the present study was to determine whether AMPK activation may be involved in β -cell death induced by cytokines or cytotoxic T-cells and might therefore represent an interesting therapeutic target to enhance the efficacy of transplantation protocols.

The nature of the immunological effectors that induce apoptosis in β -cells, and may thus lead to β -cell destruction and type 1 diabetes, is still debated. However, involvement of autoreactive T-cells and an inflammatory response in which perforin, granzyme B, FasL, TNF- α , IL-1 β , IFN- γ NO, or a combination of all of the above, has been implicated in the destruction of pancreatic β -cells (2,5,6). These molecules are likely to act in synergy to induce apoptotic signaling cascades through a caspase-3-dependent cell death (6,30). Nevertheless, it is also now recognized that endoplasmic reticulum stress, possibly triggered by IL-1 α and acting via NO formation and caspase-12 activation (43), as well as stress-activated kinases through reactive oxygen species production and loss of mitochondrial membrane potential, possibly triggered by IFN- γ /TNF- α (36), are also key factors in primary β -cell apoptosis.

In our hands, 12- to 48-h treatment of MIN6 β -cells with the cytokines TNF- α , IL-1 β , and IFN- γ led to increased apoptosis as demonstrated by enhanced caspase-3 cleavage (Fig. 1). These findings are in accordance with previous results in rat pancreatic islets (44) and clonal insulin-secreting cells (45). Although still clearly apparent (Fig. 6C and D), cytokine-induced caspase-3 cleavage in primary mouse islet (expected to be largely β -) cells was less markedly increased than TUNEL staining (Fig. 6A and B), suggesting that different apoptotic mechanisms may be triggered in primary cells versus clonal MIN6 cells. Kefas et al. (17) have previously shown that chronic treatment of MIN6 cells or rat islets with low glucose concentrations, or a sustained activation of AMPK with AICAR, also leads to programmed cell death. Correspondingly, our results show an increase in AMPK activity in response to cytokines (Fig. 3) and in apoptosis in MIN6 cells after an infection with a recombinant adenovirus expressing AMPK-CA (Fig. 2).

These changes were matched by significant decreases in ATP content and hence probably increases in AMP level, which are likely to underlie the activation of AMPK (Fig. 7). Nevertheless, it is also conceivable that increases in intracellular free Ca²⁺ concentration, and hence activation of CaMKK1 β (11,45), may also contribute.

We demonstrate for the first time that cytokine-mediated β -cell death is inhibited when the activation of

