For Diabetes 18/7/2007; DB05-1504 V2

Inhibition of AMP-activated protein kinase protects pancreatic β-cells from cytokine-mediated apoptosis and CD8$^+$ T cell-induced cytotoxicity

Audrey Riboulet-Chavey[1#], Frédérique Diraison[1#], L. Khai Siew[2], F. Susan Wong[2] and Guy A. Rutter[1].

1: Department of Cell Biology, Division of Medicine, Sir Alexander Fleming Building, Imperial College London, Exhibition Road, London SW7 2AZ, UK

2: Dept. of Cellular & Molecular Medicine, University of Bristol, School of Medical Sciences, Bristol, BS8 1TD, UK

#: Both authors contributed equally to this work

Running title: Activation of AMPK in β-cell death

Word Count: 3886

Number of tables and figures: 8

Key words: AMPK, type 1 diabetes, cytokines, pancreatic β-cells, islets, apoptosis, CD8$^+$ T cells

Corresponding author: Guy A. Rutter PhD, Professor and Head of Department of Cell Biology, Division of Medicine, Sir Alexander Fleming Building, Imperial College, London, Exhibition Road, London SW7 2AZ, UK; Tel: +44(0)20 759 43340; Fax: +44(0)20 759 43351; g.rutter@imperial.ac.uk

http://www1.imperial.ac.uk/medicine/about/divisions/medicine/cellbiology/

ABSTRACT

Objective: Apoptotic destruction of insulin-producing pancreatic β-cells is involved in the aetiology 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 post-transplantation. 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 (Null), constitutively-active AMPK (AMPK CA), or dominant-negative AMPK (AMPK DN) and exposed or not to tumour necrosis factor-α (TNF-α), interferon-γ (INF-γ) and interleukin-1β (IL-1β). Apoptosis was detected by monitoring the cleavage of caspase-3 or DNA fragmentation. The cytotoxic effect of $CD8^+$ purified T cells was examined against pancreatic islets from NOD mice infected with either Null or 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, over-expression of AMPK DN protected against the proapoptotic effect of these agents, without affecting cytokine-induced decreases in cellular ATP, and lowered the cytotoxic effect of $CD8^+$ T cells towards 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.

Abbreviations: ACC, acetyl-CoA carboxylase; AICAR, 5-amino-4-imidazolecarboxamide riboside; AMPK, AMP-activated protein kinase; AMPK CA, constitutively-active AMPK; AMPK DN, dominant-negative AMPK; CaMKK, calmodulin kinase kinase; DMEM, Dulbecco's modifed Eagle's medium; eGFP, enhanced green fluorescent protein; FasL, Fas ligand; FCS, fetal calf serum; HRP, Horseradish Peroxidase; IFN, interferon; IGRP, Islet specific glucose-6-phosphatase catalytic subunit related protein; IL, interleukin; KRB, Krebs Ringer bicarbonate medium; NO, nitric oxide; NOD, non-obese diabetic; ON, overnight; TMR, tetramethylrhodamine; TNF, tumour necrosis factor; TRITC, Tetramethyl rhodamine isothiocyanate; TUNEL, Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling.

Diabetes mellitus currently affects ~ 6 % of the population in westernised societies, an incidence expected to double by 2020 (1). Destruction of β-cells is now believed to be involved in the aetiology of both type 1 and type 2 diabetes (2). Strategies which delay or reverse the loss of β-cell mass in either case are therefore likely to be of significant therapeutic value. Furthermore, such approaches may enhance the survival of islet allograft transplanted into type 1 diabetic patients. Indeed, current human transplantation protocols involve a substantial (60-80 %) loss of functional islet mass after transplantation (3). Thus, several donors are required for successful grafting, emphasising the need to enhance β-cell survival before and after transplantation.

In type 1 diabetes, autoreactive $CD4^+$ and $CD8^+$ T cells recognize their target autoantigens (such as insulin) as peptide fragments presented by major histocompatibility complex molecules. The activated T-cells then travel to the pancreas, infiltrating (insulitis) 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 responsible for β-cell apoptosis. These all lead to the cleavage and activation of caspases by 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 (4;5).

AMP-activated protein kinase is a multisubstrate, trimeric serine/threonine kinase composed of one 63 kDa catalytic α-subunit and two regulatory subunits, β and γ (6;7). AMPK activity is regulated allosterically by AMP (8) and through reversible phosphorylation at Thr-172 of the α-subunit by upstream kinases such as LKB1 (9) or calmodulin kinase kinase (CaMKKβ) (10). AMPK is thus a sensor of cellular energy charge that is activated by the fall in ATP/AMP ratios or an elevation in free Ca^{2+} concentration (11). 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, ACC) and cholesterol (hydroxymethylglutaryl-CoA reductase, HMG-CoA reductase) biosynthesis, and the consequent activation of mitochondrial fatty acid oxidation (6;12). 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 specialised and may represent a key part of the glucose-sensing machinery of these cells (6;13).

A good deal of data has emerged in the last 3-4 years showing that sustained AMPK can exert a proapoptotic effect on a variety of cell types (14;15). Thus, work in hepatocytes (16), gastric cancer (17), neuroblastoma (18), HT-29 colon cancer (19) and chronic lymphocytic leukaemia (20) cells, has implicated AMPK activation in cell death. Taken together, these data support 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 (21), 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 an activated form of AMPK (AMPK CA) reduces the ability of syngeneic islet to reverse streptozotocin-induced diabetes, whilst a dominant-negative form of AMPK (AMPK DN) tended to enhance graft efficiency (22).

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 non-obese diabetic (NOD) mouse, which spontaneously develops diabetes remarkably similar to human type 1 diabetes (23) and constitute a good model to study this disorder. $CD8^+$ T cells are also likely to play a role in humans. Thus, in identical twins who had received a transplant from their non-diabetic co-twin, recurrent disease occurred within six weeks with $CD8^+$ T cells constituting a majority of the cells infiltrating the transplants (24). 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 (25). Finally, a number of recent studies have indicated that T cells recognising proinsulin and IGRP may be detected with high sensitivity at onset of diabetes (26).

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 (27). In addition, in the insulitis lesion in type 1 diabetes, invading immune cells produce pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interferon-γ (INF-γ) and interleukin-1β (IL-1β) and therefore constitute a key step in the pathology of type 1 diabetes (28).

Here, we demonstrate that AMPK activation is involved both (a) in regulating β-cell apoptosis induced by cytokines, and (b) in the cytotoxicity of $CD8^+$ T cells towards islets from NOD mice.

RESEARCH DESIGN AND METHODS

Materials - Collagenase, Histopaque (1077) and Hoechst (type V) were from Sigma (St Louis, MO). Mouse recombinant TNF-α, INF-γ and IL-1β were from PreproTech EC (London, UK). BM Chemiluminescence's blotting substrate (ECL), anti-rabbit and anti-mouse Horseradish Peroxidase (HRP), as well as the [^{32}P] γATP and the ^{51}chromium sulphate, were from Amersham BioSciences (Buckinghamshire, UK). Firefly luciferase was from Promega (Madison, WI), the *In Situ* Cell Death Detection Kit, tetramethylrhodamine red (TMR Red) was from Roche (Basel, Switzerland). The CD8a$^+$ T cell isolation kit was from MACS (Bergisch Gladbach, Germany). Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 media were from Cambrex (East Rutherford, NJ). All the other culture media and cell dissociation buffer were from Invitrogen (Paisley, UK). Scintillation fluid Ultima Gold LLT was from Perkin Elmer (Waltham, MA) and the scintillation 96 well plate 1450 micro beta counter was from Wallac (Turku, Finland).

Animals - Wild-type CD-I mice (20-25 g) and 8-12-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 TCR transgenic mice were generated from G9Cα cloned T cells (29) which had previously been shown to have specific reactivity to amino acids 15-23 of the insulin-β chain (30). TCR α and β founder lines were inter-crossed to produce αβ TCR transgenic mice (G9.NOD). G9Cα$^{-/-}$.NOD mice expressing T cells monoclonal for the transgenic TCR were generated by crossing the αβ TCR transgenic mice to NOD.Cα$^{-/-}$ 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-conjugated (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), or constitutively-active AMPK (AMPK CA), or dominant-negative form of

AMPK α1 ($D^{157}A$) (AMPK DN), have been described by Woods and Carling *et al.* (31;32). AMPK CA comprises the amino terminal domain (amino acids 1-312) common to both AMPKα1 and α2, and is rendered constitutively active by a $T^{172}D$ point mutation. Islets and MIN6 cells were infected at a multiplicity of infection of 100 viral particles / cell respectively overnight or 4 h and cultured for a further 48 h before experiments.

Cell culture and islet isolation - Clonal mouse pancreatic β-cells MIN6 were used between passages #18 and #30 and grown in DMEM containing 25 mmol/l glucose and supplemented with 2 mmol/l L-glutamine, 15% heat-inactivated fetal calf serum (FCS), 50 μmol/l 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. Mouse mastocytoma P815 cells were grown in RPMI 1640 medium, 5% FCS, 2 mmol/l L-glutamine, 50 μmol/l 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. Both were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Mice were killed by cervical dislocation. Collagenase [1 mg/ml in Krebs Ringer bicarbonate (KRB) medium comprising (mmol/l): 120 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 24 $NaHCO_3$, and 1 mg/ml bovine serum albumin, gassed with O_2/CO_2 (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.

$CD8a^+$ 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 (ON) in presence of the Insulin B15-23 peptide, in RPMI 1640 medium containing 5% FCS, 2 mmol/l L-glutamine and 100 U/ml penicillin and 100 μg/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 an untouched $CD8a^+$ 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 3 times with PBS before fixation with 4% (v/v) paraformaldehyde in 0.1 M NaH_2PO_4, pH 7.4. Cells were then permeabilized with 0.3% (v/v) 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 Leica SP2 laser-scanning confocal microscope or Leica SP5 MP/FLIM inverted optic confocal microscope, using a x40 or a x63 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 visualise 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 570-620 nm. Results were quantifed by densitometry.

Western (immuno-) blot Analysis - MIN6 cells or mouse islets were incubated as for measurements of caspase activity and lysed. Whole cellular extract (50 µg) was denatured for 5 min at 100°C in 2% (w/v) 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, before extraction into perchloric acid (10%, v/v). ATP was quantitated in extracts neutralized with Hepes-buffered KOH, using partially purifed firefly luciferase and photon counting, as described before (33).

Cytotoxicity assay - A chromium release assay was performed similar to that previously described (29). Islets from 8-12 week old NOD male mice were infected with either the Null adenovirus or the AMPK DN for 48 h. They were then labelled with ^{51}chromium sulphate for 1 h, as well as the P815 control cells, and washed (see Fig. 8 (a)). The purified activated $CD8^+$ T cells were added for 16 h to the islets with or without added insulin B15-23 peptide at an effector:target ratio of 20:1 (assuming 1000 cells per islet). Cytotoxicity using P815 cells coated with insulin B15-23 peptide, at an effector:target ratio of 10:1 was used as a positive control. Results were expressed as % specific lysis determined as (((cytotoxic release-Min) / (Max-Min)) x100) %, 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 ± S.E.M. of at least three independent experiments. Statistical significance was evaluated using the Student's *t* test for unpaired comparison with Bonferroni correction as appropriate. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

Effect of cytokines on β-cell apoptosis - It has previously been reported that incubation of MIN6 cells (34) or islets (35;36) 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 h, 24 h and 48 h with a combination of the three cytokines IL-1β, TNF-α and IFN-γ (Fig. 1) induced the cleavage of caspase-3, indicating the establishment of apoptosis.

AMPK activation induces β-cell apoptosis - Previous studies have shown that AMPK activators such as 5-amino-4-imidazolecarboxamide riboside (AICAR) (15), or chronic incubation of MIN6 cells or islets with low concentrations (0-3 mmol/l) of glucose (37) or metformin (38) can lead to β-cell apoptosis. Consistent with these earlier findings, when MIN6 cells were infected with the Null (Null) or the constitutively-active AMPK (AMPK CA) adenoviruses, and cultured for 24 h to 96 h, we observed a four-fold increase in the level of activated caspase-3 after 96 h in cells infected with adenovirus expressing AMPK CA compared to Null virus-expressing cells (Fig. 2). Thus, AMPK activation alone is sufficient to induce apoptosis in MIN6 cells.

Cytokines induce an increase in β-cell AMPK activity - Measured either in MIN6 cells (Fig. 3 (a)) or islets (Fig. 3 (b)), AMPKα phosphorylation at Thr-172 was markedly higher after incubation for 30 min in KRB containing low (0-3 mmol/l) glucose concentrations, than at elevated (17 mmol/l) glucose, consistent with previous findings (13). Likewise, incubation for 48 h with a combination of IL-1β, TNF-α and IFN-γ increased by six-fold the level of phosphorylation on AMPK Thr-172 at 17 mmol/l glucose in both MIN6 cells (Fig. 3 (a)) and islets (Fig. 3 (b)).

Cytokine-induced apoptosis requires AMPK activation - To determine whether cytokine-mediated apoptosis required AMPK activation, we used an adenovirus to express a dominant-negative form of AMPK (13). MIN6 cells were treated with the AMPK DN adenovirus for 48 h and then 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 4-5-fold in MIN6 cells (Fig. 4 & 5) 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 assay either in single cells (where infection with adenovirus could be confirmed on a cell by cell basis by the presence of eGFP; Fig. 5 (a)), or in cell populations (Fig. 5 (b)).

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 h or 48 h with cytokines, apoptosis was then assessed by TUNEL assay (Fig. 6). A clear, albeit non-significant ($p = 0.07$) tendency towards a decrease in apoptosis was apparent in cells expressing AMPK DN *versus* those infected with Null adenovirus. By contrast, no effect of AMPK DN was observed after 48 h of incubation with the cytokines.

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/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/AMP ratio (Fig. 7). As anticipated, cellular ATP content was decreased as glucose concentrations were elevated, consistent with a lowering of AMP levels and inhibition of AMPK (13). Interestingly, when cells were incubated for 12 h to 48 h with the cytokine combination, the cellular ATP content was decreased at both 3 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/AMP ratio, and LKB1-mediated phosphorylation of AMPKα (11). By contrast, AMPK DN 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 towards islets probably occurs via a combination of effects including cytokine-mediated killing, Fas/FasL-stimulated 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. 8 (a)) involving insulin B15-23-reactive $CD8^+$ T cells as effectors, and islets isolated from NOD mice infected either with the Null or the AMPK DN adenoviruses. Inhibition of AMPK led to a 50 % decrease of $CD8^+$ T cell cytotoxicity towards the islets (Fig. 8 (c)). Lysis of B15-23 coated P815 target cells was used as a positive control for $CD8^+$ T cell cytotoxicity (Fig. 8 (b)).

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 can 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, Fas ligand (FasL), TNF-α, IL-1β, INF-γ, NO, or a combination of all of the above, has been implicated in the destruction of pancreatic β-cells (2;5;39). These molecules are likely to act in synergy to induce apoptotic signalling cascades. Caspase-dependant cell death can be mediated by two different pathways involving either a death receptor pathway or via a mitochondrial pathway (28;39). Although cytokine-induced apoptosis is known to mediate the death receptor pathway via specific receptors, caspase-3 cleavage and therefore activation lies downstream of both pathways. In our hands, 12 h - 48 h treatment of MIN6 β-cells with the cytokines TNF-α, IL-1β 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 (40) and clonal insulin-secreting cells (41). Kefas *et al.* 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 (15). Conversely, knock-out of the α2 catalytic subunit of AMPK in mice leads β-cells to become resistant to AICAR-induced apoptosis. Correspondingly, our results show an increase in apoptosis in MIN6 cells after an infection with a recombinant adenovirus expressing a constitutively-active form of AMPK (AMPK CA; Fig. 2).