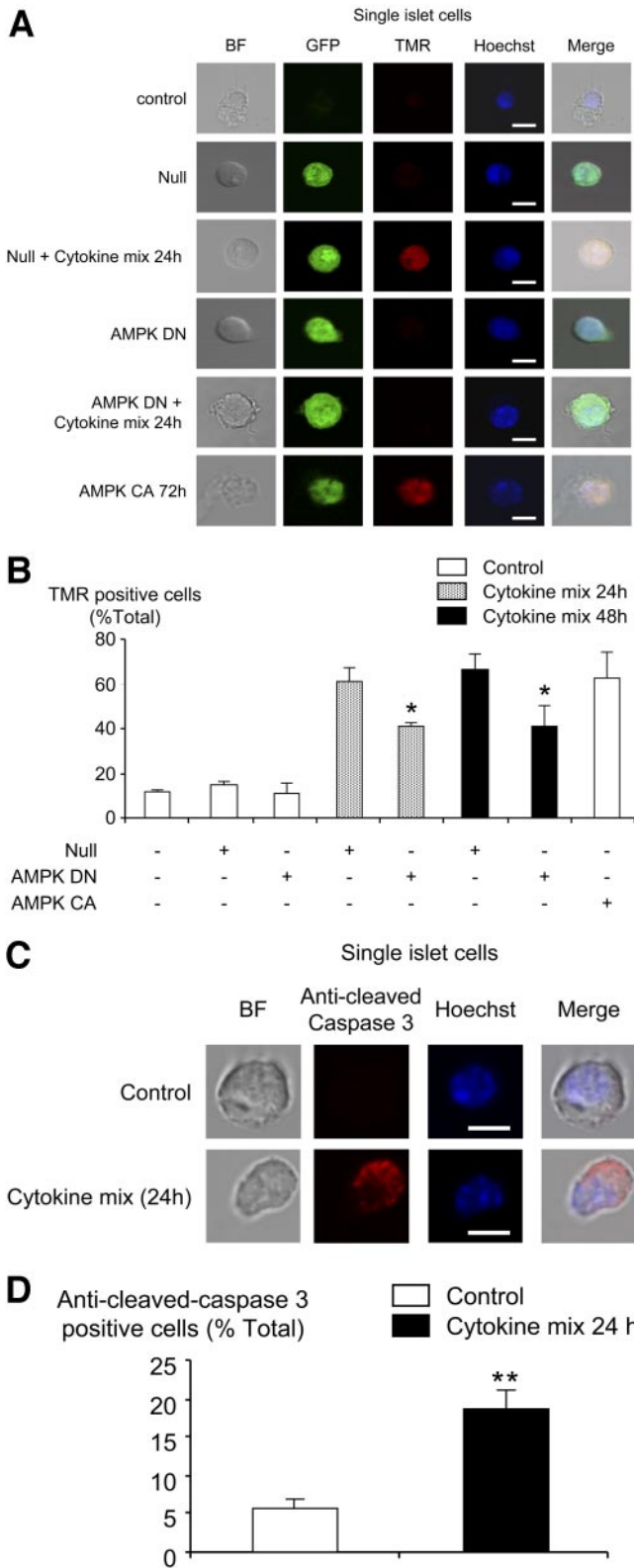


FIG. 6. Effect of dominant-negative AMPK and AMPK-CA on cytokine-mediated apoptosis in dispersed mouse pancreatic islets. **A:** Dispersed islets were infected or not with the indicated adenoviruses at 100 MOI (Null/GFP, AMPK-DN, AMPK-CA) for 48 h. Then, islets infected with Null/GFP or AMPK-DN were treated or not with cytokines, as in Fig. 1, for a further 24 or 48 h. Islets infected with AMPK-CA were maintained for a further 24 h. Apoptosis was measured by TUNEL assay as described in Fig. 4, followed by nucleus coloration with Hoechst dye (BF represents the bright field). **B:** Data from **A** given as means \pm SEM of duplicate analyses from five experiments involving 50 individual cells per condition. Scale bar, 5 μ m. * P < 0.05 for the effect of

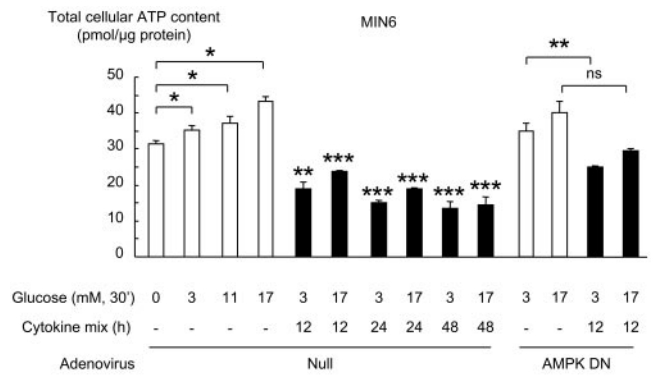


FIG. 7. Effect of cytokines on total cellular ATP content in MIN6 cells. MIN6 cells were infected with the indicated adenoviruses at 100 MOI (Null/GFP, AMPK-DN) for 48 h, before treatment with cytokines as in Fig. 1, for the indicated times. Before ATP assay, cells were incubated with the indicated concentrations of glucose for 30 min (see Fig. 3). Cells were then extracted in perchloric acid as described in RESEARCH [SCAP]DESIGN [SCAP]AND [SCAP]METHODS, before assay of total ATP. * P < 0.05, ** P < 0.01, and *** P < 0.001 for effects of glucose or cytokines compared with the corresponding controls. Data are the means \pm SEM of triplicates from observations on three separate cell cultures. Results shown are representative of three independent experiments.