We now extend these earlier findings to demonstrate that AMPK activity is increased in response to cytokines (Fig. 3), as well as to low glucose concentrations (12;13). These changes were matched by significant decreases in ATP content and hence probable 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 Ca^{2+} concentration, and hence activation of CaMKK1β (10;41), may also contribute.

Recently, it has been shown that transforming growth factor-beta-activated kinase (TAK1), a member of the mitogen-activated protein kinase kinase kinase family, can be activated by cytokines (42), and might also phosphorylate and activate AMPK in β-cells (43).

We demonstrate for the first time that cytokine-mediated β-cell death is inhibited when the activation of AMPK is blocked by over-expression of a dominant-negative form of the enzyme (Fig. 4, 5, 6), likely to inhibit complexes containing either AMPKα1 and α2 by binding to the common β1 or β2 scaffolding subunits. Thus, inhibition of AMPK decreased both caspase-3 cleavage and DNA fragmentation (as measured by TUNEL assay) in MIN6 cells (Fig. 5), and tended to decrease DNA fragmentation in islet cells (Fig. 6). Although, we were unable to identify β-cells selectively in this preparation due to the presence of three other dyes, it seems likely that the cytokine-induced changes reflect an action on β-cells rather than other islet cell types.

Interestingly, AMPK DN blunted to only a small extent the cytokine-induced changes in intracellular ATP content of MIN6 cells (Fig. 7), indicating that the pro-apoptotic pathways activated in response to AMPK may act downstream to the decline in ATP. Several mechanisms might 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 (44). This observation is consistent with the fact that the upstream kinase LKB1 (45), is a tumour suppressor mutated in Peutz-Jeghers syndrome. Importantly, recent work demonstrates that AMPK activation induces phosphorylation of the tumour suppressor p53, an event required to initiate cell-cycle arrest in G1 phase (46). Whilst this arrest was reversible in the short term, persistent activation of AMPK led to cell death. Providing alternative mechanisms, Kefas *et al.* showed that AICAR treatment of β-cells lead to c-Jun N-terminal kinase (JNK) and caspase-3 dependant apoptosis whereas Jambal *et al.* and Inoki *et al.* showed an inhibition of protein kinase B and mTOR, respectively, by AMPK and thus an inhibition of the anti-apoptotic pathway and protein synthesis (14;47;48). AMPK activation is also associated with increased mitochondrial superoxide-derived radical (ROS) production and decreased mitochondrial activity (49;50).

There has been much debate as to the mode of cytotoxity towards islet β cells mediated by $CD8^+$ T cells and it is likely that cytokines, Fas/FasL system, and granzyme/perforin all play a role in the

attack (27). We demonstrate here that AMPK may be involved in cytokine-induced apoptosis and we also show that the inhibition of AMPK decreases the cytotoxic effect of $CD8^+$ T cells (Fig. 8) though 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.* 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 (51). 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, these studies highlight a new intracellular signalling pathway involved in cytokine induced β-cell apoptosis. 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 towards NOD mouse islets. These data suggest that inhibition of AMPK activity may enhance the survival of islets in diabetes or after islet transplantation.

ACKNOWLEDGMENTS: 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 (Facility for Imaging and Light Microscopy, Imperial College London) for their assistance. Supported by a Fellowship from ALFEDIAM (France; A, R-C) and grants to GAR from the Medical Research Council (G0401641), the Wellcome Trust (Research Leave Fellowship and Programmes 067081/Z/02/Z and 081958/Z/07/Z) and Diabetes UK. F.S.W is a Wellcome Trust Senior Fellow in Clinical Science.

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FIGURE LEGENDS

Fig. 1. **Cytokines induce apoptosis in clonal MIN6 β-cells.** MIN6 cells were cultured with a cytokine mix containing IL-1β (1 ng/ml), TNF-α (100 ng/ml) and IFN-γ (100 ng/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). Total protein content was estimated using anti-ERK1/2 antibody. Results shown are from three independent experiments.

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) are given as means ± SEM of triplicate analyses from three independent experiments. Results were analysed by densitometry and statistical differences were assessed by unpaired Students t-test (*$p<0.05$ for effect of AMPK CA virus compared with Null virus). Scale bar, 20 μm.

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 the 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 phosphor-specific antibody. Total protein was estimated using anti-ERK1/2 antibody. Results shown are from representative of three independent experiments and statistical differences were assessed by unpaired Students *t*-test: *$p<0.05$ and **$p<0.01$ for the effect of cytokines compared without cytokines.

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 analysed by densitometry and statistical differences were assessed by unpaired Students *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) are given as means ± SEM of triplicate analyses from three independent experiments involving 100 individual cells per condition. Scale bar, 20 μm.

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 either by (a) immunocytochemistry or (b) Western blotting with anti cleaved-caspase-3 antibody. Results were analysed 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. (a) Data are given as means ± S.E.M. of triplicate analyses from three independent experiments involving 50 individual cells per condition or (b) are from three independent experiments. Scale bar, 10 μm.

Fig. 6. **Dominant-negative AMPK blocks cytokine-mediated apoptosis in dispersed mouse pancreatic islets.** (a) Dispersed islets 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 24 or 48 h. Apoptosis was measured by TUNEL assay as described in Fig. 4, followed by nucleus coloration with Hoechst dye. †p = 0.07 for the effect of AMPK DN; (b) Data from (a) are given as means ± SEM of duplicate analyses from three independent experiments involving 50 individual cells per condition. Scale bar, 10 μm.

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 time. Prior to 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 under "Research Design and Methods", before assay of total ATP. *p<0.05, **p<0.01 and ***p<0.001 for effects of cytokines compared to control at the same glucose

concentration, unless otherwise indicated. Data are the means ± SEM of triplicates from observations on three separate cell cultures. Results shown are representative of three independent experiments.

Fig. 8. **Dominant-negative AMPK decreases the cytotoxicity of insulin-reactive CD8$^+$ T cells towards NOD mouse pancreatic islets.** (a) Scheme explaining the cytotoxic assay (see Research Design and Methods). (b) P185 control cells were labelled with ^{51}chromium sulphate 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 islets: % of lysis = ((sample release-min) / (max-min))*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. 20 islets per condition were used in presence of 400 000 CD8a$^+$ T cells, assuming 1000 cells / islet, and insulin peptide was added or not at 1 μg/ml. Data are given as means ± S.E.M. of triplicate analyses from three independent experiments involving 20 individual islets per condition.

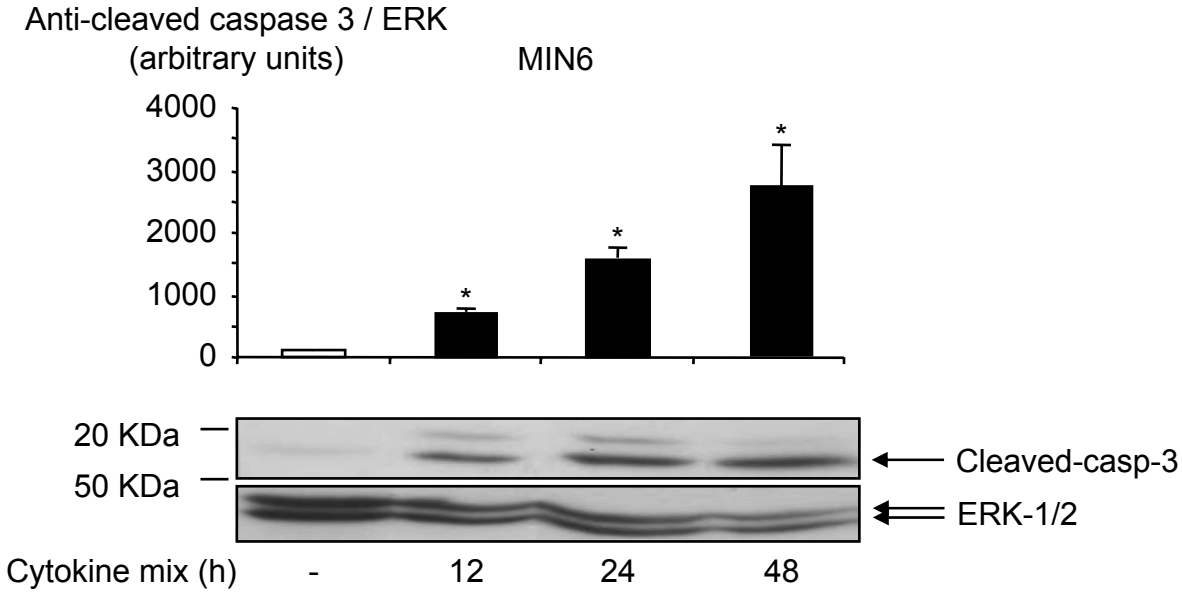

Anti-cleaved caspase 3 / ERK
(arbitrary units)
MIN6
4000
3000
2000
1000
0
*
*
*
20 KDa
50 KDa
Cleaved-casp-3
ERK-1/2
Cytokine mix (h)
-
12
24
48

Figure 1

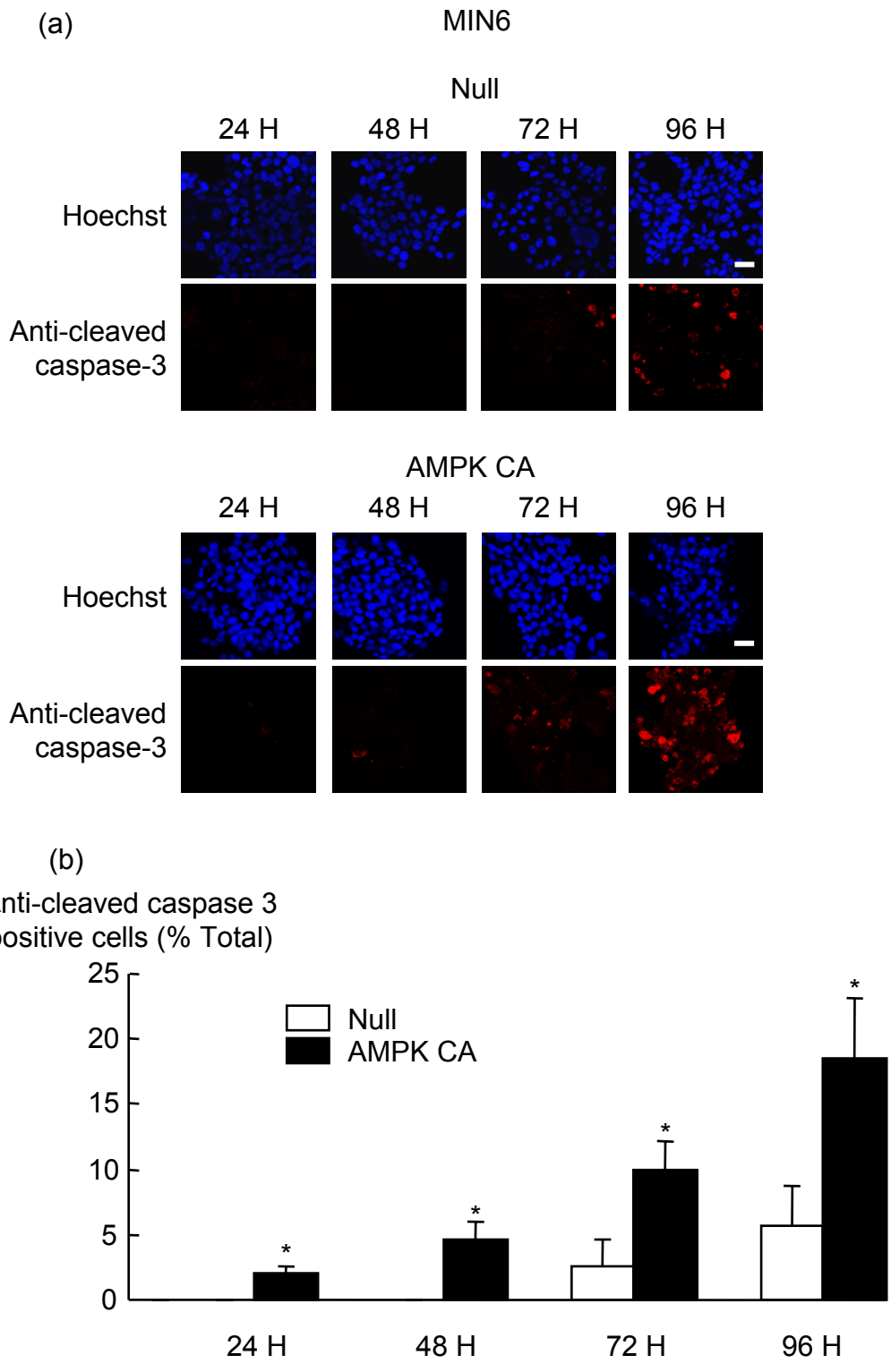
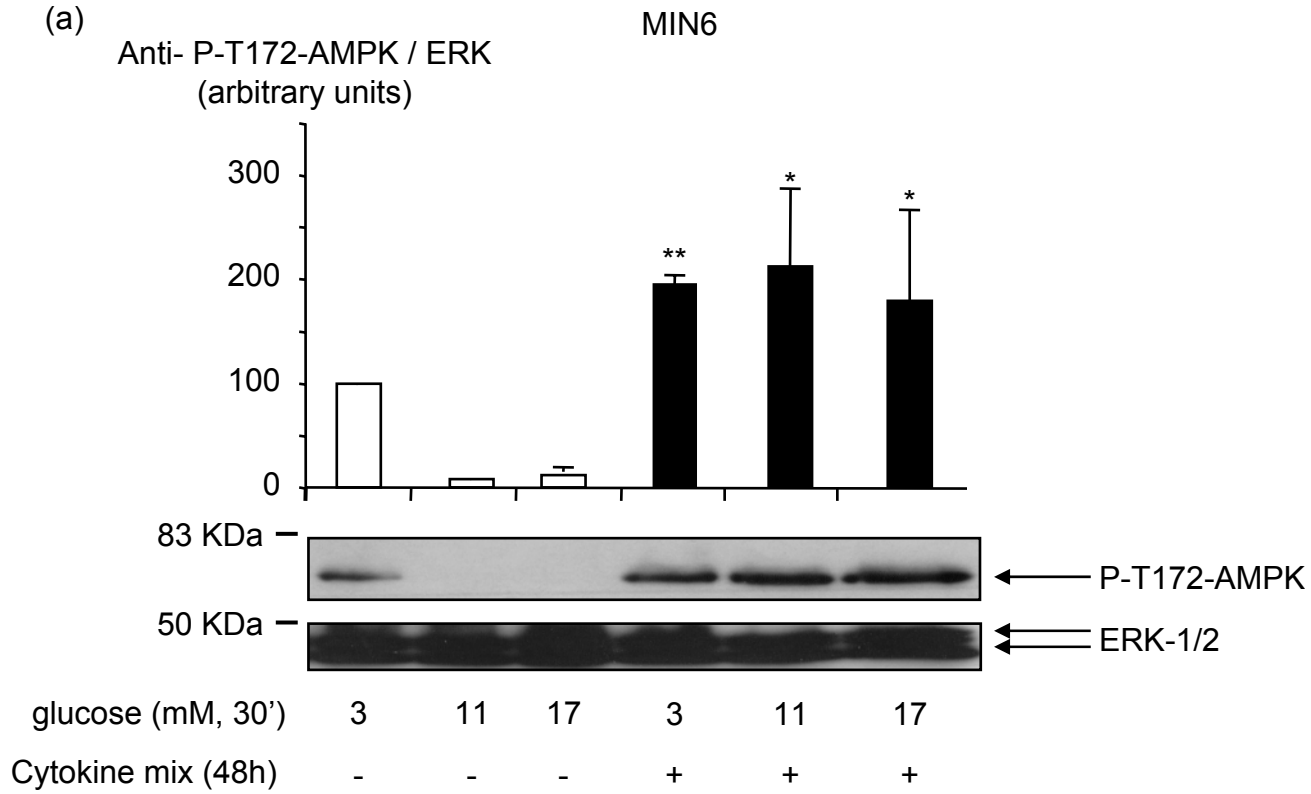