AMPK is blocked by overexpression of a dominant-negative form of the enzyme (Figs. 4, 5, and 6). Interestingly, AMPK-DN had no apparent effect on the cytokine-induced changes in intracellular ATP content, indicating that the pro-apoptotic pathways activated in response to AMPK act downstream of the decline in ATP. Several mechanisms could explain the link between AMPK and apoptosis. First, activation of AMPK may cause cell cycle arrest, as observed in prostate cancer and smooth muscle cells (46). This observation is consistent with the fact that the upstream kinase LKB1 (47) is a tumor suppressor mutated in Peutz-Jeghers syndrome. Importantly, recent work demonstrates that AMPK activation induces phosphorylation of the tumor suppressor p53, an event required to initiate cell-cycle arrest in G1 phase (48). While this arrest was reversible in the short term, persistent activation of AMPK led to cell death. Providing alternative mechanisms, Kefas et al. (16) showed that AICAR treatment of β -cells lead to a c-jun NH₂-terminal kinase (JNK) and caspase-3-dependent apoptosis, whereas Jambal et al. and Inoki et al. (49,50) showed an inhibition of protein kinase B and mTOR, respectively, by AMPK and thus an inhibition of the anti-apoptotic pathway and protein synthesis. AMPK activation is also associated with increased mitochondrial superoxide-derived radicals (reactive oxygen species) production and decreased mitochondrial activity (51,52).

There has been much debate on the mode of cytotoxicity toward islet β -cells mediated by CD8⁺ T-cells, and it is likely that cytokines, the Fas/FasL system, and granzyme/perforin all play a role in the attack (53). We demonstrate here that AMPK may be involved in cytokine-induced apoptosis, and we also show that the inhibition of AMPK

AMPK-DN with cytokines compared with Null virus in presence of cytokines. **C:** Dispersed islets were treated with cytokines during 24 h, and caspase-3 cleavage was measured by immunocytochemistry with the appropriate antibody, followed by nucleus coloration with Hoechst dye. **D:** Data from **C** given as means \pm SEM of duplicate analyses from three experiments involving 50 individual cells per condition. Scale bar, 5 μ m. ** P < 0.01 for the effect of the cytokines compared with the control without cytokines. (Please see <http://dx.doi.org/10.2337/db07-0993> for a high-quality digital representation of this figure.)