(a)
MIN6
Null
24 H
48 H
72 H
96 H
Hoechst
Anti-cleaved caspase-3
AMPK CA
24 H
48 H
72 H
96 H
Hoechst
Anti-cleaved caspase-3
(b)
Anti-cleaved caspase 3 positive cells (% Total)
25
20
15
10
5
0
Null
AMPK CA
*
*
*
*
24 H
48 H
72 H
96 H

Figure 2


(a)
MIN6
Anti- P-T172-AMPK / ERK
(arbitrary units)
300
200
100
0
**
*
*
83 KDa
50 KDa
P-T172-AMPK
ERK-1/2
glucose (mM, 30')
3
11
17
3
11
17
Cytokine mix (48h)
-
-
-
+
+
+

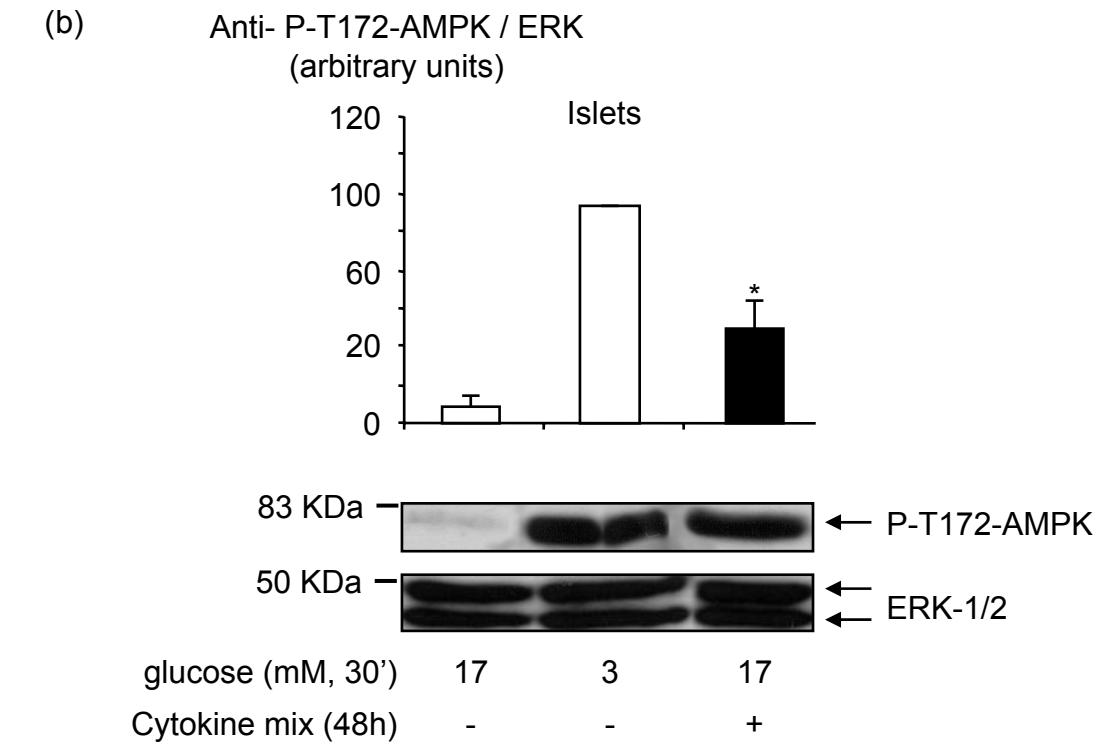

(b)
Anti- P-T172-AMPK / ERK
(arbitrary units)
Islets
120
100
60
20
0
*
83 KDa
50 KDa
P-T172-AMPK
ERK-1/2
glucose (mM, 30')
17
3
17
Cytokine mix (48h)
-
-
+

Figure 3

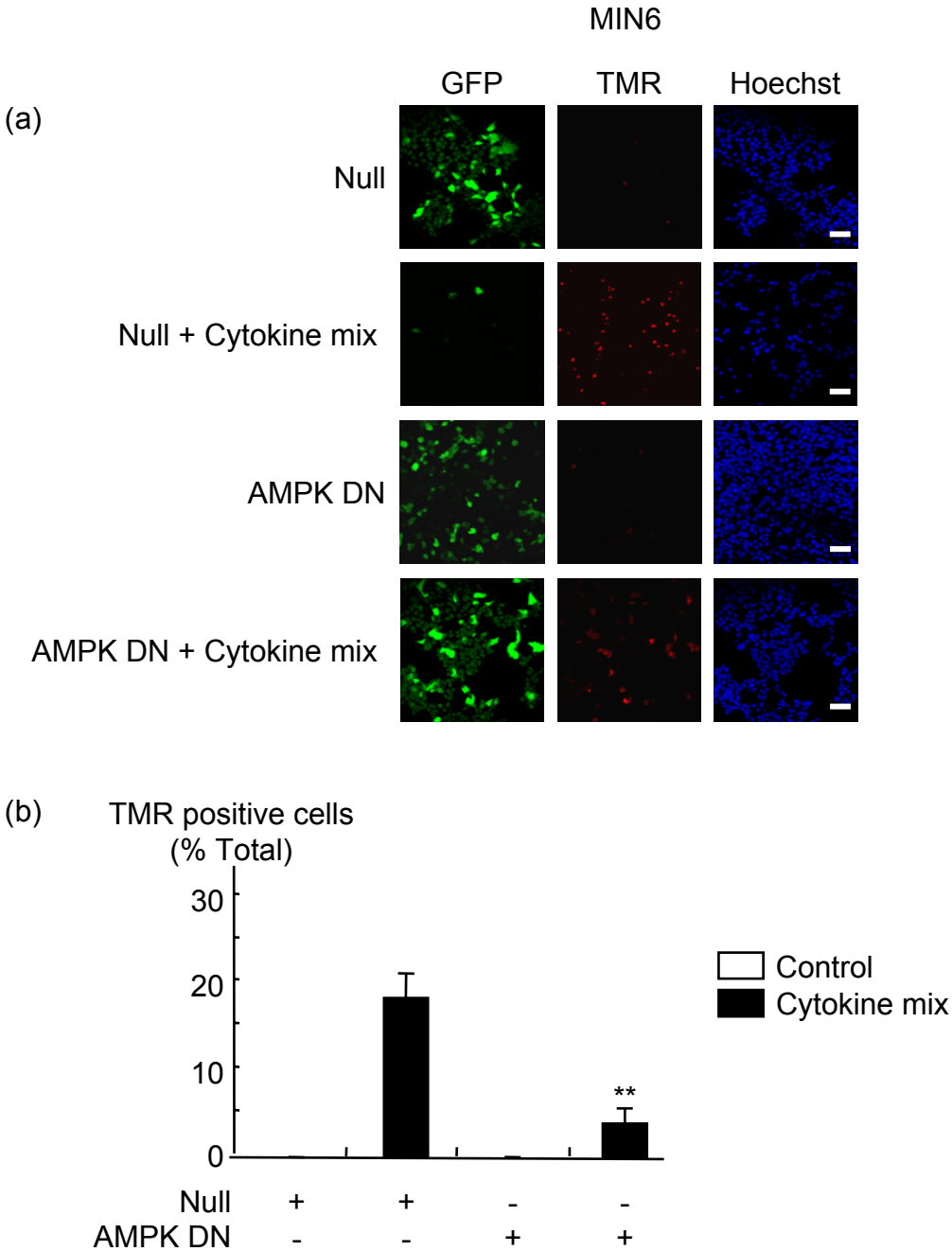

MIN6
(a)
GFP
TMR
Hoechst
Null
Null + Cytokine mix
AMPK DN
AMPK DN + Cytokine mix
(b)
TMR positive cells
(% Total)
30
20
10
0
**
Control
Cytokine mix
Null + + - -
AMPK DN - - + +

Figure 4

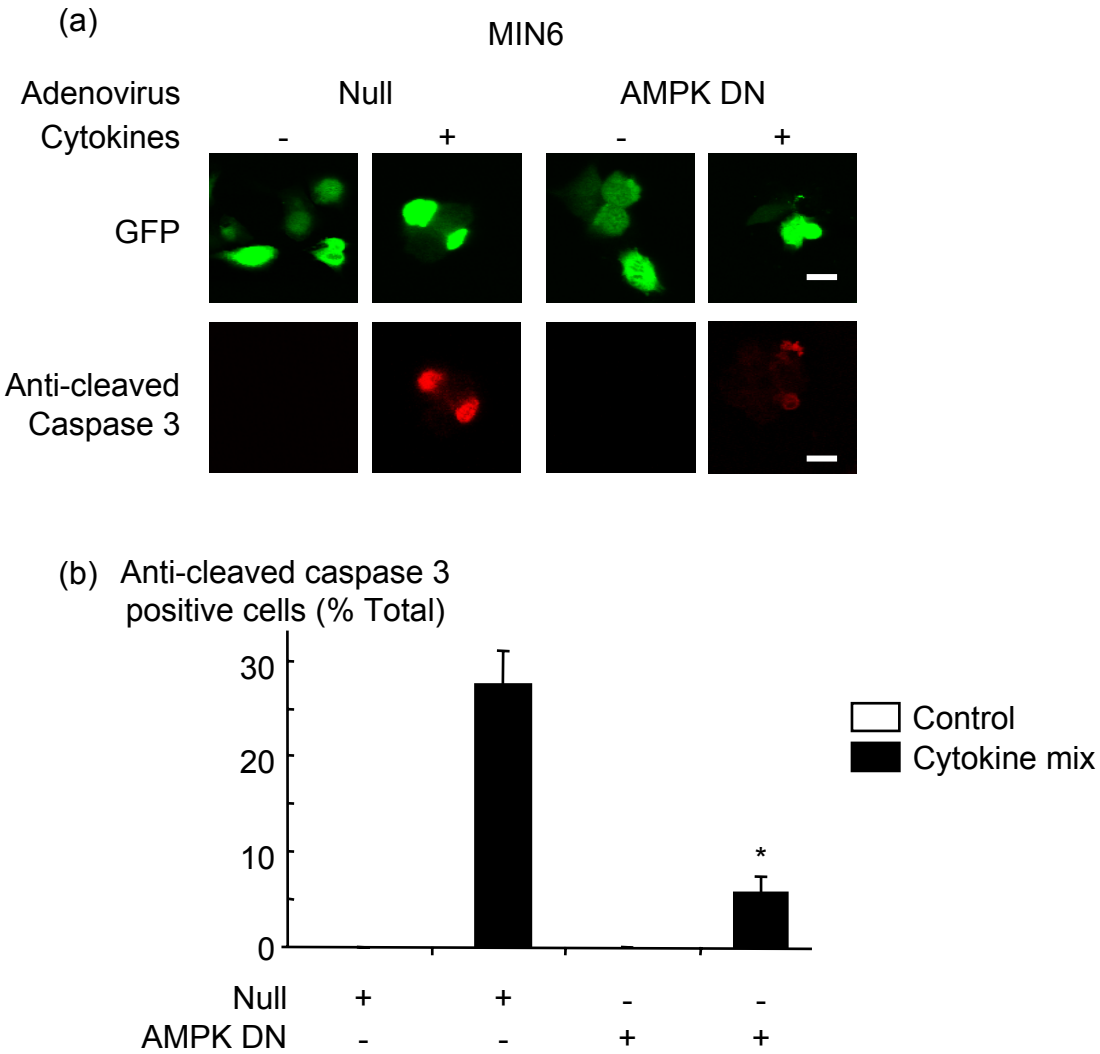

(a)
MIN6
Adenovirus
Null
AMPK DN
Cytokines
-
+
-
+
GFP
Anti-cleaved
Caspase 3
(b) Anti-cleaved caspase 3
positive cells (% Total)
30
20
10
0
*
Control
Cytokine mix
Null
+
+
-
-
AMPK DN
-
-
+
+