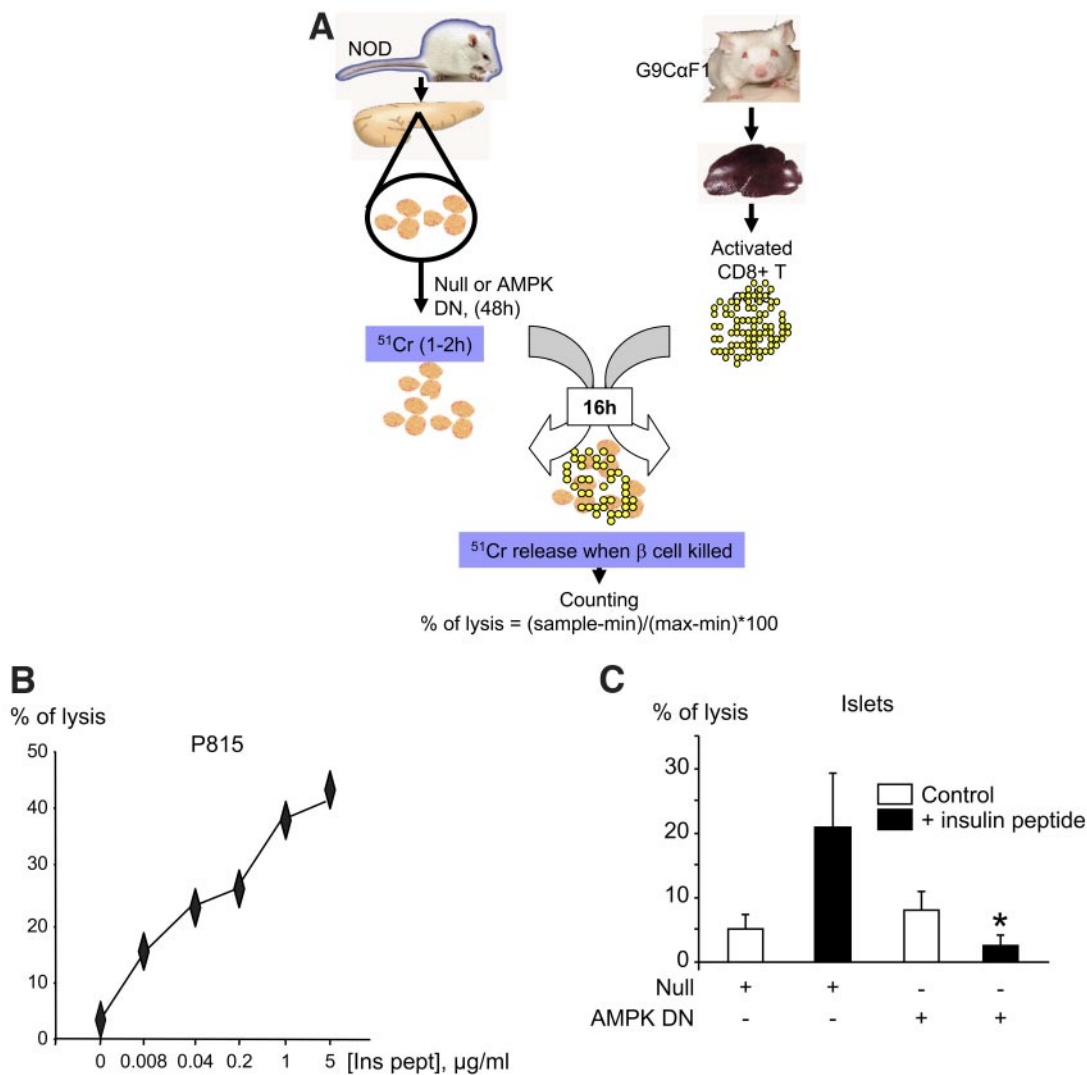


FIG. 8. Dominant-negative AMPK decreases the cytotoxicity of insulin-reactive CD8⁺ T-cells to NOD pancreatic islets. **A:** Scheme explaining the cytotoxic assay (see RESEARCH DESIGN AND METHODS). **B:** P815 control cells were labeled with chromium sulfate (^{51}Cr) for 1 h, washed, and incubated for 16 h with insulin B15–23 reactive CD8⁺ T-cells at a ratio of 1:10, in the presence of increasing concentrations of insulin B15–23 peptide. The percentage of lysis was determined measuring the gamma radioactivity released in the supernatant by the lysed cells: $\% \text{ of lysis} = [(\text{sample release} - \text{min}) / (\text{max} - \text{min})] \times 100$. **C:** The same procedure was used with islets from NOD mice, which were infected with the AMPK-DN adenovirus at 100 MOI for 48 h. A total of 20 islets per condition were used in presence of 400,000 CD8⁺ T-cells, estimating 1,000 cells/islet, and insulin peptide was added or not at 1 $\mu\text{g/ml}$. * $P < 0.05$ for the effect of AMPK-DN virus in the presence of the peptide compared with Null virus. Data are given as means \pm SEM of triplicate analyses from three independent experiments.

decreases the cytotoxic effect of CD8⁺ T-cells (Fig. 8), although this finding alone is insufficient to pinpoint which aspect of T-cell action was affected. This question will need further elucidation, for example by blocking each of these pathways using cells from mice expressing targeted mutations, or with blocking antibodies. It has recently been shown by Suzuki et al. (54) that ARK5, a novel AMPK catalytic subunit family member of AMPK, whose activation is directly regulated by Akt, leads to the resistance of colorectal cancer cells to Fas-induced apoptosis by negatively regulating procaspase-6. Taken with our own findings, the latter result suggests that activation of different AMPK family members may exert quite distinct effects on cell survival.

In summary, we demonstrate for the first time that AMPK is involved in regulating β -cell apoptosis induced by cytokines and that AMPK is involved in CD8⁺ T-cell cytotoxicity toward NOD mouse islets. These data suggest

that inhibition of AMPK activity may enhance the survival of islets in diabetes or after islet transplantation.

ACKNOWLEDGMENTS

This study was supported by a fellowship from ALFEDIAM (France) (to A.R.-C.) and grants to G.A.R. from the Medical Research Council (G0401641) the Wellcome Trust (Programmes 067081/Z/02/Z and 081958/Z/07/Z), and Diabetes UK. G.A.R. was a Wellcome Trust Research Leave Fellow, and F.S.W. was a Wellcome Trust Senior Fellow in Clinical Science.

We thank Drs. Gabriela da Silva Xavier, Isabelle Leclerc, and Richard Smith for discussion and Dr. Sarah Richards, Dr. Laura Parton, Rebecca Rowe, Dr. Magalie Ravier, Stephen Chapman, Alan Leard (Bristol MRC Imaging Facility), and Dr. Martin Spitaler (Imperial College

London, Facility for Imaging by Light Microscopy) for their assistance.

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