Figure 5

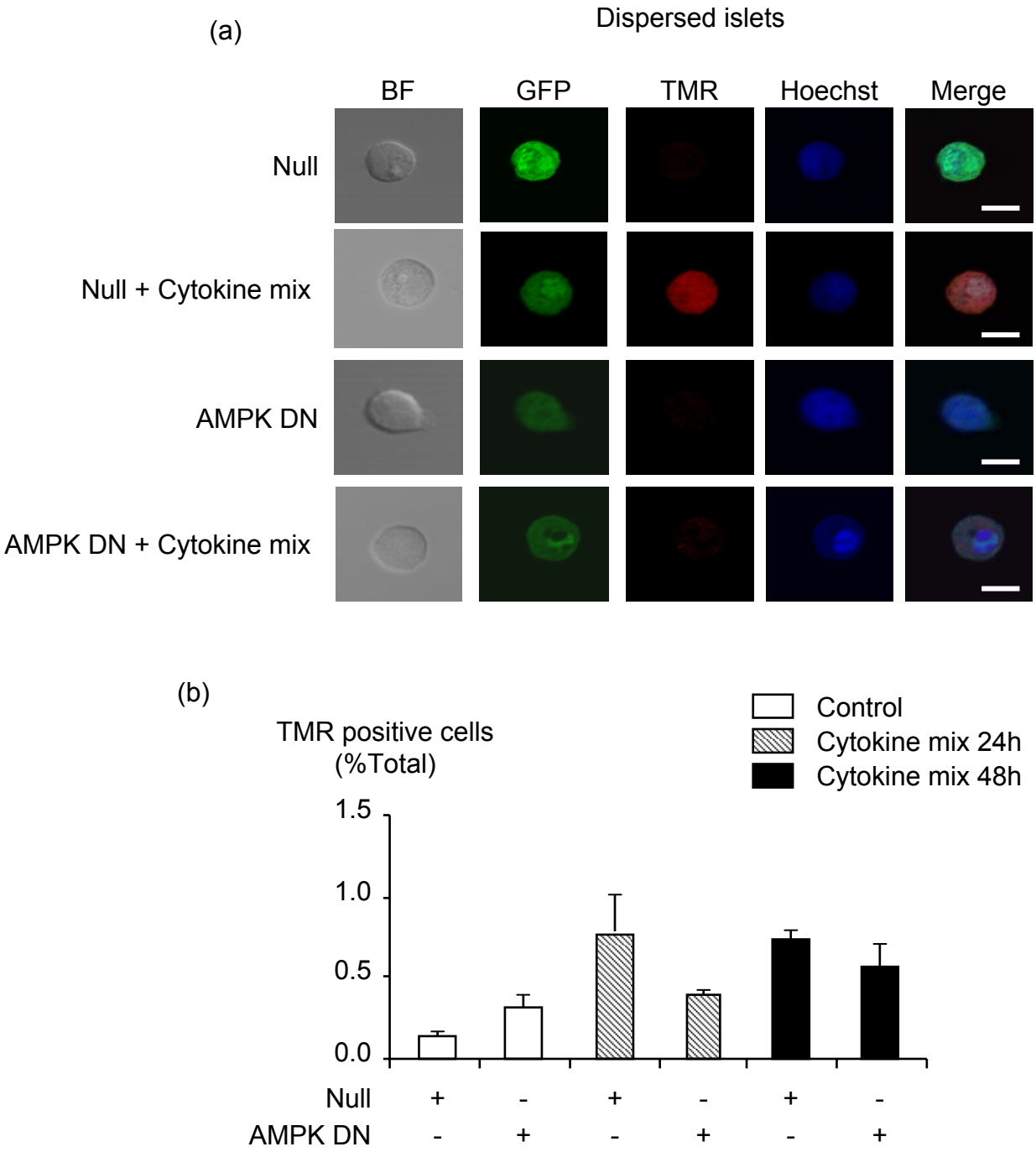

(a)
Dispersed islets
BF
GFP
TMR
Hoechst
Merge
Null
Null + Cytokine mix
AMPK DN
AMPK DN + Cytokine mix
(b)
TMR positive cells
(%Total)
Control
Cytokine mix 24h
Cytokine mix 48h
1.5
1.0
0.5
0.0
Null + - + - + -
AMPK DN - + - + - +

Figure 6

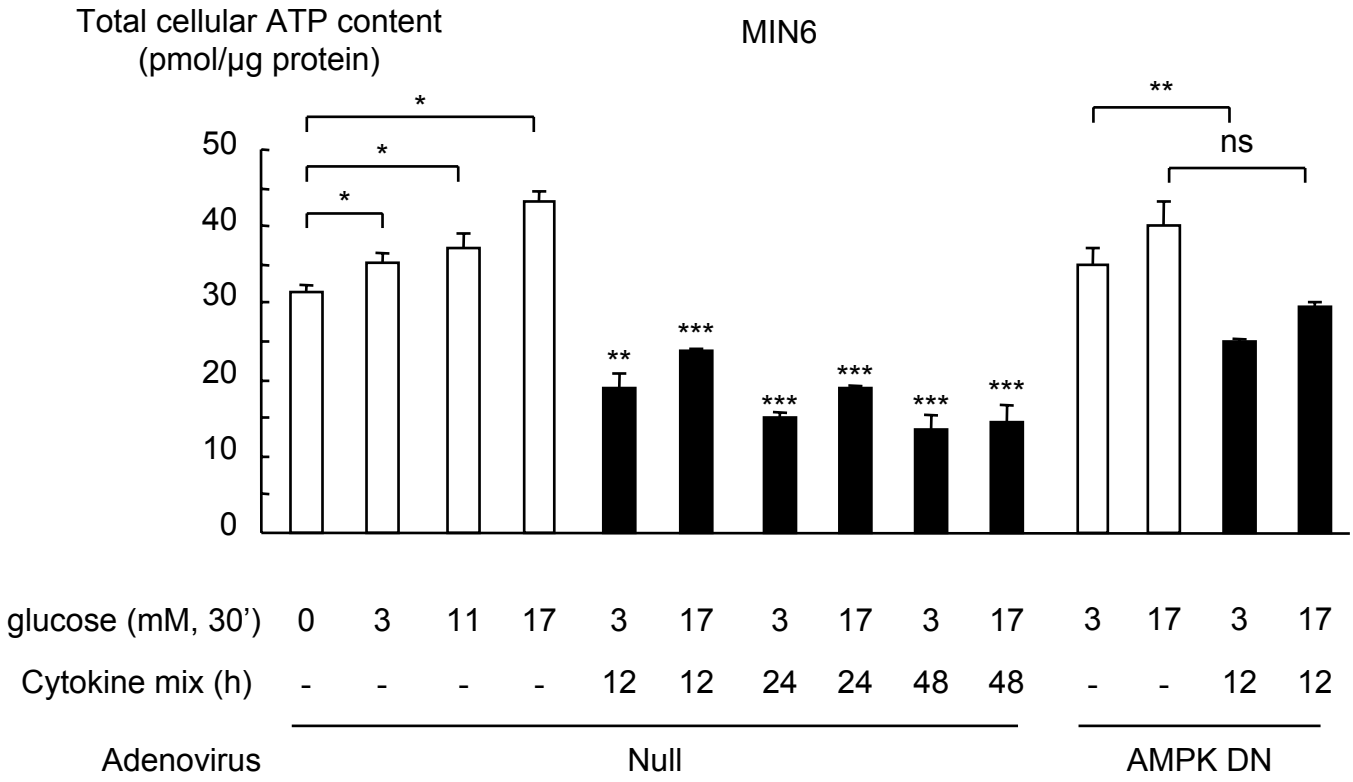
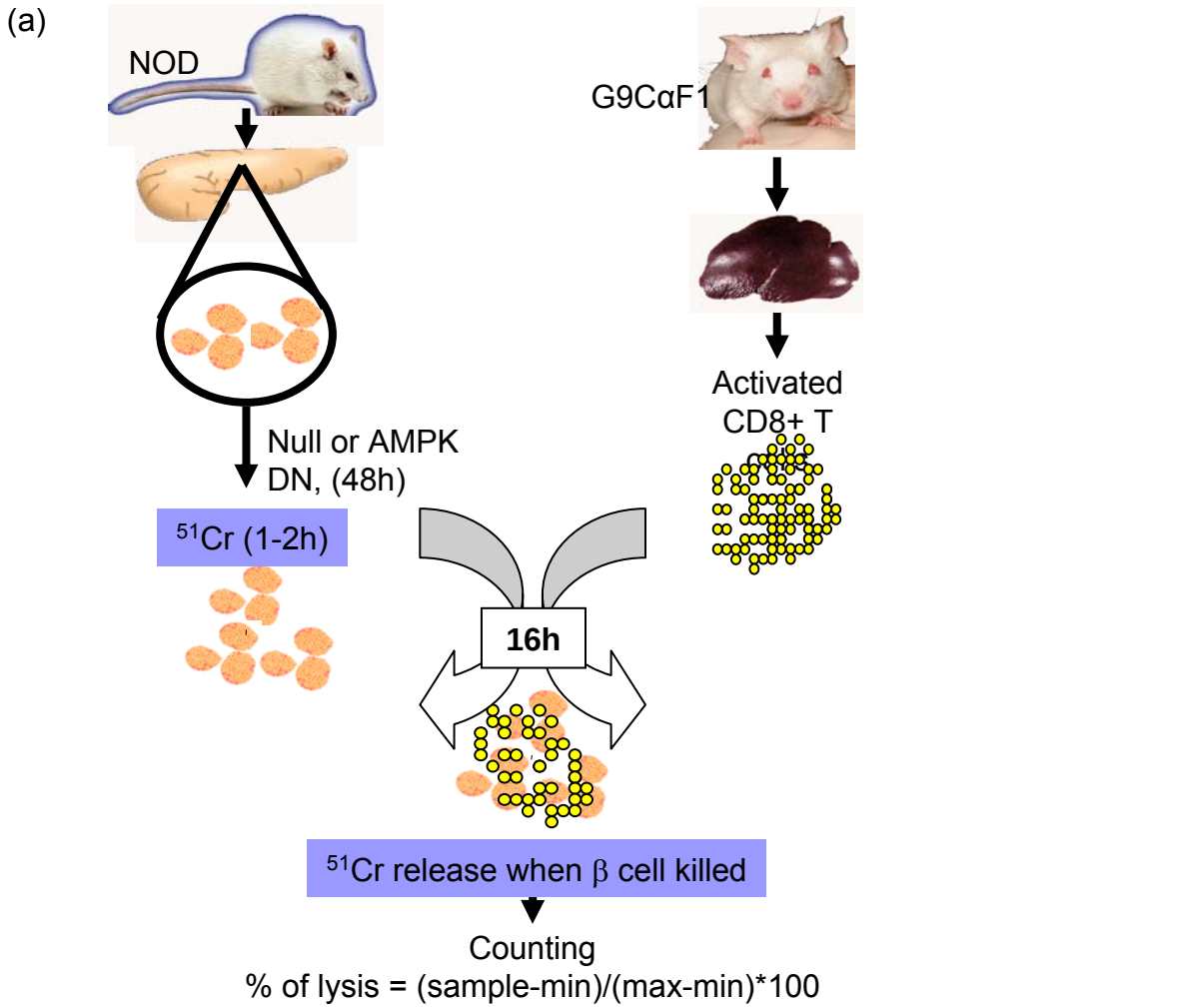
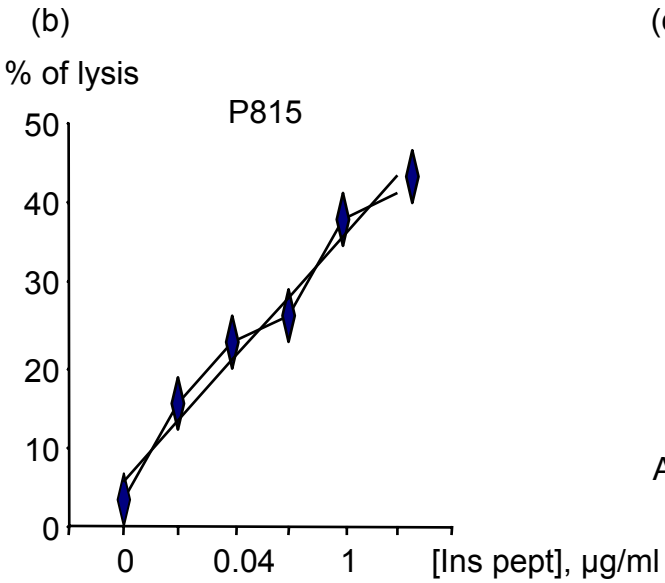

Total cellular ATP content
(pmol/μg protein)
MIN6
50
40
30
20
10
0
*
*
*
**
***
***
***
***
**
ns
glucose (mM, 30')
0 3 11 17 3 17 3 17 3 17 3 17 3 17
Cytokine mix (h)
- - - - 12 12 24 24 48 48 - - 12 12
Adenovirus
Null
AMPK DN

Figure 7


(a)
NOD
G9CαF1
Activated
CD8+ T
Null or AMPK
DN, (48h)
51Cr (1-2h)
16h
51Cr release when β cell killed
Counting
% of lysis = (sample-min)/(max-min)*100


(b)
% of lysis
P815
50
40
30
20
10
0
0
0.04
1
[Ins pept], µg/ml

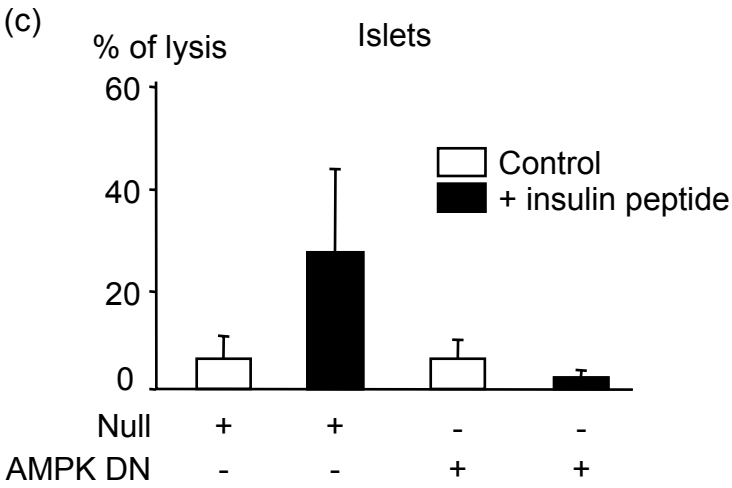
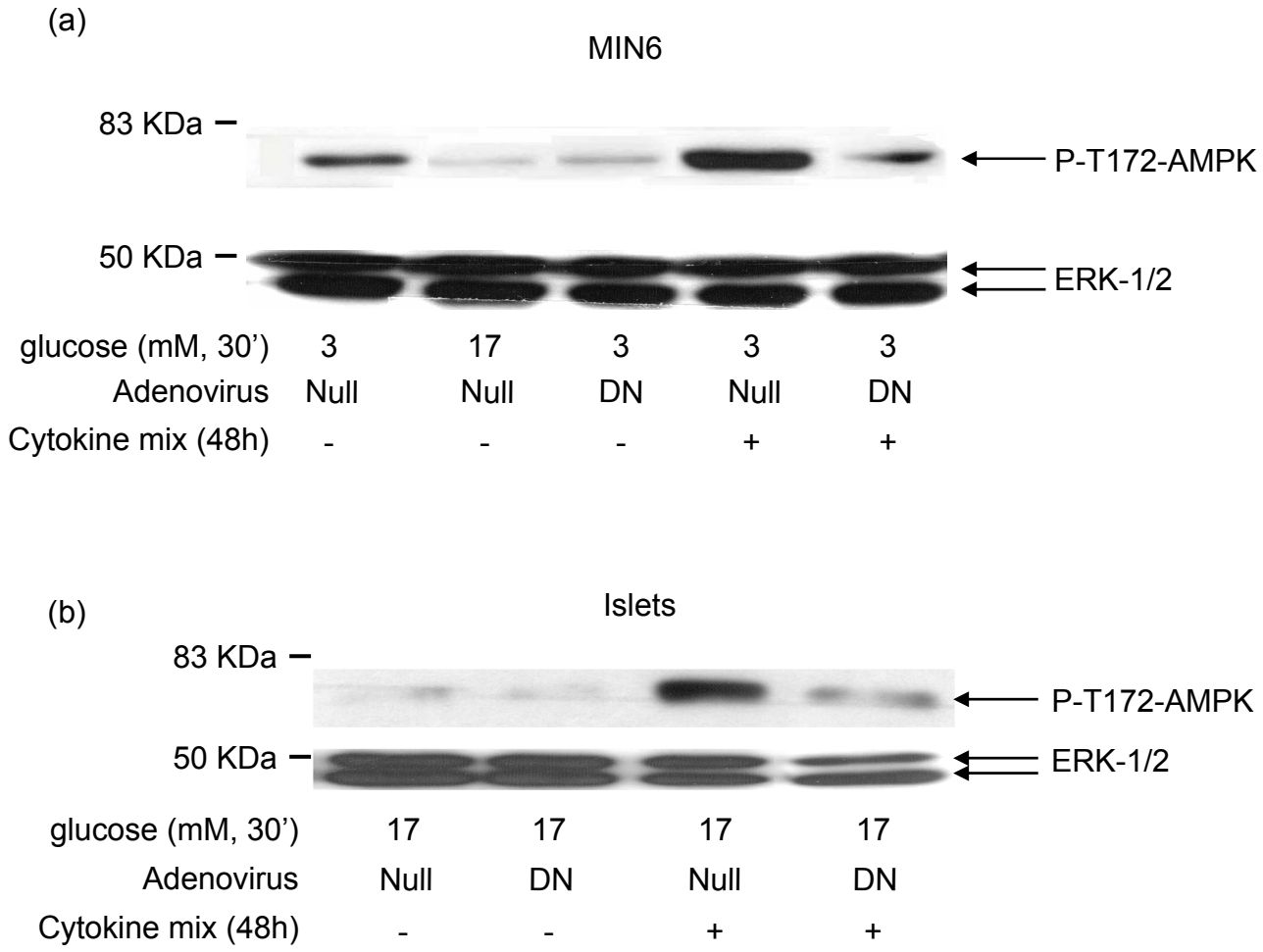

(c)
% of lysis
Islets
60
40
20
0
Control
+ insulin peptide
Null + + - -
AMPK DN - - + +

Figure 8



Supplementary figure